

Freezing and Storing *Ditylenchus dipsaci* in Liquid Nitrogen

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Abstract: After 18 months of storage at -150°C , some larvae of *Ditylenchus dipsaci*, which had been treated in a 7.5% solution of dimethyl sulphoxide and cooled to -25°C before storage, were still viable on thawing. Some survivors penetrated and developed normally in stems of alfalfa seedlings. Tests showed that active larvae could be frozen directly, thus eliminating the need to use the quiescent stage of this nematode previously thought necessary for successful storage at cryogenic temperatures. The method described is suitable for long-term storage of *D. dipsaci* and may, with slight modifications, be used to preserve other plant-parasitic nematodes. **Key Words:** liquid nitrogen refrigeration, nematode survival, cold storage.

Over the years, techniques have been developed in which a few nematode species have survived for relatively short periods of from a few seconds to several days at extremely low temperatures of -77°C to -269°C (1, 3, 4, 5, 10, 11, 12, 13). In most experiments, the nematodes used were partially dehydrated or in a quiescent state before being subjected to the temperatures.

A long-term practical method for storing nematodes was developed by Hwang (6), in which four species of free-living and mycophagous nematodes were successfully stored for several months in a liquid-nitrogen refrigerator. Also, her method was used successfully to store a plant parasite, *Ditylenchus dipsaci* (Kühn) Filipjev (7). This paper details the physical conditions under which long-term storage of viable *D. dipsaci* was accomplished and outlines the method used after thawing to demonstrate that the larvae invaded alfalfa seedlings, matured and reproduced.

MATERIALS AND METHODS

Larvae and adults of *D. dipsaci* used in the freezing experiments were cultured on alfalfa "callus" (*Medicago sativa* L. 'Du Puits') by Krusberg's method (8). Nematodes were extracted from callus in a Baermann funnel,

rinsed in tap water, and suspended in 7.5% aqueous solution (v/v) of dimethyl sulphoxide (DMSO) just prior to freezing. A 0.2-ml suspension of the nematodes, containing 150-500 larvae and adults, was pipetted into 1.2-ml glass ampoules, and the ampoules heat-sealed. Ampoules were placed for 10 min in the internal chamber of a prototype freezer which had been cooled to -5°C . Temperature was monitored by a programmed temperature controller (Canalco, Rockville, Md.). After the 10-min period, the temperature was lowered at a rate of $2^{\circ}\text{C}/\text{min}$ to -25°C . The frozen ampoules were placed immediately into a liquid-nitrogen bath and held until placed in a liquid-nitrogen refrigerator for long-term storage.

Nematodes were stored for 1, 30, and 540 days. After each storage period, nematodes were thawed at 38°C in the ampoules. The ice disappeared in approximately 30 sec. Ampoules were opened immediately, and nematodes were rinsed into 4 ml of Heller's solution (KCl-2.24 g; NaCl-1.2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -0.74 g; NaH_2PO_4 -0.33 g; CaCl_2 -0.26 g; Ferric chelate-0.1 g; distilled H_2O -1.0 liter; pH 5.7). After incubation for 24 h at 21°C , only active larvae were considered viable and counted. The same number of *D. dipsaci* were extracted from fresh cultures and used as controls in infectivity experiments.

Pathogenicity of surviving larvae was tested on 'Du Puits' alfalfa seedlings. Seeds were placed 2 cm apart and 1 cm from the top of a moist blotter (10×24 cm) (Fig. 1). Seeds and blotter were covered with paper toweling the same size as the blotter, and placed upright in a tray ($27 \times 31 \times 14$ cm). Each blotter was separated from another in the tray by a wall, of moist vermiculite 2 cm wide and 10 cm high. After 24 h at 18°C , blotters were removed from the trays, and the toweling folded down to expose germinating seeds. By a modification of Bingefors and Eriksson's

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method (2), viable nematodes, plus any debris resulting from freezing, were suspended in 1% hydroxyethyl cellulose, and in a 0.1-ml drop, containing 15-30 viable larvae, was pipetted onto a given seedling.

After seedlings had been inoculated and covered, blotters were again returned to the trays, which then were covered for 48 h at 18 C to provide high humidity and time for nematode penetration. Trays were uncovered for 7-14 days at 18 C under a light intensity of 2,690 lux for a 12-h day to allow for plant growth and for nematode disease development.

