Tolerance and Uptake of 1,2-Dibromoethane by Nematode-Trapping Fungi

R. Mankau and J. Imbriani

Department of Nematology, University of California, Riverside 92502.
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ABSTRACT

The nematode-trapping fungi, *Arthrobotrys arthrobotryoides*, *A. conoides*, *A. oligospora*, *A. dactyloides*, *Dactylaria haptotyla*, *Monacrosporium doedycoides*, and *M. gephyropagum* tolerated up to 700 ppm 1,2-dibromoethane (EDB) in culture media. EDB dosages below 300 ppm generally did not reduce growth of the fungi, and *A. arthrobotryoides* was apparently stimulated by 200-300 ppm EDB. Uptake of ^{14}C-labeled EDB by the fungi was demonstrated by the presence of from 0.5 to 14% of total specific activity (dpm) in mycelia after optimal growth periods for the various species. Varying percentages of labeled carbon remained bound to cell fragments that had undergone extraction with boiling ethanol, ultrasonic disruption, and centrifugation. *Arthrobotrys conoides* produced $^{14}CO_2$ from a ^{14}C-EDB substrate within 3 days after introduction of the biocide. EDB probably has no adverse effect on predacious hyphomycetes, as low concentrations of EDB appear to be rapidly degraded by these fungi, and dosages much higher than effective nematicidal field rates are not lethal.

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Additional key words: ethlene dibromide.

The effectiveness of nematode-trapping fungi in reducing plant-parasitic nematode populations has not been clearly demonstrated. Nor is the biology of these fungi in the soil well understood. However, there is some evidence that they can contribute to the reduction of undesirable nematode species (4). They easily capture and destroy in vitro large numbers of nematodes which come in contact with their specialized trapping organs, and they appear to be widespread and active in soil (2). If nematode-trapping fungi are important as natural enemies of nematodes, then the effect on these fungi of the widely used nematicide, 1,2-dibromoethane (EDB), which has only moderate fungicidal properties (5, 7, 8, 9), may have significance in nematode control programs.

We report herein the results of an investigation of the tolerance of a group of nematode-trapping fungi with several types of trapping organs to levels of EDB which include and greatly exceed field dosages normally used to control nematodes. An apparent slight stimulation of some species by low dosages of EDB prompted studies on the uptake of ^{14}C-labeled EDB by the fungi.

MATERIALS AND METHODS.—The nematode-trapping fungi used in these experiments were isolated from citrus grove soil at the University of California, Riverside, and included various combinations of the following: *Arthrobotrys arthrobotryoides* (Berlese) Lindau; *A. conoides* Drechs.; *A. oligospora* Fres.; *A. dactyloides* Drechs.; *Dactylaria haptotyla* Drechs.; *Monacrosporium doedycoides* (Drechs.) Cooke; and *M. gephyropagum* (Drechs.) Subram. *Rhizoctonia solani* Kuehn was included in one experiment for comparative purposes. A basic medium composed of 3.6 g $NaNO_3$, 1.0 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 30.0 g glucose, and 3.0 g yeast extract (Difco) in 1 liter of distilled water was used throughout. Glucose was deleted from the medium in experiments concerned with uptake by the fungi of 1,2-dibromoethane (EDB). Suspensions of blended mycelial fragments of the fungi were added to the medium in 125-ml flasks or 4-oz (120-ml) prescription bottles with Teflon-lined screw caps. Fifty ml of medium was used in all units, and treatments were replicated five times unless otherwise noted.

Fungus tolerance to the fumigant was measured by adding pure laboratory-synthesized EDB to replicate flasks. The EDB was added aseptically with a microliter syringe 24 hr after the fungi were added. Initially, *A. arthrobotryoides*, *A. conoides*, *A. oligospora*, *A. dactyloides*, *D. haptotyla*, and *M. gephyropagum* were tested for 15 days in media containing EDB concentrations of 21, 55, 105, 168, 195, and 249 ppm. These amounts roughly approximate levels effected by soil applications of the fumigant for nematode control. A second experiment involved exposing *A. arthrobotryoides*, *A. conoides*, and *A. oligospora* to 100, 200, 300, 400, and 500 ppm EDB for 15 days. In a third experiment, *A. arthrobotryoides*, *A. dactyloides*, and *M. gephyropagum* were exposed to media that contained EDB at 100-ppm increments up to 700 ppm for 20 days. Mycelial mats were collected on Whatman No. 1 filter paper under suction, rinsed, then oven-dried.

