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An Alternative Gelling Agent for Culture and Studies of Nematodes, Bacteria, Fungi, and Plant Tissues

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Abstract: Pluronic F127 polyol, a block copolymer of propylene oxide and ethylene oxide, was studied as an alternative to agar in culture media for nematodes, bacteria, fungi, actinomycetes, and plant tissues or seedlings. At a polyol concentration of 20% w/v, the culture media, semi-solid at room temperature (22 C) but liquid at lower temperatures, had minimal effects on the test organisms. Most of the fungi and bacteria grew as well in 20% polyol as in 1.5% agar media; however, various species of nematodes and plant seedlings or tissues exhibited differential sensitivities to different concentrations of the polyol. In cases where the organisms were unaffected, the polyol media had certain advantages over agar, including greater transparency and less contamination under nonaseptic conditions. Polyol media have potentially greater ease for recovery of embedded organisms or tissues inside the media by merely shifting to lower temperatures.

Key words: plant tissue culture, pluronic polyol, polyglycol, soil micro-organism, solidifying agent.

The study of nematode-plant interactions would be facilitated by the availability of a transparent synthetic substrate in which both nematodes and roots could be observed directly. Water agar has served this purpose, but it has the disadvantage that plant roots or nematodes in the interior of the agar may not be observed or recovered easily. Also, deliberate embedding of intact nematodes or plant tissues is not possible without raising the temperature to 45 C, which is detrimental to many nematodes (10). Pluronic F127 polyol, a block copolymer of propylene oxide and ethylene oxide, can liquefy and gel at temperatures not harmful to nematodes. The exact temperature of phase transition depends on the polyol concentration (6). At certain concentrations, a solution of pluronic polyol is a gel at room temperature but a liquid at a lower temperature. Such a characteristic may have many applications in nematology, plant pathology, and microbiology. For example, the polyol could be used as an embedding and solidifying agent in culture media for enrichment, isolation, and maintenance of plant-parasitic nematodes, pathogens, or other soil micro-organisms. The objectives of this study were 1) to determine by using selected test organisms a concentration of polyol that could maintain a semisolid medium while having minimal effects on the growth of plants and micro-organisms, and 2) to evaluate the suitability of such a concentration of polyol as a general gelling substrate for in vitro culturing of nematodes, soil micro-organisms, and plant seedlings. The initial test organisms used were the bacterium *Bradyrhizobium japonicum,* the nematode *Meloidogyne incognita,* the fungus *Botrytis cinerea,* and the plant species *Lemna minor, L. obscura, Lycopersicon esculentum* cv. Rutgers, *Medicago sativa* cv. Drummer Boy, and *Avena sativa* cv. Astro. The duckweeds were selected for the experiment because they are indicator plants used in phytotoxicity studies (3).

Materials and Methods

Preparation of polyol media: Pluronic F127 polyol (MW 12,500) was obtained from BASF Wyandotte (Parsippany, NJ). The polyol was placed in water or the desired liquid medium and left overnight in a refrigerator at 4 C to ensure complete dissolution. It was sterilized when necessary by autoclaving at 121 C for 15 minutes, chilled to 4 C, dispensed, and then warmed to the incubation temperature. When the medium was in a liquid or semisolid phase it was inoculated with various organisms.

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Organisms: Bradyrhizobium japonicum strains I110 and 32H1 were obtained from Nitragin Company (Milwaukee, WI), and *Pseudomonas syringae* was from Dr. S. Beer, Cornell University. *Bursaphelenchus xylophilus* and *Aphelenchus avenae,* originally obtained from Dr. R. I. Bolla, University of Missouri, St. Louis, and Dr. B. Tsai, University of California at Riverside, respectively, were maintained on *Botrytis cinerea* on potato dextrose agar. *M. incognita,* race 1, was maintained in Rutgers tomato plants in the greenhouse, whereas *Pratylenchus penetrans,* originally from an apple orchard in Wayne County, New York, was propagated in Drummer Boy alfalfa callus. *Caenorhabditis elegans* strain N2 was obtained from Dr. K. Kemphues at Cornell University. Other fungi and actinomycetes were obtained from the Cornell Teaching Collections. Seeds of most plants were obtained from W. Atlee Burpee Company (Fresno, CA); seeds of *Glycine soja* were obtained from Dr. J. S. Huang, North Carolina State University. The duckweeds *L. minor* and *L. obscura* were obtained from Dr. G. R. Leather, USDA at Frederick, Maryland. Callus tissues of garlic (*Allium sativum*) were obtained from V. Bringi, Cornell University, or started from seeds growing in BDS nutrient agar supplemented with 1 mg/liter 2,4-D (2).