Seedlings that showed swelling at the base of petioles of cotyledons were counted as nematode-infected. All seedling tops were excised and stained in a 0.1% (w/v) cotton-blue and lactophenol mixture. Seedlings were destained in lactophenol and examined under a microscope at $\times 35$ magnification to determine if eggs and larvae were present.

RESULTS AND DISCUSSION

In a preliminary study, 7.5 and 10% (v/v) solutions of DMSO were equally effective as cryoprotective agents in preserving motile stages of *D. dipsaci* at -150 C. Without the DMSO treatment, no motile nematodes survived freezing. Also, the rate of cooling, from room temperature to -25 C, at approximately 2 C/min was an effective prestorage treatment that increased survival at cryogenic temperatures (7).

Survival rate at -150 C for 1, 30, and 540 days, averaged 35, 27, and 23% of the population, respectively (Fig. 2). In one experiment, as few as 6% of a population survived storage for 540 days, whereas in another experiment nearly 100% survived storage for 1 day.

In bioassay tests on alfalfa seedlings, some surviving nematodes penetrated the host, mated, and incited symptoms of the stem disease (Table 1). However, even though equal numbers of viable larvae were used, frozen nematodes did not incite stem disease symptoms as frequently as did freshly extracted nonfrozen larvae. Apparently, freezing injuries prevented some nematodes from entering seedlings or lowered their reproductive rate in seedlings. Part of the decline in incidence of stem disease might be attributed to the DMSO treatment, in that a substantial decrease was noted in disease incidence when nematodes were treated with DMSO without freezing (Table 1). However, in this latter treatment, nematodes used without freezing were subjected to DMSO at 21 C for 3 h, whereas frozen nematodes were subjected to DMSO at 21 C for less than 30 min. Frozen nematodes were placed in Heller's solution immediately after thawing, thus effectively reducing the time at room temperature in the DMSO concentrate. Because nematodes would be much more active metabolically at 21 C than at -150 C, they would be more subject to injury from DMSO at the higher temperature.

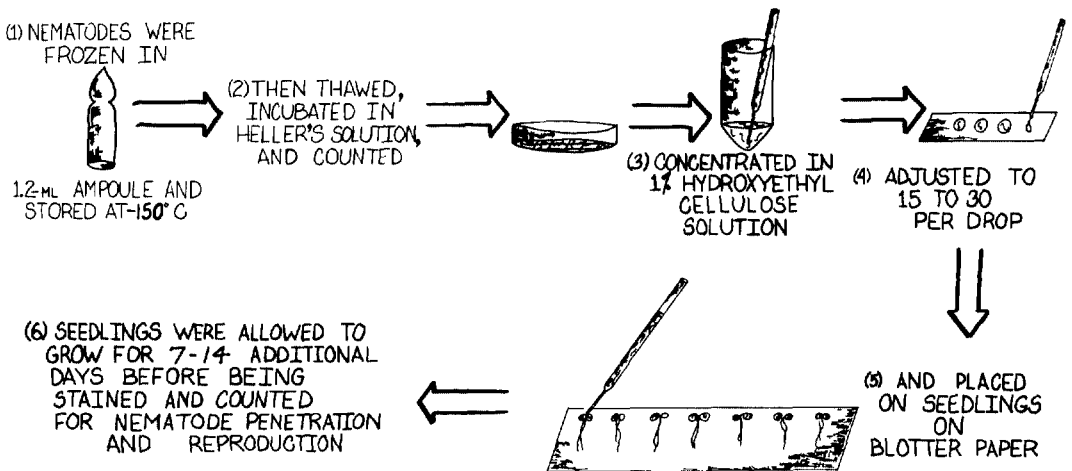


FIG. 1. Flow diagram of steps followed in freezing (liquid nitrogen), thawing, and infectivity-testing in alfalfa seedlings.

The chi-square test for comparison of proportions in independent samples indicated that the control treatment differed significantly from the treatments of DMSO and freezing. When the data were analyzed on a time series of 1, 30, and 540 days, a chi-square of 37.02 (2 d.f., $P > 0.005$) was found. An examination of these components showed that most of the significance was from the day-one treatment. This established that most freezing injuries occurred during initial freezing and that there was no significant increase in nematode mortality during storage for 30 or 540 days.