EDB uptake was determined with ^{14}C-labeled, 1,2-dibromoethane prepared by New England Nuclear Corporation and diluted to a specific activity of 8.95×10^{-3} mc/mmole. Only flasks with Teflon-lined screw caps were used for radioactive studies. Assays for EDB uptake involved using suction to collect the mycelia on Whatman No. 1 filter paper (4.25 cm) and washing the mycelial mats with two 50-ml portions of 1% acetone. Preliminary trials indicated that this procedure was sufficient to rinse mycelia free of extracellular radioactive residues. In one experiment, mycelial mats were extracted with three 50-ml portions of boiling 95% ethanol after the 1%-acetone washes. All washings and ethanol extractions were collected separately, and two 1.0-ml aliquots from each replicate were assayed for radioactivity. Mycelium that had been washed in acetone and

ethanol was rinsed into the cup of a Raytheon 200-w, 10 kc, sonic oscillator (Model DF-101) with 50 ml of 1% acetone, and sonicated for 20 min. Two 1-ml samples of the mycelium sonicate were counted. The remaining sonicate was centrifuged 20 min at 12,100 g in a Sorvall RC-2 centrifuge at 4 C. The pellet and the supernatant were counted, and corrections made for volume. To facilitate counting, the pellet was suspended by shaking in the scintillation cocktail just before counting.

The specific activity of various samples was determined in a Packard Tri-Carb liquid scintillation spectrometer, Model 3214. The scintillation cocktail was composed of: 7 g 2,5-diphenyloxazole (PPO); 0.3 g 2,2-p-phenylenebis (5-phenyloxazole) (POPOP); 100 g napthalene; 100 ml methanol; and 20 ml ethylene glycol in 1 liter of dioxane. Efficiency was determined by using an external standard in most cases. Internal spiking was used when there was a possibility of internal quenching. All samples were counted for 10 min, and data are given in disintegrations per min (dpm).

The effect of EDB on CO_2 evolution by $A.$ $conoides$ in culture was studied by incubating the fungus in 2 μliters EDB in 50 ml yeast extract-basal salts medium in the main chamber of a side-arm flask designed by Bartha & Pramer (1). The flasks and media were sterilized by autoclaving them, and the ascarite filters were sterilized in an ethylene oxide sterilizer; the entire apparatus then was assembled aseptically. The ascarite filters were removed aseptically, EDB and the fungus inoculum were added, and the filter was flamed and replaced. KOH in the side-arms was removed daily and titrated with 0.05 N HCl by procedures appropriate to the flasks (1). CO_2 evolution first was measured in six replicate flasks containing EDB, and compared with a control without EDB. In a subsequent test, ^{14}C-labeled EDB was added to the medium, which then was inoculated with $A.$ $conoides$ (six flasks) or not inoculated (six flasks). CO_2 absorbed in the KOH in each flask was precipitated as $BaCO_3$ with 1.0 ml of 1.0 N $BaCl_2$. The precipitate was centrifuged, rinsed once in distilled water, and recentrifuged. The $BaCO_3$ was suspended in scintillation solvent in scintillation vials to which about 1.0 cc Cabosil paste was added. $Ba^{14}CO_3$ in the vials was counted by the liquid scintillation techniques described previously.

RESULTS.—*Tolerance to EDB.*—In the initial experiment, up to 249 ppm EDB in the medium did not affect the growth of *A. arthrobotryoides* and *A. dactyloides.* Only growth by *D. haptotyla* was clearly suppressed by 55 to 249 ppm EDB; growth of *A. conoides, A. oligospora,* and *M. gephyropagum* at 249 ppm EDB was only slightly less than that of the controls. A slight stimulation of growth, which was statistically insignificant, occurred with *A. arthrobotryoides* and *A. dactyloides* when these were tested at 21 ppm EDB.