Growth of micro-organisms and plant tissues: Bradyrhizobium japonicum was cultured in yeast extract mannitol broth, and colony-forming units (CFU) were subsequently enumerated by the dilution plate method on yeast extract mannitol agar or polyol medium (9). Fungal plugs were grown in polyol or agar media containing 10% cornmeal, potato dextrose, or V-8 juice (8). *C. elegans* was cultured on *Escherichia coli* strain OP50 (1), *B. xylophilus* and *A. avenae* were propagated on *B. cinerea,* and *P. penetrans* was on alfalfa callus in White's (7) agar or polyol medium. Duckweeds were cultured in E medium (3). Most of the growth studies were performed in 10-cm-d or 6-cm-d plastic petri dishes. Plant tissues were grown either in modified White's (7), Gamborg's B5 (5), or BDS (2) media. Increase in numbers (bacteria, nematodes, and duckweeds) or culture diameter (fungal or actinomycete mycelia and plant tissues) was measured at appropriate time intervals.

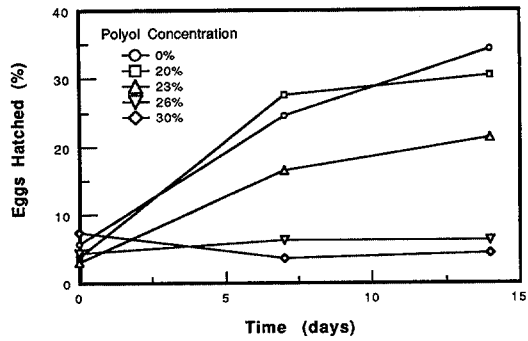

FIG. 1. Effect of Pluronic F127 polyol concentration on hatching of *Meloidogyne incognita* eggs. Each point is a mean of five replicates.

Germination of seeds: Seeds were surface sterilized for 5 minutes consisting of 20% commercial bleach, 10% alcohol, and 70% water. The seeds were placed in 2.5 ml polyol solution at 10 C in 6-cm-d petri dishes which were then incubated at 22 C. Germination on Whatman paper (No. 1) moistened with an equal volume of water served as controls. In some cases, the seeds were germinated and grown in polyol or agar medium supplemented with ⅛ strength of Hoagland's solution (9).

Hatching and responses of nematodes: Experiments were conducted in 3.5-cm-d or 6-cm-d plastic petri dishes containing 2.5 or 10 ml of various concentrations of polyol, respectively. *M. incognita* eggs or juveniles (J2) (1,000/ml) were suspended in the liquid phase of the polyol, and the petri dishes were incubated at 25 C (semisolid phase). In some cases, egg hatching in 1.5% agar was used as a control. Egg hatching was estimated by counting the number of empty egg shells. Juvenile responses were estimated by counting intact, coiled, and distorted J2. A J2 was considered coiled if the length of its body formed a loop of 360° and distorted if it lost its structural integrity and shriveled.

