Killing and Preserving Nematodes in Soil Samples with Chemicals and Microwave Energy[1]

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Abstract: Three basic procedures for treating nematode-bearing soil samples for international shipment or from areas under quarantine were tested for their killing effect and recovery of nematodes by sugar flotation for diagnostic and advisory purposes. These were: fumigation with methyl bromide followed by storage at −15 C; microwave treatment (2450 MHz, 630 w, 2-5 min) followed by addition of FAA + picric acid or 5% Formalin; and adding chemical preservatives (FAA + picric acid, 5% Formalin, NaN_3, and 2-phenoxyethanol) directly to the soil. Larvae of *Heterodera glycines* in eggs within cysts were stimulated to hatch by 2-min exposure to microwaves, and an exposure of 5 min was required to kill them. Soil type and moisture significantly affected microwave effectiveness. Direct saturation of soil samples with preservative chemical solutions (FAA + picric acid or 5% Formalin) was most effective, and often increased the number of nematodes recovered. The high concentration (2%) of NaN_3 required for soil sterilization is too hazardous for routine work. NaN_3, therefore, is not recommended for this purpose. *Key words:* extraction methods, quarantines, *Heterodera glycines, Meloidogyne incognita, Helicotylenchus dihystera, Tylenchorhynchus claytoni, Xiphinema americanum.*

Only a few techniques for killing and preserving nematodes prior to routine extraction have been described (5, 6). Minderman (8) investigated the value of embedding and sectioning soils in population studies of nematodes, but encountered many difficulties. High numbers of identifiable nematodes have been extracted shortly after treatment of soil with methyl bromide (Barker *et al.*, unpublished data). Furthermore, freezing soil at −15 C kills most nematodes, which then can be extracted readily by sugar-flotation methods (3). Elmiligy and De Grisse (5) recently showed that adding hot fixative (4% Formalin with 0.1% glycerine) prior to storage of soil samples did not affect recoveries of nematodes by centrifugal flotation. This led to hypotheses that other procedures might be developed to kill and preserve nematodes prior to extraction from soil with sugar-flotation procedures. Such techniques would be valuable to insure that pests under international or interstate quarantines would not be spread in soil samples.

This investigation was initiated to determine the suitability of such methods for use in nematode diagnostic and advisory programs. Specific experiments included: (i) use of high concentration of methyl bromide, followed by freezing at −15 C; (ii) use of microwave energy to kill the nematodes, followed by immediate extraction, storage at −15 C, or the addition of preservatives; and (iii) adding chemical preservatives directly to the soil.

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MATERIALS AND METHODS

Soil preparation and assay procedures. All lots of soil were pre-mixed in either a concrete mixer or a sample splitter. Unless indicated otherwise, 150-cc samples of soil were treated with three or four replicate samples per treatment. The types of soils and nematode species used are given in each experiment.

Nematodes were extracted by the sugar-flotation-sieving procedure described by Byrd *et al.* (4), except when the Baermann funnel procedure was used to detect motility. Fifty cc of soil were used for most extractions, and all nematode data were converted to numbers per 500 cc of soil. For bioassays, 1-month-old Floradel tomato [for *Meloidogyne incognita* (Kofoid and White) Chitwood] or 1-week-old Lee soybean seedlings (for *Heterodera glycines* Ichinohe) were transplanted to 10-cm clay pots containing 150 cc of test soil per plot. Silica sand was used to fill the remaining space in these pots. Bioassay plants were harvested and nematode populations determined after 6 weeks.

Fumigation with methyl bromide. Immediately prior to fumigation, individual soil samples were placed in 500-cc paper boxes

which were sealed in an 80-liter metal can; and 454 g of methyl bromide were released in this container. After 16- to 20-hr exposure to methyl bromide, samples were placed in plastic bags and frozen at −15 C. When nematodes from soil samples were not counted shortly after extraction, fixative (FAA + picric acid) was added.

Microwave energy. An Amana Radarange®, microwave oven model RR-2 (Amana Refrigeration, Inc., Amana, Iowa 52203) which emits 2450 MHz (630 w) was used in these experiments. Preliminary tests showed that volume of soil and type of container greatly influenced kill of nematodes at a given exposure time. A 3-min exposure of 150 cc of soil 2 cm deep in sealed 700-cc cellophane bags approximately 25 cm from the emitter killed all *M. incognita* larvae. This container and volume of soil were used as standards in subsequent experiments. Exposure times varied with given experiments as indicated. In some experiments, soil samples were processed immediately after treatment, and in others they were frozen or a chemical fixative was added.