When *A. conoides, A. oligospora,* and *A. arthrobotryoides* were grown in media containing 0, 100, 200, 300, 400, or 500 ppm EDB for 15 days, growth was similar to the controls for *A. conoides*
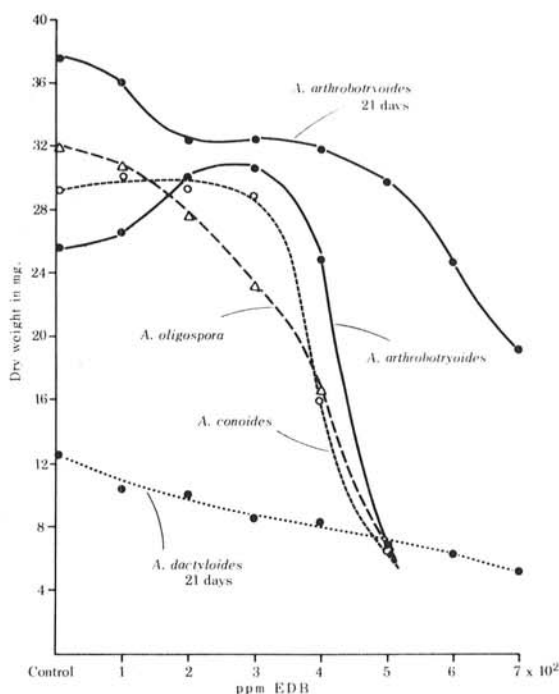

Fig. 1. The effect of increasing concentrations of 1,2-dibromoethane (EDB) on growth of *Arthrobotrys arthrobotryoides, A. conoides,* and *A. oligospora* in 15 days, and on growth of *A. arthrobotryoides* and *A. dactyloides* in 21 days. Dry weight of *A. arthrobotryoides* at 200 and 300 ppm after 15 days is significantly more than that of controls at $P = .01$.

and *A. oligospora* at 100-300 and 100-200 ppm EDB, respectively (Fig. 1). Growth of *A. arthrobotryoides* was stimulated at 200 and 300 ppm and was like that of the controls at 400 ppm. EDB at 500 ppm distinctly suppressed growth of all three species, but did not kill any of the fungi.

Arthrobotrys arthrobotryoides, A. dactyloides, and *M. gephyropagum* were tested by the same procedure, except that EDB up to 700 ppm and 21 days' incubation were used. Growth of *A. dactyloides* was decreased slightly with increasing EDB concentration, but was still 42% of that in controls at 700 ppm EDB. Growth of *A. arthrobotryoides* was not suppressed at concentrations below 500 ppm, and was 51% of that in controls at 700 ppm; growth in 100-400 ppm EDB was not significantly different from that of controls. Stimulation of growth at 200-300 ppm was not discerned in this experiment; the additional growth associated with a longer incubation period may have masked any stimulatory effects of EDB apparent early in the incubation period. Results for *M. gephyropagum* were inconsistent in this experiment; it was determined, however, that this fungus tolerated up to 700 ppm EDB, and apparently was stimulated by low concentrations of the fumigant.

Uptake of ^{14}C-labeled EDB.—The apparent stimulation of fungus growth, in some instances by low concentrations of EDB, led to attempts to

discern EDB uptake or utilization by nematode-trapping fungi. In several preliminary experiments with ^{14}C-labeled EDB at the rate of 2 μliters/50 ml medium, only 0.5-14.0% of the total disintegrations per min (dpm) were associated with the mycelium of the various fungus species, whereas most of the activity remained in the medium. These results were verified in a test in which *A. arthrobotryoides, A. conoides,* and *M. doedycoides,* when grown for 31 days in a medium containing 2 μliters ^{14}C-EDB/50 ml of medium, incorporated 13.9, 4.4, and 6.4%, respectively, of the total dpm into the hyphae. In still another experiment, *A. arthrobotryoides, A. conoides,* and *M. doedycoides* were cultured in the absence of EDB until the mycelial mats were well developed (13 days), and then ^{14}C-EDB was added to replicate flasks at rates of 2 μliters or 7 μliters/50 ml, and shaken well. After 4 days' incubation, incorporation of label by mycelium of *A. arthrobotryoides, A. conoides,* and *M. doedycoides* averaged only 0.2, 0.7, and 0.8%, respectively, of the total dpm at the 2 μliter (87 ppm) level; at 7 μliters (320 ppm), no incorporation was detected for the mycelia of any of the fungi. These results indicated that incorporation of EDB into hyphae apparently occurred only at low concentrations which were not inhibitory, and only during active growth phases of the fungi.

