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Cryopreservation of *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*

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Abstract: A method for the cryopreservation of third-stage infective juveniles (IJ) of *Steinernema carpocapsae* and *Heterorhabiditis bacteriophora* was developed. Cryoprotection was achieved by incubating the nematodes in 22% glycerol (*S. carpocapsae*) or 14% glycerol (*H. bacteriophora*) for 24 hours, followed by 70% methanol at 0 C for 10 minutes. The viability of *S. carpocapsae* frozen in liquid nitrogen as 20 µl volumes spread over cover slip glass was > 80%. Survival of *H. bacteriophora* frozen on glass varied from 10 to 60% but was improved to > 80% by replacing the glass with filter paper. Cryopreservation and storage of 1-ml aliqots of *S. carpocapsae* IJ resulted in > 50% survival after 8 months; pathogenicity was retained and normal in vitro development took place. Trehalose and glycerol levels increased and glycogen levels decreased during incubation of *S. carpocapsae* IJ in glycerol. Normal levels of trehalose, glycerol and glycogen were restored during post freezing rehydration.

Key words: cryopreservation, desiccation, entomopathogenic nematode, *Heterorhabditis bacteriophora*, nematode, *Steinernema carpocapsae*.

Entomopathogenic nematodes in the genera *Steinernema* and *Heterorhabditis* are effective biological control agents for a variety of insect pests. Members of each species are maintained as separate geographical isolates. The growing number of researchers working on these organisms would benefit from a method of cryopreservation for the maintenance of these isolates. The development of a liquid culture method (1) and large-scale fermentation processes for these nematodes (5,9) has also led to a need for a method of preservation of nematode inocula for in vitro cultures. We describe herein a cryopreservation method applicable to both genera.

MATERIALS AND METHODS

Infective juveniles (IJ) of *S. carpocapsae* Weiser strain All and *H. bacteriophora* Poinar (syn. *H. heliothidis* Khan, Brooks, & Hirschmann) strain HP88 were produced in liquid culture in 150-liter fermentors (3). They were stored in aerated deionized water at a concentration of 2.5×10^5 IJ/ml for periods not exceeding 3 months.

An initial experiment was performed on IJ of *S. carpocapsae* to determine an optimum time for incubation in glycerol. Infective juveniles were suspended in 22% (w/v) glycerol at a nematode concentration of 2.5×10^5/ml. They were maintained as 2-mm-deep suspensions in culture flasks at 25 C for 24, 48, and 72 hours. In all subsequent experiments, IJ of both species were maintained in glycerol for 24 hours. Glycerol concentrations used for *H. bacteriophora* were 10, 11, 12, 14, 15, 16, 17, 18, and 22%. Nematode suspensions in volumes greater than 10 ml were maintained in 250-ml flasks (25 ml/flask) on a rotary shaker at 100 rpm.

Following incubation in glycerol the nematodes were centrifuged at 1,000 rpm and the glycerol supernatant was removed. The nematodes were resuspended in ice-cold 70% methanol and maintained on ice for 10 minutes. They were subsequently recentrifuged, and the nematode concentration was adjusted to 10^6/ml by removal of methanol. After both glycerol and methanol incubations, subsamples of nematodes were resuspended in 0.85% saline and maintained (24 hours) for viability determination.

Aliquots (20 µl) of nematode suspension in methanol were spread over glass slivers (5 × 40 mm, prepared by breaking cover slips precooled on an ice-cold aluminum block). The slivers were plunged directly into liquid nitrogen, giving a cooling rate of approximately 5,100 C/minute (6). The

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slivers were held in liquid nitrogen for 1 minute. The samples were thawed by rapidly transferring them into 5 ml of 0.85% saline at room temperature and agitating. Infective juveniles of *H. bacteriophora* were also frozen on similar-sized strips of Whatman #1 filter paper.

Aliquots (1 and 0.5 ml) of *S. carpocapsae* IJ suspended in methanol were transferred to 3-ml cryotubes which were affixed to storage canes and submerged in liquid nitrogen within the freezer. Cryotubes were stored for periods of up to 8 months. Samples were thawed by adding 2 ml of 30-C saline and continuously agitating until the pellet had completely thawed. The IJ suspension was then removed and diluted a further 10-fold with saline.

Infective juvenile viability was assessed 24 hours after thawing by microscopic observation of motility and response to probing. The mean percentage of viable nematodes + SD was calculated from triplicate samples.

