Dosage Response of the Vesicular-Arbuscular Mycorrhizal Fungi Glomus fasciculatus and G. constrictus to Methyl Bromide

J. A. Menge, D. E. Munnecke, E. L. V. Johnson, and D. W. Carnes

Assistant Professor, Professor, Research Assistant, and Postdoctoral Associate, respectively; Department of Plant Pathology, University of California, Riverside, CA 92521.

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ABSTRACT

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Propagules of the vesicular-arbuscular mycorrhizal fungus *Glomus fasciculatus* were fumigated with 3,000, 6,000, and 12,000 μliter methyl bromide (MB)/liter of air for periods varying from 1.5 to 48 hr. Propagules fumigated with MB at a CT (concentration × time) less than 84,000 remained viable and were capable of infecting sudangrass. *Glomus fasciculatus* did not reproduce on the roots of sudangrass if inoculum was fumigated with 12,000 μliter MB/liter air for 7, 12, and 15 hr (CT = 84,000, 144,000, and 180,000, respectively) or 6,000 μliter MB/liter air for 24 hr (CT = 144,000). *Glomus fasciculatus* remained viable when fumigated at 3,000 μliter MB/liter air for 48 hr (CT = 144,000). Dry weight of sudangrass was significantly (P = 0.05) reduced when seedlings were inoculated with *G. fasciculatus* inoculum which had been subjected to 12,000 μliter MB/liter air for 3 hr or longer (CT = 36,000) when compared to dry weight of sudangrass which received nonfumigated inoculum. Ninety percent of the chlamydospores of *G. fasciculatus* and *G. constrictus* failed to germinate when subjected to 12,000 μliter MB/liter air for 6 hr or more (CT = 72,000). No chlamydospores of either mycorrhizal fungus germinated when subjected to 12,000 μliter MB/liter air for longer than 8 hr (CT = 96,000). *Glomus fasciculatus* and *G. constrictus* are both more sensitive to MB fumigation than most soilborne plant pathogens. Furthermore, mycorrhizal fungi can readily be destroyed by MB fumigation in the top 45-cm of soil by most commercial MB fumigations.

Additional key words: soil microbiology, plant nutrition.

Fumigation of nurseries, greenhouse soil, and some field soils, is a widespread practice which frequently is necessary to prevent infection of plants by soilborne pathogens. Fumigation of nursery and greenhouse soil often is required before plants can be certified by a state as disease-free. Most fumigations with methyl bromide (MB) stimulate growth of plants because of this elimination of soilborne pests (15). However, stunting of plants grown in MB fumigated soils has been observed repeatedly throughout the USA (1, 3, 4, 5, 7, 8, 9, 12, 13, 14, 19, 22, 23, 24, 25, 26), and in New Zealand (21), Spain (19), Peru (19), South Africa (17), and Venezuela (19). Stunting following fumigation with MB has been reported with citrus (7, 9, 13, 14, 17, 19, 24, 25, 26), cotton (8), peach (12), soybean (23), white clover (21), and hardwood tree species (1, 3, 4, 5, 22).

The addition of phosphate or inoculation with mycorrhizal fungi can eliminate the problem of stunting in MB fumigated soil (7, 9, 14, 26). Absorption of phosphorus and other minerals by plants is enhanced considerably by mycorrhizal fungi, and the lack of mycorrhizal fungi frequently results in mineral deficiencies (7, 9). Reduced populations of mycorrhizal fungi following fumigation with MB have been documented (22). Kleinschmidt and Gerdemann (9) concluded that the primary cause of stunting following fumigation was inadequate nutrition brought about by the destruction of mycorrhizal fungi.

Knowledge of the sensitivity of specific mycorrhizal fungi to fumigation with MB will enable better understanding of the stunting problem following fumigation. The purpose of this study was to define the sensitivity of two vesicular-arbuscular mycorrhizal fungi, *Glomus fasciculatus* (Thaxter) Gerd. and Trappe and *Glomus constrictus* Trappe to MB.