All the predacious fungi were grown separately in a medium containing 2 μliters ^{14}C-EDB/50 ml medium. Three replications were prepared for each fungus. The rapidly growing species, *A. arthrobotryoides* and *A. conoides,* were incubated 10 days, whereas the slower growing species, *A. dactyloides, M. doedycoides, M. gephyropagum,* and *D. haptotyla,* and the rapidly growing *A. oligospora,* for comparison, were incubated 16 days. Most of the total dpm were associated with the culture filtrates. Dpm for the second acetone wash equaled background radiation levels; this result was obtained consistently. Of the total activity recovered for each fungus group, ca. 8% were associated with hyphae of *A. conoides,* and 2.3% with *A. arthrobotryoides* hyphae. The average percentage of the total activity associated with the hyphae of *A. oligospora, A. dactyloides, M. doedycoides, M. gephyropagum,* and *D. haptotyla* was 7.4, 1.9, 1.2, 0.5, and 0.6, respectively. The most uptake occurred in the rapidly growing species, *A. arthrobotryoides, A. conoides,* and *A. oligospora,* all of which produced a greater mass of mycelium than did the other species. The comparatively low activity associated with the hyphae of the other four species was nevertheless above the background level and statistically significant.

In subsequent experiments, glucose was omitted from the medium, which then had only yeast extract and ^{14}C-EDB present as carbon sources. In a test in which all species were grown in this medium, *A. conoides, A. oligospora,* and *A. arthrobotryoides* were incubated for 7 days, and *R. solani* for 10 days. Use of *R. solani* made possible a comparison of uptake by a common nonpredacious soil fungus with that of the nematode-trapping species; results were identical with several of the predacious species. Average counts per min recovered from samples of media filtrates, washes, and mycelia are given in Fig. 2-a. Most of the activity (97-99%) occurred in the media filtrates and in washes, but significant residual radiation remained in the mycelia; substantially greater activity was associated with *A. conoides* than with the other three species, which had similar counts. Data for *A. dactyloides, D. haptotyla, M. gephyropagum,* and *M. doedycoides,* which were incubated for 19 days, are given in Fig. 2-b. As much as 93-99% of the activity occurred in the media filtrates and in washes. EDB-uptake by the mycelia of these four species was approximately the same, but slightly higher for *A. dactyloides,* which also was the most rapidly growing fungus of this group. ^{14}C in the medium was successfully rinsed from the mycelium by the first 1% acetone wash (Fig. 2-a, b). Sonicated mycelia of all species also were centrifuged at 12,000 g to separate soluble and ribosomal fractions (supernatant) from cell wall, nuclei, and mitochondrial material (pellet). Of the radiation remaining in mycelial fractions (Table 1), ca. 80-90% of the total counts were associated with the supernatant, but a

TABLE 1. Distribution of ^{14}C label in fractions of fungus mycelium disrupted by ultrasonication, then centrifuged at 12,000 g

Fungus	Total activity[a] recovered			
	In supernatant		In mycelial pellet	
	dpm	% of total	dpm	% of total
Arthrobotrys arthrobotryoides	4,059	91.5	377	8.5
A. oligospora	3,508	78.4	967	21.6
A. conoides	7,832	86.7	1,200	13.3
A. dactyloides	9,637	90.3	1,036	9.7
Monacrosporium doedycoides	4,180	91.8	371	8.2
M. gephyropagum	6,558	94.6	373	5.4
Dactylaria haptotyla	4,411	86.8	1,393	13.2
Rhizoctonia solani	3,676	93.0	186	7.0