Diffusion of chemicals: Dry paper discs (0.5 cm) impregnated with 0.1% $HgCl_2$ in water, 1 ppm avermectin consisting of 80% B_{1a}

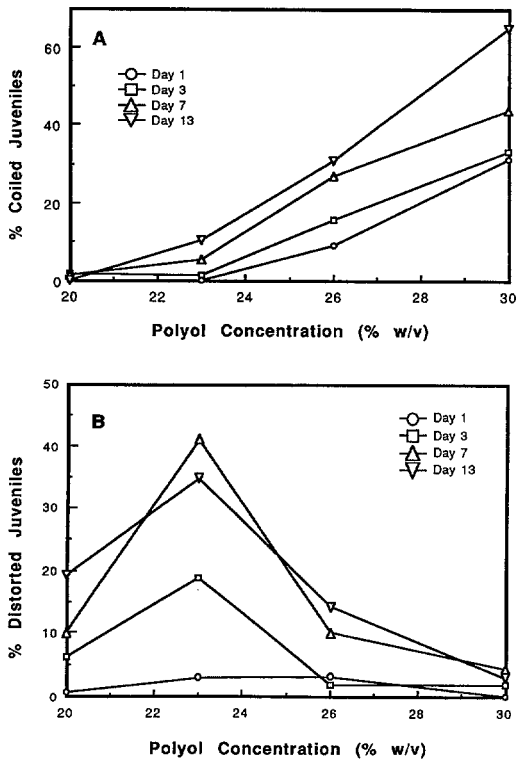

FIG. 2. Effect of Pluronic F127 polyol concentration on *Meloidogyne incognita* juveniles (J2). A) Proportion of coiled J2. B) Proportion of distorted J2. Each point is a mean of five replicates.

and 20% B_{1b} (Merck and Company, Philadelphia, PA) in chloroform, or 100 ppm oxamyl in methanol were air dried. The discs were placed on top of 20% w/v polyol plates (semisolid) in which *M. incognita* eggs (1,000/ml) had been embedded under nonaseptic conditions. Live nematodes or empty egg shells were counted after incubation at 22 C for 1 and 7 days.

RESULTS

Effects on nematodes: No significant difference ($P = 0.05$) was observed between the proportion of eggs hatched in 20% w/v polyol and in 1.5% agar (Figs. 1, 2). Egg hatching was inhibited at polyol concentrations of 23% and above (Fig. 1). *M. incognita* J2 embedded in the polyol exhibited three types of responses: no observable changes, coiling, and distortion. Usually, coiled J2 were not distorted during the span of the experiment.

The proportion of coiled J2 increased with increased polyol concentration and time of exposure (Fig. 2A). At 20% polyol, few J2 coiled but some were distorted (Fig. 2A, B). The maximum proportion of distorted J2 occurred at 23% polyol. At higher polyol concentrations (i.e., 26% and above), the proportion of distorted J2 was relatively low (Fig. 2B); however, the high proportion of coiled J2 (Fig. 2A) indicated that coiling somehow protected the structural integrity of the nematode. Most J2 were immobilized by the high viscosity of 30% polyol.

Effects on bacteria and fungi: The colony-forming units of 3-day-old cultures of *B. japonicum* were determined in media containing 0% (1.5% agar), 20%, 25%, or 30% polyol (Table 1). In general, bacterial colonies became visible more slowly in the polyol media than in the agar medium; however, the numbers of colony-forming units after 10 days at 22 C were not significantly affected by the polyol concentrations. Radial growth of *B. cinerea* in polyol potato dextrose or *Phytophthora infestans* in V-8 media was not affected until polyol concentrations exceeded 25% (data not shown).

Effects on plants: As with *M. incognita* egg hatch, increasing polyol concentration inhibited duckweed proliferation (Fig. 3). At 10% polyol, the two species of duckweed

TABLE 1. Effect of Pluronic F127 polyol concentration on the number of colony-forming units ($\times 10^{-6}$) of *Bradyrhizobium japonicum* strains.

Strain	Polyol concentration			
	0%	20%	25%	30%
32H1	65.0 ± 8.2 a	65.0 ± 6.6 a	56.0 ± 4.5 a	67.3 ± 6.1 a
I110	102.3 ± 11.0 b	87.7 ± 12.5 b	106.3 ± 8.5 b	90.0 ± 17.8 b

Numbers followed by the same letters within a row are not significantly different ($P = 0.05$) according to Duncan's new multiple-range test.