Beginning with the 1959 report of Lovelock and Bishop (9), DMSO has been shown repeatedly to be a suitable cryoprotective agent for a number of bacterial and fungal species and some organs and tissues of higher animals. Consequently, it comes as no surprise that DMSO affords some protection for motile larvae of *D. dipsaci* against freezing injuries. However, DMSO does not appear to protect a *Meloidogyne* sp. (Sayre and Hwang, unpublished). Also, there is considerable variability in the amount of freezing injury in different experiments. The variability undoubtedly lies in not controlling precisely all conditions during freezing and thawing of nematodes and/or their physiological state.

Some conditions governing survival of nematodes include the following: (i) age and vigor of nematode populations before freezing; (ii) time nematodes are in contact with DMSO; (iii) concentration before and after freezing; (iv) rate of freezing; and (v) rate of thawing. The relative importance of these conditions in determining ultimate survival rate of nematodes is not precisely known. In our study, only conditions 4 and 5 were regulated to any extent; generally, we used a slow freezing rate coupled with fast thawing. This is the usual procedure suggested in the literature to obtain maximum survival of animal cells stored in liquid nitrogen. However, when young colonies of more vigorous nematodes were used, survival rate was usually higher. The concentration of DMSO toxic to *D. dipsaci* has not been established, nor has the influence of sublethal doses, which may impair the nematode's viability. Obviously, there is a definite need to refine these conditions so that improved nematode survival in storage can be achieved.

With modifications, methods might be developed that would apply to several other

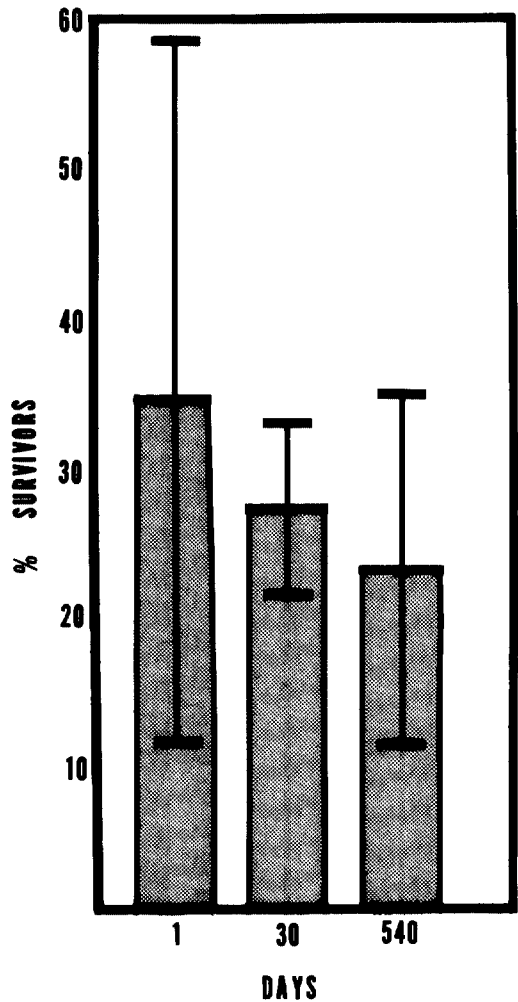


FIG. 2. Percent survivors in populations of *Ditylenchus dipsaci* stored for 1, 30, and 540 days at -150°C . The bar and vertical line represent respectively the average percent survivors and 95% confidence limit.

TABLE 1. Effects of storage at -150°C on the ability of *Ditylenchus dipsaci* to penetrate and incite stem disease in alfalfa seedlings.

Nematode treatments	% disease per storage time (days)		
	1	30	540
In 7.5% DMSO, frozen	62.3	40.4	37.1
In 7.5% DMSO ^a , not frozen	53.7	64.4	...
Control ^b , not frozen	78.9	95.1	67.7

^aNematodes freshly extracted from alfalfa callus were incubated in 7.5% DMSO for 3 h before being placed on seedlings.

^bNematodes were extracted from alfalfa callus over a 24-h period and used immediately.

plant-parasitic nematodes. There is a real need for the long-term preservation of plant-parasitic nematodes, and cryogenic storage may be the best method. First, the effect of spontaneous mutation is avoided, and second, the tedium of continuous culturing is no longer necessary. Primarily for these reasons, a search for a suitable storage method is a worthwhile goal in plant nematology.

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