Since soil moisture influences the effects of microwave treatments (2), experiments were conducted to determine the effects of soil moisture on rates of kill. Soil moisture levels ranged from 4.5% (oven dry weight basis) to saturated soil. Since cysts of *H. glycines* imbibe water slowly, some samples infested with this nematode were saturated and incubated for 3 days at 15 C prior to treatment.

Adding chemical preservatives directly. Several fixatives and other toxic or preservative chemicals were evaluated; these included 0.01-4% NaN_3, 5% Formalin, FAA + picric acid, and 2-4% 2-phenoxyethanol. In one experiment, the value of adding hot water 90-100 C to soil prior to adding fixative (FAA + picric acid) or Formalin, as compared to cold preservatives, was investigated. Fifty cubic centimeters of soil were used in these experiments. These samples were placed in closed containers and stored at room temperature. Nematode extractions were made weekly or 1 month after treatment unless otherwise indicated.

Potato-dextrose agar, water agar, and nutrient agar dilution plates (1:10 to 1:1,000) of soils from each treatment were made at 4 weeks after treatment to determine the relative rates of kill of fungi and bacteria. Plates were rated for microbial growth after incubation at room temperature for 2 weeks.

RESULTS

Fumigation with methyl bromide. When nematodes were extracted from soil immediately after being fumigated with methyl bromide, or after subsequent storage at −15 C, numbers of dead but identifiable *M. incognita* larvae or *Tylenchorhynchus claytoni* Steiner were not significantly different from the nonfumigated control in most instances (Table 1). When compared to nontreated soil, these treatments increased numbers of *Helicotylenchus dihystera* (Cobb) Sher recovered (Table 1).

Microwave energy. Larvae of *M. incognita* were rendered noninfective by 1.5-min

TABLE 1. Effect of fumigation with methyl bromide and subsequent storage at −15 C on nematode recovery by sugar-flotation sieving.[a]

Storage time (weeks)	Nematode (No./500 cc of soil)		
	M. incognita[b]	*H. dihystera*[b]	*T. claytoni*[c]
0 (not frozen)	45,450	10,100[d]	106
1	45,450	14,275[d]	375[e]
2	43,800	13,025[d]	181
3	44,150	10,650[d]	106
4	44,000	11,500[d]	81
Control (no treatment)	49,400	8750	119
LSD .05	NS	718	156
.01		977	NS
C.V.	12%	17%	66%

[a]Soil treated in 80-liter container with 454 g methyl bromide for 20 hr.
[b]Norfolk sandy loam.
[c]Appling loamy sand.
[d]Significantly different from control at 1% level.
[e]Significantly different from control at 5% level.