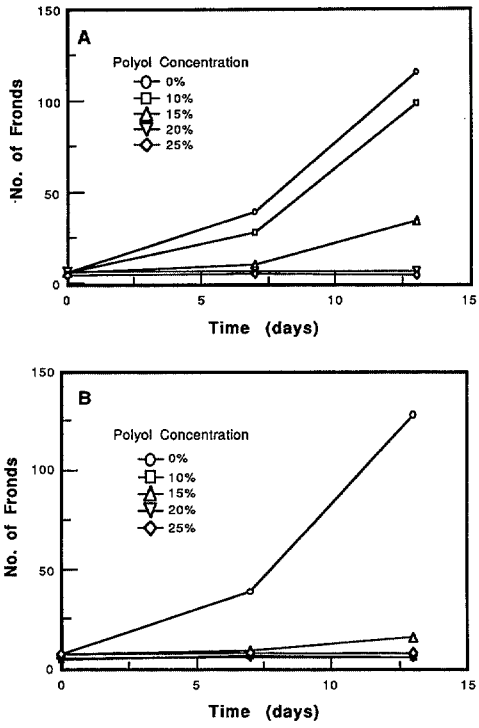

FIG. 3. Effect of Pluronic F127 polyol concentration on frond production by duckweeds. A) *Lemna minor*. B) *Lemna obscura*. Each point is a mean of five replicates.

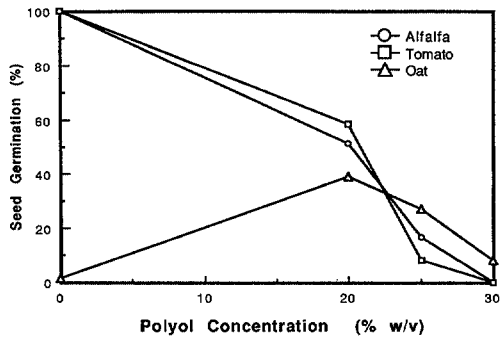

FIG. 4. Effect of Pluronic F127 polyol concentration on seed germination of alfalfa (*Medicago sativa* cv. Drummer Boy), oat (*Avena sativa* cv. Astro) and tomato (*Lycopersicon esculentum* cv. Rutgers) after 10 days at 22 C.

exhibited a differential sensitivity to the polyol, with *L. obscura* more sensitive than *L. minor*. With either species, a polyol concentration of 15% or above was inhibitory.

High concentrations of the polyol usually inhibited seed germination, with the extent of inhibition depending on the species. For tomato and alfalfa, seed germination after 10 days decreased with increasing polyol concentration. Oat seed germination was better in 20% polyol than in moistened Whatman paper during the same period (Fig. 4).

Growth of other micro-organisms and plant seedlings: The bacteria *A. rhizogenes* and *P. syringae* were greater in number in the polyol medium than in the agar medium (Table 2). Bacterial colonies were more uniform in size and shape in the polyol medium than in agar. The actinomycete *Streptomyces scabies* grew in 20% polyol medium and in agar medium. Depending on the species, nematodes were or were not sensitive to 20% polyol. *A. avenae* grew better in polyol, whereas *B. xylophilus* and *C. elegans* grew better in agar. Of particular interest was the fact that the plant-parasitic nematode *Pratylenchus penetrans* reproduced on alfalfa callus equally well in polyol and agar medium. Fungi grew at least as well in 20% polyol as in 1.5% agar media (Table 3). In general, there was less aerial growth, radial striation, and transverse zonation for the mycelium in polyol than in agar.

Similar to the effects on the test plants (Fig. 4), 20% polyol inhibited germination of bird's-foot trefoil, cabbage, marigold, mungbean, and radish seeds but enhanced germination of beet and okra seeds (Table 4). Seeds of millet and wild soybean (*Glycine soja*) grew in 20% polyol for 4 weeks under nonsterile conditions in diluted Hoagland's solution with 70 ppm NO_3^- (Fig. 5).