MATERIALS AND METHODS

Fumigation of Glomus fasciculatus inoculum in soil.—Inocula of *G. fasciculatus* consisted of mixtures of soil and roots from sudangrass (*Sorghum vulgare* Pers.) which were grown for 105 days after inoculation with *G. fasciculatus*. The inoculum contained chlamydospores, vesicles, arbuscules, and hyphae of *G. fasciculatus*. Inoculum was inserted into small gauze bags (10 g/bag). Ten bags of inocula contained an average of 49.8 (range 45.5-59.5) chlamydospores of *G. fasciculatus*/g as determined by wet-sieving (6) and counting. Bags of inocula were placed in 250-ml flasks and covered with a loamy sand. The soil was adjusted to 12% moisture content (w/w). The flasks were attached to the manifold of a MB fumigation apparatus (10). The apparatus assured fumigation with a constant flow of MB. Desired concentrations of MB were monitored frequently with a gas chromatograph. Methyl bromide was bubbled

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through water to assure adequate humidity, passed through the soil, and removed through an exit tube at the top of the flask. Controls were identical to MB treatments except that only air was passed through the inocula. Inocula were subjected to concentrations of 12,000 μliter MB/liter air at nine time periods from 1.5-15 hr; 6,000 μliter MB/liter air at eight time periods from 3-24 hr; or 3,000 μliter MB/liter air at eight time periods from 6-48 hr (Table 2). Five bags of inocula were treated at each concentration × time (CT).

Sudangrass seedlings which were germinated and grown in autoclaved sand for 14 days were transplanted into 1-liter styrofoam cups (one seedling per cup) containing a loamy sand which had been autoclaved previously for 1 hr on 2 successive days. Fumigated inoculum from 1 bag was added around the roots of one sudangrass seedling at transplanting. Sudangrass was fertilized with 250 ml of half-strength Hoagland's solution each week to assure adequate growth of all plants. After 105 days in a glasshouse at 23-35 C, soil and roots from each cup were chopped with a razor blade, mixed thoroughly by shaking in a plastic bag and added to a nematode elutriator (2) to extract the chlamydospores of *G. fasciculatus*. The elutriator floats chlamydospores and roots from the soil with a mixture of water and air. Chlamydospores from 10% of the sample were collected and counted using a ×12 dissecting microscope.

To determine the persistence of killed chlamydospores after treatment, some bags of inocula were autoclaved for 30 min and others were fumigated with propylene oxide for 48 hr and subsequently used to inoculate sudangrass seedlings as described above. To determine if MB had any residual toxic effect upon infection or production of chlamydospores by *G. fasciculatus* or on the growth of sudangrass, inocula fumigated with 12,000 μliter MB/liter air for 15 hr were mixed with nonfumigated inocula and used to inoculate sudangrass. Roots were later examined for infection by mycorrhizal fungi as described in the following experiment.

In a second experiment, inocula were exposed to 12,000 μliter MB/liter air at seven time periods from 1.5-15.2 hr (Table 3). Then the inocula were used to inoculate sudangrass transplanted to a loamy sand in 10-cm diameter clay pots. Six replicate plants received inocula treated at each time period. The plants were not fertilized except for receiving 107 mg NH_4NO_3 and 68 mg KNO_3 per pot on two occasions. This low fertility regime encouraged maximum disparity in growth between mycorrhizal and nonmycorrhizal plants. After 105 days, an 85-g sample of soil and roots from each pot was wet-sieved (6), and the number of chlamydospores of *G. fasciculatus* were counted. Ten root pieces 1 cm long from each sudangrass plant were stained in lactophenol cotton-blue (20) and examined for infection by *G. fasciculatus* fungi. Dry weights of sudangrass also were obtained.