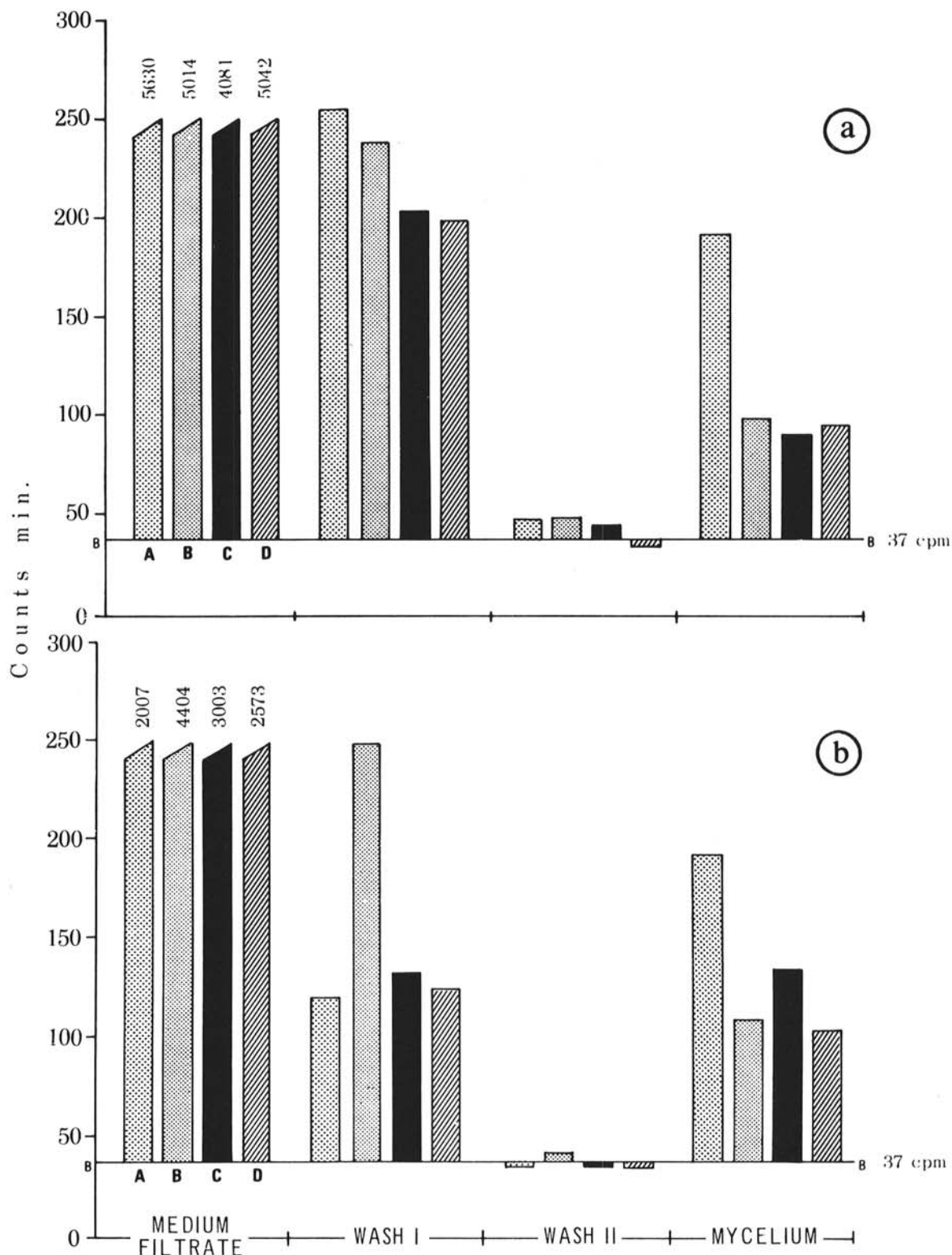[a] Counts corrected for volume as average disintegrations per minute per mycelial mat.

Fig. 2. Average disintegrations per minute (dpm) above background (B) in samples of medium filtrates, 1% acetone washes and in sonicated mycelia. **a)** *Arthrobotrys conoides* (A), *A. oligospora* (B), *A. arthrobotryoides* (C), and *Rhizoctonia solani* (D); **b)** *A. dactyloides* (A), *Dactylaria haptotyla* (B), *Monacrosporium gephyropagum* (C), and *M. doedycoides* (D). The graph scale precluded accurate representation of dpm values for medium filtrates; therefore, the specific values are indicated above the respective bars.
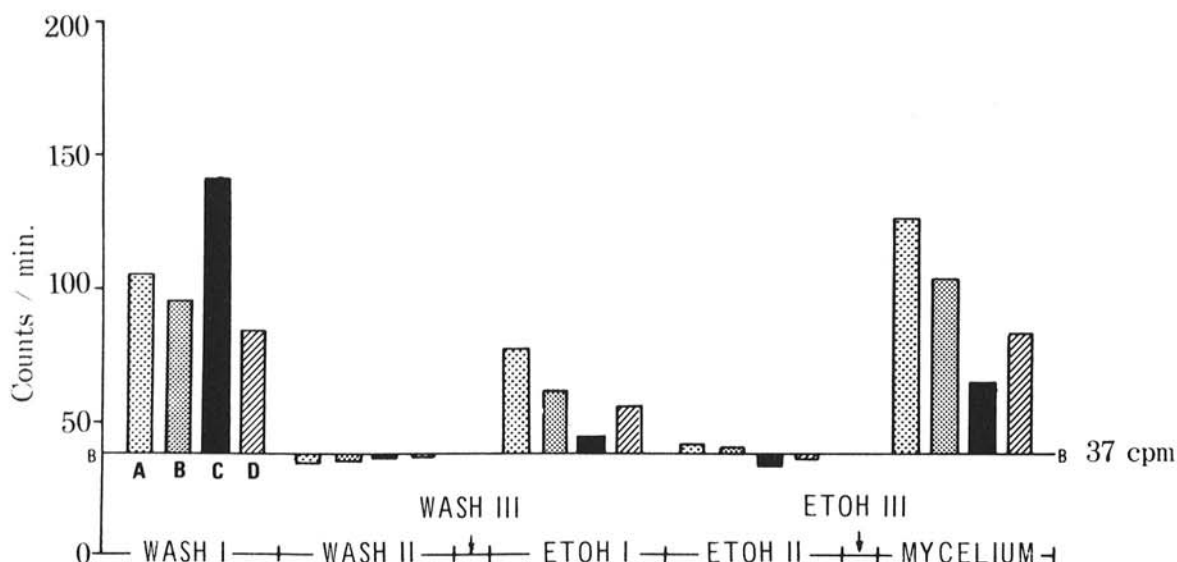
Fig. 3. Average disintegrations per minute (dpm) above background (B) in samples of 1% acetone (wash I-III) and boiling ethanol (I-III) rinses and in sonicated mycelia of *Arthrobotrys dactyloides* (A), *A. arthrobotryoides* (B), *A. conoides* (C), and *Monacrosporium gephyropagum* (D) grown in ^{14}C-1,2-dibromoethane-yeast extract-basal salts medium for 28 days. Acetone wash III and ETOH rinse III were at background levels.

