

RESEARCH NOTES

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Efficient Preservation of Root-knot and Cyst Nematodes in Liquid Nitrogen¹

A. C. TRIANTAPHYLLOU,² AND E. MCCABE³

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Temperatures below -130 C (the recrystallization point of ice) are known to assure long-term, and possibly indefinite, preservation of certain biological specimens (8,9). Successful cryopreservation, however, almost always requires 1) an appropriate pretreatment of the specimens with substances (cryoprotectants) that minimize intracellular and (or) intercellular crystal formation, 2) a precisely controlled rate of cooling of the specimens, at least during the early stages of freezing, and 3) a controlled rate of thawing. Different organisms usually require different protocols for pretreatment and speed of cooling and thawing, and these protocols are established empirically.

Successful cryopreservation in liquid nitrogen has been reported for free-living and mycophagous nematodes of the genera *Panagrellus*, *Turbatrix*, *Caenorhabditis*, and *Aphelenchoides* (3) using dimethylsulfoxide (DMSO) as a cryoprotectant. The same cryoprotectant was not as successful with juveniles of the plant-parasitic nematode *Ditylenchus dipsaci* and was unsatisfactory for second-stage juveniles (J2) of a

Meloidogyne spp. (6). A solution of glycerol with certain salts is routinely used for cryopreservation of *Caenorhabditis elegans* (7), but the method is not effective for preservation of *Meloidogyne* spp. (unpubl.).

Ethylene glycol has been known as an efficient cryoprotectant at least since 1952 (5). In 1981, Ham et al. (2) and James (4) reported a simple technique that uses ethylene glycol as an internal cryoprotectant for cryopreservation of the animal parasites *Schistosoma mansoni* and *Onchocerca microfilariae*. The same technique tested later by Bridge and Ham (1) on *Meloidogyne graminicola* J2 proved to be moderately effective. Basically, the technique involves incubation of the J2 in 10% ethylene glycol at 37 C for 15 minutes followed by incubation in 40% ethylene glycol at 0 C for 30-45 minutes. Subsequently, a drop of J2 suspension is spread on a coverslip sliver which is plunged directly into liquid nitrogen. For thawing, the coverslip slivers are transferred from the liquid nitrogen to vials containing water at 37 C.

We have tested various modifications of the same technique on J2 and eggs of several *Meloidogyne* and *Heterodera* species in order to develop a more efficient and practical method for long-term cryopreservation of valuable collections and genetic stocks of these nematodes. We have studied the effects of the length of exposure of J2 and eggs to various concentrations of ethylene glycol at 37 C and at room temperature (23-27 C) on the viability and infectivity of J2 after freezing. Similarly, we have experimented with various methods of supporting the J2 during freezing in an

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² Professor, Department of Genetics, North Carolina State University, Raleigh, NC 27695.

³ Research Technician, Department of Plant Pathology, North Carolina State University, Raleigh, NC 27695.

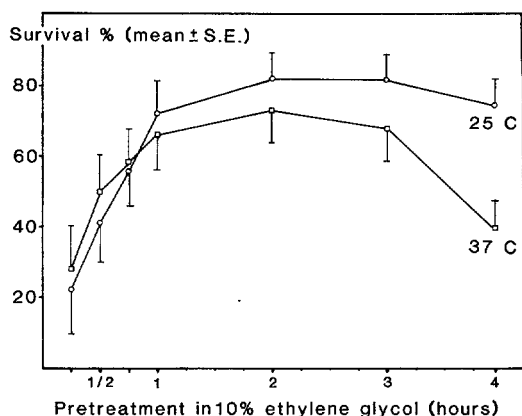


FIG. 1. Survival of *Meloidogyne incognita* second-stage juveniles pretreated in 10% ethylene glycol at 25 or 37 C for various time intervals before they were frozen in liquid nitrogen according to the procedure recommended in this paper. Survival (relative to the control) is expressed as the percent of moving juveniles 12 hours after thawing.

effort to find a substitute for the delicate coverslip slivers used by previous investigators. In summary, the results of these investigations are as follows:

1. Pretreatment with a concentration of 10% ethylene glycol resulted in better J2 survival than did concentrations of 5, 15, or 20%.

2. One to three hours of exposure of J2 to 10% ethylene glycol gave better results both at 37 C and at room temperature than did shorter or longer exposures (Fig. 1). Also, pretreatment at room temperature gave slightly better J2 survival than pretreatment at 37 C.

3. None of the above pretreatments gave satisfactory results with untreated eggs or eggs treated for 5, 10, 15, 20, or 30 minutes in 0.5% NaOCl to dissolve egg membranes and facilitate permeation by ethylene glycol. Only occasionally did a few J2 (less than 0.1%) hatch from frozen eggs after thawing.

4. Strips of chromatography paper or thick filter paper proved to be satisfactory for supporting J2 during freezing. Glass hematocrit tubes or plastic straws (ca. 1 mm i.d.) were also satisfactory but resulted in a slightly reduced rate of J2 survival compared with paper strips.

5. Thawing in water at room tempera-

ture gave a slightly better rate of J2 survival than did thawing at 37 C.

A description of the recommended procedure for freezing and thawing *Meloidogyne* and *Heterodera* juveniles follows.

1. A concentrated suspension of freshly hatched J2 is transferred to a small (1.5–2 ml), preferably round bottom glass vial. After the J2 have settled to the bottom, the excess water is removed with a fine-tipped pipet.

2. A small quantity of 10% (v/v) ethylene glycol (about five times the volume of the J2) is added to the vial at room temperature.

3. One to three hours later the vial is transferred to a bath of crushed ice and an equal quantity of ice cold 70% ethylene glycol is added to the vial so that the final concentration of ethylene glycol is about 40%.