Fumigation of Glomus fasciculatus and G. constrictus chlamydospores.—Soil and roots from pots containing sudangrass infected with *G. fasciculatus* were divided into 0.5-kg portions and suspended in 2 liters of water. The suspended material was screened through a series of sieves (1.0, 0.25, and 0.045 mm), disrupted in a Waring Blendor for 3 min, and filtered through four layers of gauze. Chlamydospores of *G. fasciculatus* were partially concentrated in the filtrate. Chlamydospores were deposited on 10-μm nylon mesh and surface-disinfested by dipping the nylon mesh into 2% chloramine T and 200 μg streptomycin/ml of water for 30 sec. The spores were rinsed twice in sterile distilled water and approximately 200 spores were deposited on Whatman No. 1 filter paper which was folded, placed in a 125-ml flask, and fumigated with 12,000-12,300 μliter MB/liter air at 10 time periods (200 spores/time period) from 0-12 hr (Table 4). Chlamydospores serving as the control were treated with air only for 12 hr. In one experiment, the filter papers (spore side down) were placed on agar plates. In a second experiment, the chlamydospores were washed from the filter papers onto the agar plate with sterile distilled water to obtain a better distribution of spores. The agar used was 3% water agar containing 0.05% thiamin. The plates were incubated at 24-25 C in the dark for 10-15 days and germination of chlamydospores which were free of debris or other fungi was determined visually using a ×25 dissecting microscope.

RESULTS

Fumigation of Glomus fasciculatus inoculum in soil.—A few chlamydospores of *G. fasciculatus* were found in soil surrounding all sudangrass seedlings which received inoculum of *G. fasciculatus*. A small number of chlamydospores (0.2-0.5 chlamydospores/g of soil) were even recovered from around sudangrass plants which received inoculum that was fumigated for 15 hr with 12,000 μliter MB/liter air, fumigated with propylene oxide, or autoclaved (Table 1), even though no evidence of root infection was observed from these treatments. Since chlamydospores were not found in soil which did not receive mycorrhizal inoculum (Table 1), it was concluded that these small numbers of chlamydospores were the remnants of the original inocula which were killed but remained intact throughout the experiment. As a result, the numbers of chlamydospores of *G. fasciculatus* produced on sudangrass roots were adjusted for these dead chlamydospores by subtracting 0.5 chlamydospores/g of soil from all counts of

TABLE 1. The number of chlamydospores from soil surrounding roots of sudangrass which received *Glomus fasciculatus* inoculum[a] treated in several ways

Treatment	Chlamydospores from soil surrounding sudangrass roots/g dry wt soil
Autoclaved (30 min)	0.2
Propylene oxide (48 hr)	0.5
No inoculation	0.0
Methyl bromide (12,000 μliter/liter air for 15 hr) + nonfumigated inoculum	15.1
Air only 1.5 hr	11.3
Air only 48 hr	16.3

[a]Inocula consisted of 10 g samples of soil, roots, and chlamydospores from pots of sudangrass inoculated with *G. fasciculatus*. Inoculum contained an average of 49.8 chlamydospores of *G. fasciculatus*/g of inoculum. Five replicate samples of inocula received each treatment.

chlamydospores. With this adjustment, numbers of chlamydospores per gram of soil correlated very closely ($r = 0.98$; $P = 0.01$) with the amount of *G. fasciculatus* hyphae per centimeter of sudangrass root in the second experiment.

Growth of sudangrass and the survival of *G. fasciculatus* were not affected by residual methyl bromide (Table 1). This was shown by comparing sudangrass which received nonfumigated mycorrhizal inoculum with sudangrass which received a mixture of MB fumigated and nonfumigated inoculum. An average of 15.1 chlamydospores/g soil were produced in soil surrounding roots of sudangrass which received a mixture of nonfumigated and fumigated (12,000 μliter MB/liter air for 15 hr) inoculum. This number was not significantly different from the number of chlamydospores formed in soil around roots of sudangrass that received inoculum exposed to air only (11.3-16.3 chlamydospores/g soil) (Table 1).

Inocula of *G. fasciculatus* which were fumigated with MB at a CT less than 84,000 remained viable and were capable of infecting sudangrass, as evidenced by the production of chlamydospores on roots (Table 2). *Glomus fasciculatus* did not reproduce on the roots of sudangrass when fumigated with 12,000 μliter MB/liter air for 7, 12, and 15 hr (CT = 84,000, 144,000, and 180,000) or 6,000 μliter MB/liter air for 24 hr (CT = 144,000) (Table 2). Inoculum of *G. fasciculatus* remained viable when fumigated at 3,000 μliter MB/liter air for as long as 48 hr (CT = 144,000) (Table 2).