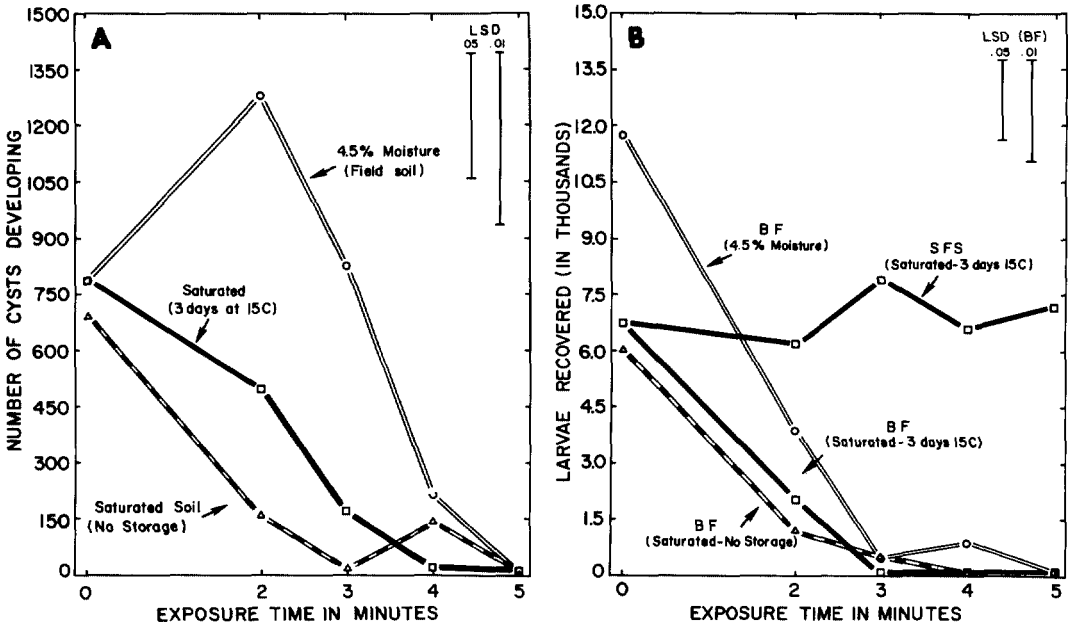

FIG. 1. Inactivation of *Heterodera glycines* by microwave energy. **A.** Effect of soil moisture and exposure time on rates of kill as measured by a bioassay; **B.** Effect of exposure time on larval recovery by Baermann funnel (BF) and sugar-flotation sieving (SFS). LSD values may be used to compare effects of moisture treatments vertically and effects of exposure time for a given moisture treatment horizontally (Differences with extraction by SFS were usually nonsignificant).

exposure to 630 w (2450 MHz) microwave energy. Their recovery from soil by sugar flotation was reduced from 20,038 to 14,900 after 1.5-min treatment, and to 3925 after 4.5-min exposure.

Heterodera glycines was extremely resistant to microwave energy. In Norfolk sandy loam at 4.5% moisture, an exposure of 2 min actually resulted in greater numbers of cysts developing in a bioassay (Fig. 1-A). A 5-min exposure killed all stages of this nematode as determined by the bioassay, except that a few cysts developed in one replicate. Pretreatment saturation of the soil with water greatly increased the killing effect of microwave energy on this nematode, but incubation at 15 C enhanced the effectiveness very little.

Numbers of larvae extracted differed somewhat from bioassay results. Baermann funnel extraction of treated soil indicated that 3-min irradiations were sufficient to kill *H. glycines* which differed considerably from the bioassay (Fig. 1). Regardless of exposure time, similar numbers of identifiable specimens could be extracted by sugar-flotation sieving (Fig. 1-B). A 4-min exposure was sufficient to kill all stages of *H. glycines* in saturated muck soil (data not included). The population density, however, was low in this soil.

Adding chemical preservatives directly. All test concentrations of NaN_3, 5% Formalin, or

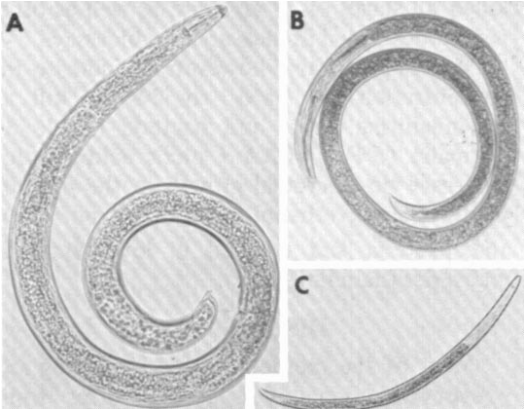

FIG. 2. Effects of adding chemical preservatives to soils on the morphological detail of nematodes. **A.** *Helicotylenchus dihystera* recovered from soil treated with cold FAA + picric acid; **B.** *Xiphinema americanum* recovered from soil treated with 5% Formalin; and **C.** *Tylenchorhynchus claytoni* recovered from a soil treated with hot water prior to adding Formalin.

TABLE 2. Effects of chemical soil treatments and storage time on nematode recovery by sugar-flotation extraction.

Treatment (chemical)	Nematode and time in storage after treatment (No./500 cc of Appling loamy sand)					
	M. incognita		*T. claytoni*		*H. dihystera*	
	0-time	4 weeks	0-time	4 weeks	0-time	4 weeks
Formalin (5%)	2788	2413[b]	331	375	381	188
Fixative[a]	2638	2163[b]	313	250	306	188
NaN_3 (1%)	2456	2800[b]	250	350	313	588[b]
Control	2106	1394[c]	225	200[c]	300	138[c]
LSD						
.05	NS	552	NS	NS	NS	194
.01		751				264
C.V. (%)	20	17	29	36	24	49

[a]FAA plus picric acid.
[b]Significantly different from control at 1% level.
[c]Mean of all other treatments significantly greater than control.