significant level of counts in the pellet residue indicated that some of the labeled carbon may have been incorporated into cell wall or heavier cellular components. Although the results for all species were similar, slightly more activity was recorded for pelleted material of those species which grew most rapidly.

In an additional EDB-uptake test, *A. dactyloides, A. arthrobotryoides, A. conoides,* and *M. gephyropagum* were incubated 28 days in ^{14}C-EDB medium without glucose, and dpm were determined for mycelia that received three 1%-acetone washes and three boiling-ethanol extractions. Radioactivity significantly above background levels was retained in the mycelial mats of all four species (Fig. 3). Mean radioactivity recovered for culture media filtrates were: *A. dactyloides*, 3,055 dpm; *A. arthrobotryoides*, 1,720; *A. conoides*, 2,809; and *M. gephyropagum*, 1,355. That amount of total radioactivity retained by the mycelia after the 1%-acetone washes and subsequently extracted with boiling ethanol was 33.5% for *A. arthrobotryoides*, 22.3% for *A. conoides*, 31.8% for *A. dactyloides*, and 39.6% for *M. gephyropagum*. Approximately two-thirds of the total activity, therefore, remained tightly bound up in the hyphae.

Effect of EDB on CO_2 evolution by A. conoides.—The effect of 2 µliters EDB (88 ppm) in 50-ml yeast extract-basal salts medium without glucose on the evolution of CO_2 by *A. conoides* was measured daily for 20 days. The same medium without EDB served as a control. *Arthrobotrys conoides* produced two peaks of CO_2 evolution that were distinctly greater than that for controls, one on the 1st day of incubation and a second at 7 days. However, total CO_2 evolved over 20 days was not significantly different from that for the controls.

After 20 days, the average dry weight of the mycelia from the EDB medium was 31.1 mg, and that from the controls was 28.5 mg. The differences were only slightly less than that required for significance at the 5% level. These results indicated that EDB may stimulate fungus growth, or that it may be utilized very rapidly by the fungus.

In a similar experiment, ^{14}C-EDB labeled medium without glucose was added to six flasks and inoculated with *A. conoides*, and another six flasks received only the medium. Flasks with *A. conoides* evolved labeled CO_2 over the first 3 days, with peak production on the 2nd day (Fig. 4). Labeled CO_2 production returned to approximately background levels after 4 days. No labeled CO_2 was produced in the control flasks.

Media from additional flasks that contained labeled EDB and the fungus were analyzed for ^{14}C-activity in addition to that recovered as labeled-CO_2. Both the medium and the ambient atmosphere in the flasks were also analyzed for EDB by gas chromatography. Approximately 4% of the total ^{14}C-activity added to the flasks remained in the medium, and only about one-half that amount remained as unmodified EDB (ca. 2 ppm) after culture periods similar to those used in the other experiments reported here. Ethylene was detected, but specific amounts could not be determined.