In the second experiment, the dry weights of sudangrass and the numbers of *G. fasciculatus* chlamydospores produced on the roots of sudangrass were inversely correlated with the dose of MB to which the inoculum had been subjected (Table 3). The dry weights of sudangrass which received mycorrhizal inocula fumigated with 12,000 μliter MB/liter air for 3 hr or longer (CT>36,000) were significantly less than dry weights of sudangrass receiving nonfumigated inoculum (Fig. 1, Table 3). Sudangrass plants receiving inoculum fumigated with 12,000 μliter MB/liter air for 10 hr or more (CT>120,000) were extremely nutrient deficient and many died (Table 3). The number of chlamydospores of *G. fasciculatus* produced in the soil surrounding the roots of sudangrass was significantly reduced when inoculum was fumigated with 12,000 μliter MB/liter air for 3 hr or more (CT>36,000) and no chlamydospores were produced in soil surrounding the roots of sudangrass when the inoculum was fumigated with 12,000 μliter MB/liter air for 10 or more hr (CT>120,000) (Table 3). A few hyphae of *G. fasciculatus* were observed in the roots of the four plants which survived after receiving inoculum fumigated with MB for 10 and 15.2 hr. The amount of hyphae in these plants was less than 4% of the amount of hyphae in roots of plants that had received nonfumigated inoculum.

Fumigation of Glomus fasciculatus and G. constrictus chlamydospores.—Ninety percent of the chlamydospores of *G. fasciculatus* and *G. constrictus* failed to germinate when subjected to 12,000-12,300 μliter MB/liter air for 6 or more hr (CT>72,000-73,800) (Table 4). No chlamydospores of either mycorrhizal fungus germinated when subjected to 12,000-12,300 μliter MB/liter air for longer than 8 hr (CT>96,000-98,400) (Table 4). Germination of nonfumigated spores was 84% for *G. fasciculatus* and 87% for *G. constrictus*.

DISCUSSION

Glomus fasciculatus and *G. constrictus* were highly sensitive to methyl bromide. The LD_{90} CT values of MB are 72,000-74,000 for *G. fasciculatus* and *G. constrictus*. These fungi are approximately twice as sensitive to MB as *Phytophthora parasitica* and *P. cinnamomi*, about four times more sensitive to MB than *Verticillium albo-atrum*,

TABLE 2. Effects of various doses of methyl bromide upon survival of infective propagules of *Glomus fasciculatus*[a]

Concentration of methyl bromide (μliter/liter air × 10^{-3}) × length of exposure (hr) (CT)	Survival[b] at methyl bromide concentration (μliter MB/liter air)		
	3,000	6,000	12,000
0	+	+	+
18	+	+	+
21	+	+	+
24	+	+	+
33	+	+	+
45	+	+	+
60	+	+	+
84	+	+	−
144	+	−	−
180			−

[a]Propagules were contained in 10-g samples of soil and roots of sudangrass which contained chlamydospores, vesicles, arbuscules and hyphae of *G. fasciculatus*. Five replicate bags of inocula were treated at each concentration × time.

[b]The symbol "+" indicates survival of inoculum as indicated by the production of *G. fasciculatus* chlamydospores around sudangrass roots inoculated with fumigated inocula, and "−" indicates destruction of inoculum since no chlamydospores of *G. fasciculatus* were produced around sudangrass roots inoculated with fumigated inocula.

TABLE 3. Dry weights of sudangrass which received inoculum[a] of *Glomus fasciculatus* fumigated with various doses of methyl bromide and the number of *G. fasciculatus* chlamydospores from soil surrounding the roots of the sudangrass

12,000 μliter methyl bromide/liter air × 10^{-3} × length of exposure (hr) (CT)	Chlamydospores from soil surrounding sudangrass roots/g dry wt of soil (no.)[b]	Dry wt sudangrass (g)[b]
Air only	25.5 A	30.2 A
18	33.3 A	20.3 AB
24	17.9 A	13.0 ABC
36	4.1 B	8.9 BC
48	5.7 B	1.6 C
72	0.6 B	0.3 C
120	0.0 B	...[c]
182.4	0.0 B	...