FAA + picric acid gave recoveries of *M. incognita, T. claytoni,* and *H. dihystera* as high or higher than nontreated controls (Tables 2, 3). Adding cold preservative chemicals directly to the soil caused some distortion in nematodes (Fig. 2). However, the morphological detail of specimens from either of the latter treatments was usually better than in those receiving a pretreatment with hot water followed by addition of fixative or Formalin. Numbers of *M. incognita* and *H. dihystera* in treated soil changed less with time in storage at room temperature than did nontreated controls. Concentrations of NaN_3 as low as 15-20 ppm killed all stages of *H. glycines*, as well as other nematodes tested as measured by bioassays.

It was necessary, however, to increase the concentration of NaN_3 to 2% to kill all soil-inhabiting fungi and bacteria (Table 3). Formalin (5%) or FAA + picric acid solution also gave no microbial growth in the dilution plates. Phenoxyethanol failed to kill most fungi and bacteria and resulted in low nematode recoveries (data not given).

DISCUSSION

Most of the methods tested for killing and preserving nematodes in soil offer some promise. The use of methyl bromide with subsequent freezing is satisfactory, but time-consuming, and the quality of specimens recovered is variable.

Some nematodes, such as *M. incognita*, were easily killed by exposure to microwaves. *H. glycines*, however, was extremely resistant to this treatment. The basic structure of the cyst is probably responsible for this. Species of this genus have been found to be very resistant to ultrasonics (7) and gamma irradiation (10). Townshend (9) found that gamma irradiation stimulated emergence of *H. schachtii* larvae in some treatments. A similar phenomenon could be responsible for the increased number of

TABLE 3. Effect of concentration of NaN_3 on recovery of *Meloidogyne incognita* and residual microflora in soil.

Concentration of NaN_3	No. larvae/500 cc of soil[a]	Residual microflora[b]	
		Fungi	Bacteria
Experiment I:			
1.0%	2800[c]	–	++
0.1%	2488[c]	+	++
0.01%	2675[c]	++	++
0 (control)	1394	++	+++
LSD .01	751		
Experiment II:			
4%	10,100[c]	–	–
2%	7,917	–	–
1%	9,817[c]	–	+
0 (control)	5,892	++	++
LSD .01	3,810		

[a]Preserved and living nematodes (control) extracted by sugar-flotation sieving after storage for 1 month (Appling loamy sand).
[b]Based on relative growth of various soil-inhabiting fungi and bacteria on dilution plates containing potato-dextrose agar (for fungi) and nutrient agar (for bacteria) from soil stored 1 month. Minus (–) = no growth; + = slight growth; ++ = moderate growth; +++ = much growth.
[c]Significantly different from control at 1% level.

cysts of *H. glycines* that developed in our bioassay soil (4.5% moisture) exposed to microwaves for 2 min.

The drastic effects of soil type and soil moisture on the effectiveness of microwave energy in killing cyst nematodes make this method unreliable with the exposure times tested. The findings of Baker and Fuller (2) showed that soil type and soil moisture also greatly affected the rate of kill of plant-pathogenic fungi which was partially due to differences in soil temperatures. The lower rate of kill in saturated soil incubated at 15 C, as compared to soil treated immediately after saturation (Fig. 1-A) apparently was due to the soil having a lower initial temperature in this treatment. A longer exposure time than that used in this study would be required for this method to be a reliable regulatory procedure. Such extended exposures would reduce the numbers of identifiable nematodes, since 4.5-min exposures drastically reduced the numbers of *M. incognita* larvae recovered. Based on our work with *H. glycines*, and that of Baker and Fuller (2), the prospects of microwave energy becoming a practical means of nematode control (1) are not encouraging.

The most uniform and reliable results were obtained by simply adding chemical preservatives directly to the soil. In addition to providing identifiable specimens when extracted by sugar-flotation methods, the addition of 2% NaN_3, 5% Formalin, or FAA + picric acid actually resulted in increased recoveries of nematodes as compared to nontreated controls in most cases. This increased recovery probably is due to individual dead specimens having an increased buoyancy when placed in sugar solution. Although adding chemicals such as Formalin directly to the soils causes some distortion of the nematodes, they may be identified readily. These chemicals affected the morphological characters of the nematodes less than exposure to methyl bromide or to microwaves. The use of hot chemicals reduces the distortion (5).

Thus, 5% Formalin or fixative not only kills and preserves nematodes in soil, but such treatments also kill fungi and bacteria. Such treatments may be used to stabilize nematode populations in storage prior to extraction (5). They should be of equal value for treatment of nematode-infested soil samples moving from areas under quarantine or in international shipments. The high concentration (2%) of NaN_3 required for soil sterilization is too hazardous for routine work. NaN_3, therefore, is not recommended for this purpose.

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