Suitability for plant tissue culture: Alfalfa seedlings formed callus in Gamborg's B5 medium in polyol as well as in agar media. Garlic callus tissues derived from different species in BDS medium (2) showed a differential sensitivity to 20% polyol. Callus from cultivated Elephant garlic grew as well in polyol as in agar, whereas callus from wild garlic was inhibited by polyol (Fig. 6). Calli from various onion cultivars in BDS medium also exhibited a similar differential sensitivity to the polyol (data not shown).

Suitability of polyol as a substrate for diffusion: After 2 weeks, 20% polyol was less

TABLE 2. Comparison of growth of various organisms in agar and Pluronic F127 polyol media at 22 C.

	Unit of growth measurement	Growth	
		20% gel	1.5% agar
Agrobacterium rhizogenes†	CFU × 10^{-6}	103 a	72 b
Pseudomonas syringae†	CFU × 10^{-6}	46 a	18 b
Streptomyces scabies‡	Radial growth (cm)	26 a	33 a
Aphelenchus avenae§	Number	238 a	81 b
Bursaphelenchus xylophilus§	Number	3,355 a	16,625 b
Pratylenchus penetrans‖	Number	753 a	761 a
Caenorhabditis elegans¶	Number	440 a	2,396 b

Numbers followed by the same letters within a row are not significantly different ($P = 0.05$) according to Student's *t*-test.
† Nutrient broth medium, growth after 5 days.
‡ Yeast malt dextrose medium, growth after 14 days.
§ *Botrytis cinerea* on potato dextrose medium, growth after 2 weeks, Pi = 5.
‖ Alfalfa callus in White's medium, growth after 5 weeks, Pi = 50.
¶ *E. coli* in Nematode Growth medium, growth after 10 days, Pi = 5.

prone to contamination than 1.5% agar (data not shown) and was therefore a suitable substrate for examination of diffusion of chemicals under nonaseptic conditions. Water soluble chemicals readily diffused from the discs into the polyol gel and inhibited hatching of root-knot nematode juveniles from the eggs (Table 5). In addition, an outward progression of zones of inhibition from disc impregnated with rifampicin (250 μg/ml) or streptomycin sulfate (500 μg/ml) against a lawn of *A. rhizogenes* or *B. japonicum* were observed (data not shown).

DISCUSSION

The selected test organisms indicated that increased concentration of the polyol caused increased detrimental effects on nematodes, fungi, plant tissues, and, to a lesser extent, bacteria. The minimal polyol concentration (20%) that gels at room temperature inhibited some organisms, particularly *M. incognita* and duckweed; however, some other nematode species such as *P. penetrans* and plant tissues such as Elephant garlic grew in 20% polyol media without observable detrimental effects. The reasons for the sensitivity are not clear. One of the possibilities may be related to the water binding properties of the polyol gel, since the coiling response of *M. incognita* J2 appeared similar to the anhydrobiotic coiling described in other nematodes (4). These phenomena themselves are worth studying because the polyol, being a hy-

TABLE 3. Comparison of growth of various fungi in agar and Pluronic F127 polyol media at 22 C.

Fungi†	Days after inoculation	Radial growth (cm)	
		20% polyol	1.5% agar
Alternaria solani	2	3.2 ± 0.1	2.0 ± 0.2
Botrytis cinerea	2	4.5 ± 0.1	4.1 ± 0.2
Colletotrichum spp.	9	4.6 ± 0.3	4.0 ± 0.3
Fusarium oxysporum	2	2.9 ± 0.1	2.6 ± 0.1
Macrophomina phaseolina	1	3.2 ± 0.2	2.0 ± 0.1
Monilinia fructicola	4	4.7 ± 0.3*	3.4 ± 0.2*
Phytophthora cactorum	2	5.6 ± 0.1*	3.1 ± 0.1*
Rhizoctonia solani	2	7.3 ± 0.1*	9.0 ± 0.2*
Sclerotinia minor	2	5.2 ± 0.1	4.9 ± 0.1
Trichoderma sp.	2	4.6 ± 0.2	4.1 ± 0.2
Verticillium sp.	1	5.4 ± 0.3*	3.3 ± 0.1*

Each number is the mean of eight replicates and standard deviation.
* Significantly different at $P = 0.05$ according to Student's *t*-test.
† All fungi were grown on potato dextrose medium except *P. cactorum* which was grown on V-8 juice medium.