4. Forty to sixty minutes later, about 70 μ l of J2 suspension is drawn from the vial with a micropipet and delivered onto a strip of chromatography paper (about 6 \times 41 mm) held from one end with a pair of fine-point, preferably self-locking, forceps.

5. The strip of paper is quickly submerged into liquid nitrogen contained in an open styrofoam container (Fig. 2). After a few seconds, when bubbling of the liquid nitrogen ceases around the forceps, the paper strip is lowered into a 1.5-ml cryogenic vial which had been placed earlier into the liquid nitrogen, inside a larger vial. The larger vial is attached to the lower end of a piece of heavy wire or plastic rod that serves as a handle (Fig. 2). Several paper strips supporting frozen J2 can be accommodated in the same cryogenic vial before the vial is transferred to a holder of a liquid-nitrogen freezer for long-term storage. The transfer must be done quickly to ensure that the paper strips inside the small cryogenic vial are constantly immersed in the rapidly evaporating liquid nitrogen.

6. Thawing is carried out by quickly transferring a paper strip from the liquid nitrogen into a vial containing about 20–30 ml of water at room temperature. (Note: Unless the paper strip is submerged in water within 10–15 seconds after it has been re-

moved from the liquid nitrogen, slow thawing takes place and the J2 die.) Live J2 start moving within 1 hour from thawing and can be used immediately as inoculum for propagation on a host plant.

This procedure consistently gave 50–90% survival of J2, as determined by the number of J2 that were moving 12 hours after thawing.

Bioassays were carried out by inoculating tomato or soybean seedlings, respectively, with J2 of *Meloidogyne* spp. or *Heterodera glycines* that had survived a few seconds to 2 hours freezing. These tests demonstrated that overall infectivity and development of such J2 was about 30–50% lower than that of nonfrozen J2 (controls), as determined by the number of adult females recovered from roots inoculated with a given number of J2. When *Meloidogyne* spp. J2 obtained from single paper strips stored for 3–6 months in liquid nitrogen were used as inoculum, they abundantly infected tomato roots and developed to adult, egg-producing females. About 25 populations tested in this manner gave 30–200 egg-producing females within 45 days from inoculation (first generation). Thus a single paper strip, prepared according to the procedure described here, provided a sufficient number of infective J2 for safe establishment of a greenhouse culture.

Satisfactory results were obtained with *Meloidogyne incognita*, *M. javanica*, *M. arenaria*, *M. hapla*, *M. microtyla*, *M. hispanica*, and *M. microcephala*, as well as *Heterodera glycines*, *H. schachtii*, and *H. trifolii*.

Based on other reports (8,9) and the principle that there is no biological activity at -196°C , the temperature of liquid nitrogen, we predict that freezing for at least 10 and possibly 100 years or more may not substantially reduce J2 survival and infectivity. Also, genetic damage caused by natural levels of background ionizing radiation is expected to be very low.

Supplementary information

We used 1.5-ml cylindrical cryogenic vials or ampules (internal dimensions 10×40 mm) that can accommodate about six paper strips. The paper strips were cut out

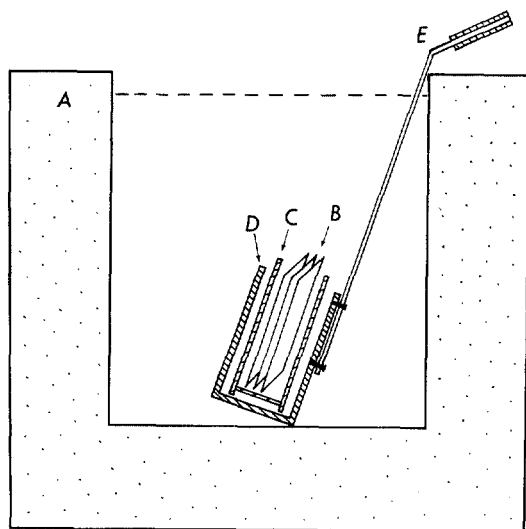


FIG. 2. Simple apparatus for freezing nematodes. Styrofoam container (A) filled with liquid nitrogen. Paper strips (B) supporting the nematodes are inside a cryogenic vial (C) that is resting in a larger vial (D) equipped with a handle (E).

of a thick "Whatman 3 mm Chr" chromatography paper available in rolls 38 mm wide. The cuts were made at an approximate 30-degree angle in order to produce rectangular strips about 0.6 mm wide and 41 mm long, i.e., 1 mm longer than the depth of the vials. The extra length of the strips facilitates later removal of individual strips using fine-point forceps and keeps the caps loosely screwed on the vials allowing free exchange of liquid nitrogen or nitrogen vapors between the interior and the exterior of the vial during storage in the liquid nitrogen freezer. Vials of different size and corresponding sizes of paper strips may be used, depending on the storage system of the liquid nitrogen freezer available in each lab. Also, different types of chromatography or filter papers may be satisfactory, provided that strips from such papers do not curl during freezing; they must remain flat for easy storage of several strips in each vial.

If only a few J2 are available for storage (e.g., fewer than 10,000), it may be difficult to estimate the amount of 10% ethylene glycol (about five times their volume) needed in step #2. In such cases, a larger amount of 10% ethylene glycol (e.g., 0.8 ml) can

be added and when the J2 have settled to the bottom of the vial, one-half of it (i.e., 0.4 ml) can be drawn out with a fine pipet. Then, in step #3, 0.4 ml of 70% ethylene glycol is added. In general, excessive dilution of the final J2 suspension should be avoided in order to have as many J2 as possible on each paper strip. It is also recommended that one or two paper strips from each sample be thawed immediately after freezing in order to test and record the rate of J2 survival of the sample.

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