DISCUSSION.—From an applied aspect, the apparent differences in tolerance to EDB among the predacious hyphomycetes tested here has little importance, as they all tolerated direct exposure to dosages considerably higher than field rates. Hence, there appears to be little possibility that nematicidal dosages of EDB can destroy whatever degree of biological control of nematodes is contributed by nematode-trapping fungi in soil. When EDB was
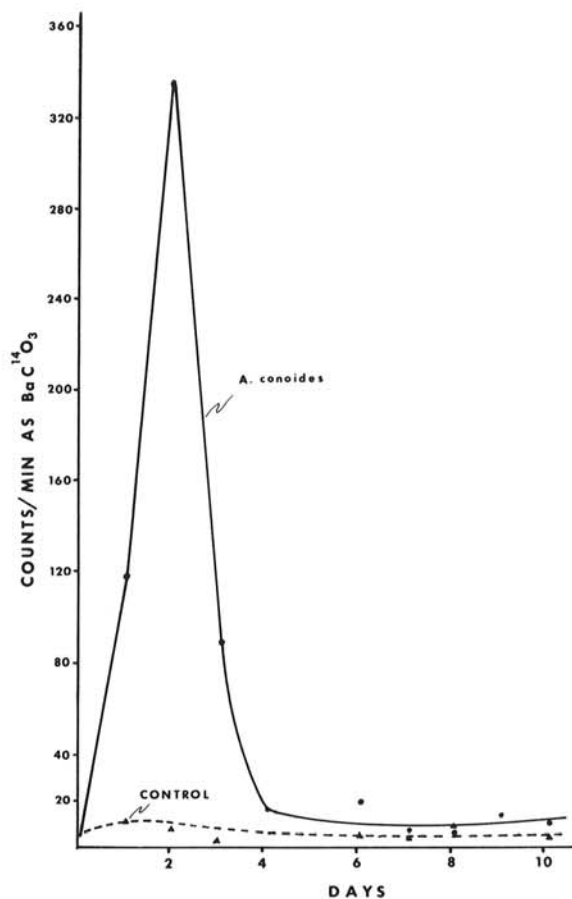
Fig. 4. $^{14}CO_2$ produced from ^{14}C-1,2-dibromoethane (2 μliters/50 ml medium) by *Arthrobotrys conoides* expressed as average disintegrations per minute derived from CO_2 precipitated with 1.0 N BaCl$_2$. Controls were noninoculated.

added to the culture media of predacious fungi, statistically significant reduction in growth did not occur until EDB concentrations reached 300 ppm, 400 ppm, and 500 ppm, respectively, for *A. oligospora*, *A. conoides*, and *A. arthrobotryoides* incubated for 15 days. *Arthrobotrys arthrobotryoides* exhibited growth stimulation at 200 ppm EDB that was significant at $P = .01$ (Fig. 1). The growth of *A. dactyloides*, however, declined significantly at 100 ppm, and decreased gradually with increasing EDB concentration during 21 days' incubation. When *A. arthrobotryoides* was incubated in media with up to 700 ppm EDB for 21 days, growth did not decline until a level of 500 ppm was reached but the stimulatory effect at 200 ppm was no longer apparent (Fig. 1). Relatively high dosage rates of EDB have reduced soil fungus populations in soil fumigated under laboratory conditions (7, 8), but most studies

with this biocide indicate that it has only moderate fungitoxicity, particularly to plant-pathogenic fungi (5, 9). Nematode-trapping fungi apparently were not reduced in field soil fumigated with EDB at nematicidal dosage rates (6). On the other hand, EDB-saturated air was completely fungistatic; however, the fungi resumed growth even after 9 days' exposure, when they were removed from contact with EDB (6).

The presence of ^{14}C in the mycelium of all species when labeled-EDB was used in the culture medium and the accumulation of ethylene in the medium indicate that the nematicide is taken up and probably is metabolized by the fungi. Most of the labeled carbon in the EDB which is not lost to the atmosphere apparently ends up in a metabolite in a medium, as from 93-99% of the total activity recovered was found in the culture filtrates (Fig. 2-a, b), and little undecomposed EDB remained in the medium. The findings of Castro & Belser (3) that EDB is dehalogenated in soil within a few weeks, and our data on CO_2 evolution, both indicate an ability of the fungi to degrade the compound.

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