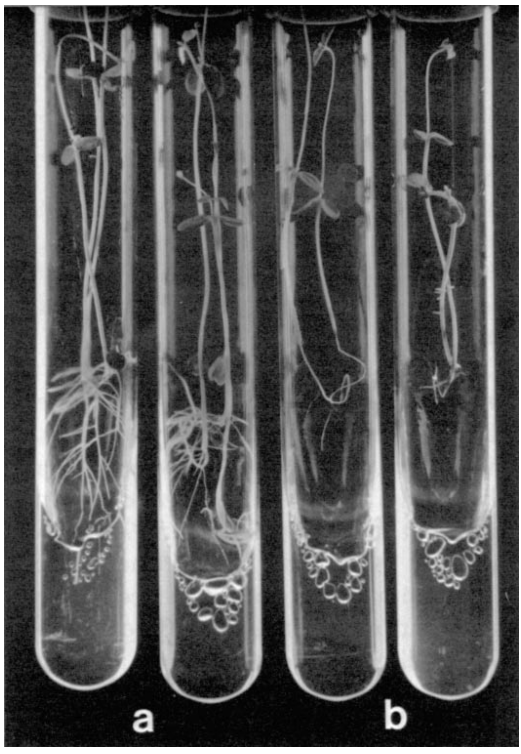

FIG. 5. Growth of seedlings of *Glycine soja* in Pluronic F127 polyol medium after 4 weeks. The medium was prepared in Hoagland's solution, ⅛ strength, with (a) and without (b) 70 ppm NO_3^-.

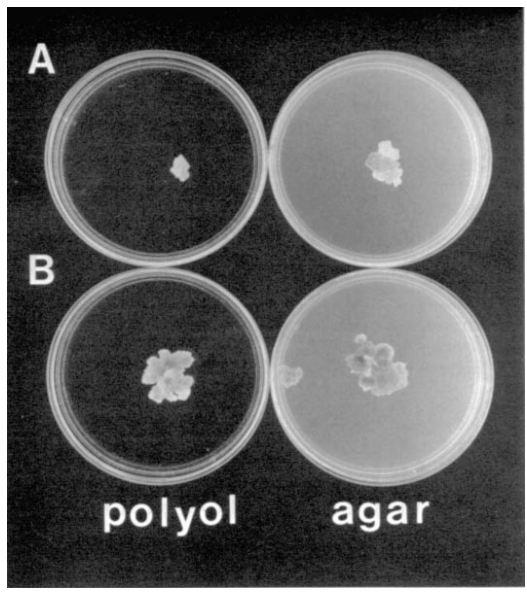

FIG. 6. Comparison of growth of garlic callus in BDS medium containing 20% Pluronic F127 polyol and 1.5% agar after 4 weeks at 22 C. A) Wild garlic. B) Cultivated garlic (*Allium sativum* cv. Elephant).

groscopic compound like polyethylene glycol, might be used to elucidate the mechanisms of drought resistance for plants and survival for nematodes. The sensitivities of nematodes to the polyol may also be related indirectly to the quality of growth of plant tissues or micro-organisms upon which the nematodes fed, or to the presence of toxic or inhibitory metabolites resulting from metabolism of the polyol components by these organisms. Further examination of these possibilities is needed.

Water soluble molecules diffused readily

TABLE 4. Comparison of seed germination percentages on 20% Pluronic F127 polyol and Whatman paper No. 1.