[a]Inocula consisted of 10-g samples of soil and roots of sudangrass which contained chlamydospores, vesicles, arbuscules and hyphae of *G. fasciculatus*. Six replicate plants received inoculum treated for each time period.

[b]Values in the same column not followed by the same letter are significantly different ($P = 0.05$).

[c]Majority of the plants died.

and about nine times more sensitive to MB than *Sclerotium rolfsii* when exposed to 12,000 μliter MB/liter air (18). Therefore, it would be impractical to reduce the dose of MB to allow beneficial mycorrhizal fungi to survive MB fumigations. If mycorrhizal fungi survive, soilborne pathogens would survive also.

It would be more practical to fumigate efficiently to kill soilborne pathogens and then to reinfest the soil with mycorrhizal fungi after fumigation. Reinfestation of fumigated soils with mycorrhizal fungi is a practical solution to the stunting problem following fumigation (16).

Apparently, mycorrhizal fungi in soil can be destroyed by as little as 12,000 μliter MB/liter air for 7 hr (CT = 84,000) and stunting of plants may occur after doses of 12,000 μliter MB/liter air for 3 hr (CT = 36,000). However, mycorrhizal fungi consistently survived low concentrations of MB (3,000 μliter MB/liter air) for over 48 hr (CT = 144,000). Most field fumigations with MB at 450-560 kg/ha are sufficient to destroy mycorrhizal fungi in the top 1 m of soil, however mycorrhizal fungi at greater depths may survive because concentrations of MB at depths greater than 1-m often do not reach 3,000 μliterMB/liter air for longer than 48 hr (11).

Small amounts of infection by *G. fasciculatus* were found in a few plants inoculated with inoculum of *G. fasciculatus* which received doses of MB (12,000 μliter MB/liter air) as high as CT = 182,400, indicating that it is very difficult to destroy 100% of the population of mycorrhizal fungi in soil.

TABLE 4. Effect of various doses of methyl bromide upon germination of *Glomus fasciculatus* and *G. constrictus* chlamydospores

12,000-12,300 μliter MB/liter air × 10^{-3} × length of exposure (hr) (CT)	Germination[a]	
	G. fasciculatus (%)	*G. constrictus* (%)
(12 hr air)	84	87
24	84	...[b]
36	63	...
48	62	47
60	20	15
72	8	3
84	6	3
96	0	0
120	0	0
144	0	0

[a]Values are averages of counts from 100 chlamydospores in each of two experiments.

[b]Three dots indicate treatments not included in experiment.

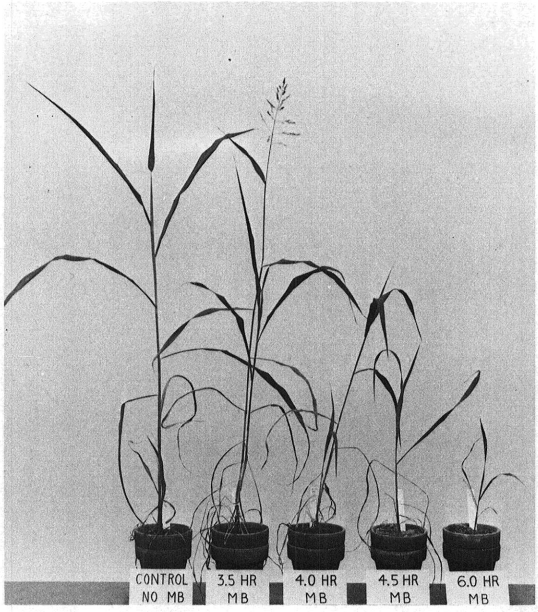

Fig. 1. Sudangrass seedlings which received inoculum of *Glomus fasciculatus* fumigated with 12,000 μliter methyl bromide/liter air for varying lengths of time. Stunting of sudangrass is evident as inoculum is subjected to higher doses of methyl bromide.

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