Plant	Germination after 6 days at 22 C	
	20% polyol	Whatman paper
Beet (*Beta vulgaris* cv. Perfect Detroit)	51.2 ± 8.2*	7.8 ± 0.9*
Bird's-foot trefoil (*Lotus corniculatus* cv. Empire)	4.8 ± 0.6*	27.6 ± 3.1*
Cabbage (*Brassica oleracea* var. *capitata* cv. Titanic)	52.8 ± 6.8*	71.0 ± 10.3*
Corn (*Zea mays* cv. Yellow Golden Cross)	85.2 ± 8.8	70.6 ± 8.3
Marigold (*Tagetes* sp.)	48.4 ± 6.1*	92.0 ± 5.8*
Mungbean (*Vigna radiata* cv. Berken)	40.0 ± 3.2*	100.0 ± 12.0*
Okra (*Abelmoschus esculentus* cv. Emerald)	41.0 ± 4.2*	7.0 ± 0.8*
Pearl millet (*Pennisetum glaucum* cv. Gahi)	96.6 ± 1.9	98.0 ± 2.0
Radish (*Raphanus sativus* cv. Champion)	3.5 ± 0.4*	7.0 ± 0.8*
Sudan grass (*Sorghum* × *Drummondii* var. *sudanense* cv. Piper)	14.0 ± 2.0	14.0 ± 1.5
Tobacco (*Nicotiana tabacum* cv. Sampson)	97.0 ± 1.4	96.6 ± 1.1
White clover (*Trifolium repens* cv. Regal)	70.8 ± 8.0	88.0 ± 6.2

Each number is the mean of five replicates and standard deviation.
* Significantly different at $P = 0.05$ according to Student's *t*-test.

TABLE 5. Diffusion of selected chemicals through Pluronic F127 polyol medium, as determined by their effects on hatching of *Meloidogyne incognita* eggs.

Chemical	Hatching percentage	
	1 day	7 days
Avermectin†	2.2 ± 0.6	2.1 ± 0.4
$HgCl_2$	3.1 ± 0.7	3.0 ± 0.4
Oxamyl	0.9 ± 0.1	2.3 ± 0.5
H_2O	2.9 ± 0.5	38.7 ± 8.6

Each number is the mean of four replicates and standard deviation.

† Mixture of 80% avermectin B_{1a} and 20% avermectin B_{1b}.

into and through the polyol gel. Because of the different physical and adsorptive properties of polyol and agar, diffusion rates in polyol and agar may be different. The relative ease of diffusion of biologically active metabolites from organisms growing in these two substrates should be evaluated if either substrate is to be used for the detection of such metabolites.

The clarity of the polyol and its semisolid characteristics allow not only colonies of bacteria and spores of fungi but also individual nematodes to be selected and observed with ease. Gardener and Jones (6) have used polyol media to isolate and enrich denitrifying bacteria, sulfate reducers, and methanogens.

The ability of some plant-parasitic nematodes and plant tissues to grow in polyol media has raised several possibilities. First, nematodes or plant tissues in the polyol potentially can be recovered with ease by sieving at cooler temperatures, at which the media liquefy. Second, additives such as plant hormones or antibiotics can be dispersed uniformly into the liquefied medium at some point during the experiment by lowering the incubating temperature. Responses of the nematodes or plant tissues may then be studied after resumption of the previous incubating temperature. Variants of nematodes or plant tissues resistant to the additives (e.g., a toxin) may also be selected subsequently.

Some of our experiments were conducted under nonsterile conditions (e.g., the egg hatching experiment). This was possible because polyol contained only a low level of impurities. This characteristic should facilitate the observation of nematodes without interference by contaminating micro-organisms under nonaseptic conditions for an extended period, unlike the case with agar. Interaction between the nematodes and the host and (or) other micro-organisms may also be observed in situ.

Although the polyol has the stated advantages over agar as a solidifying agent, it also has several important drawbacks. It is water soluble, and it forms only a semisolid gel. The gel may be strengthened somewhat by the addition of 5% saccharides such as glycerol (R. Heffner, BASF, pers. comm.), but the side effect of such manipulation is not known. The most important disadvantage, however, is that the polyol may have adverse effects on some nematodes and plant tissues. Therefore, the potential for employing polyol in the growth medium for a particular organism exists, but the interaction between the organism and the polyol must be studied in detail before the system can be used extensively for other biological studies.

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