The pathogenicity of thawed IJ was determined in a petri plate assay with *Galleria mellonella* (L.) larvae. Forty IJ were counted, suspended in 0.5 ml water, and dispensed onto each of 10 filter discs (Whatman #1) in inverted 5-cm petri plates. Ten *G. mellonella* larvae were placed onto each filter disc, and the plates were maintained at 22 C. Insect mortality was recorded at 2-hour intervals beginning at 32 hours postexposure. The LT_{50} was computed using probit analysis (3) and compared to control bioassays in which nonfrozen nematodes were used.

The ability of cryopreserved IJ to develop in vitro was evaluated by transferring thawed IJ onto dogfood agar slants (10) and observing development to reproducing adults after maintenance at 25 C for 5 days.

An experiment was performed to determine some of the biochemical changes that take place in the nematodes during incubation in glycerol and subsequent thawing and rehydration in saline. Infective juveniles of *S. carpocapsae* were incubated in 22% glycerol in flasks on a shaker for 24 hours as described. At 0, 8, 18, and 24 hours, triplicate subsamples of 5×10^5 IJ were taken and stored at −80 C for future biochemical analysis. At 24 hours the remaining IJ were resuspended in ice-cold methanol for 10 minutes and frozen for 1 hour in liquid nitrogen, as described for 1-ml aliquots. Three samples were transferred to −80 C for storage prior to biochemical analysis. The rest were thawed by adding 2.5 ml of 30-C saline to the cryotubes. The nematode suspensions were transferred to 7-cm tissue culture flasks and the volume was made up to 10 ml with additional saline. The nematodes were maintained at 25 C. At 10 minutes and 2, 4, 18, and 24 hours, samples were again taken and stored at −80 C. At the end of the experiment, all samples were thawed, washed five times in a 1% aqueous solution of Triton X 100, and pulse sonicated on ice for 6 minutes. Triplicate subsamples were taken and assayed for glucose, trehalose, glycogen, and glycerol, and total lipid content.

Soluble carbohydrates were extracted with 70% ethanol at 5 C for 1 hour, and the samples were then centrifuged at 10,000 *g* for 15 minutes. The ethanol was removed and retained for subsequent quantification of soluble carbohydrates. The ethanol insoluble pellet was washed once with 70% ethanol and recentrifuged. The ethanol wash was discarded, and the pellet was air dried and then dissolved in 30% sodium hydroxide at 100 C for 15 minutes. Glycogen was precipitated with 70% ethanol at 5 C overnight and then centrifuged at 10,000 *g* for 10 minutes. The ethanol was removed and discarded and the glycogen was dissolved in water. Levels of soluble carbohydrate in the ethanol and glycogen in the water were determined with the anthrone method of Dimmler et al. (2).

For glucose and trehalose determinations, subsamples of sonicate were centrifuged at 10,000 *g* for 10 minutes. Aliquots (10 μl) of the clear supernatant were assayed enzymatically for free glucose with a glucose hexokinase colorimetric reagent

TABLE 1. Survival (in percent) of *Steinernema carpocapsae* infective juveniles after incubation in 25 C 22% glycerol for 0–72 hours and freezing of 20-µl volumes in liquid nitrogen either directly or after subsequent incubation in 70% methanol at 1 C for 10 minutes.

Glycerol incubation time (hours)	Survival (standard deviation)			
	After glycerol incubation	After glycerol incubation plus freezing†	After glycerol plus methanol incubation	After glycerol plus methanol incubation plus freezing†
0	95.9 (1.4)	Not done	Not done	Not done
24	91.6 (1.1)	87.3 (2.5)	91.6 (1.1)	85 (2.6)
48	88 (4.3)	78.3 (6.3)	87 (2.6)	74 (3.0)
72	75.3 (2.1)	59.6 (5.5)	66.3 (1.5)	58.3 (5.8)

Values are means of three replicates.
† Samples were frozen for 1 minute.

TABLE 2. Survival (in percent) of *Steinernema carpocapsae* infective juveniles after incubation in 25 C 22% glycerol for 24 hours and freezing of 0.5-ml and 1-ml volumes in liquid nitrogen either directly or after subsequent incubation in 70% methanol at 1 C for 10 minutes.

Sample volume (ml)	Survival (standard deviation)			
	After glycerol incubation	After glycerol incubation plus freezing†	After glycerol plus methanol incubation	After glycerol plus methanol incubation plus freezing†
0.5	97.3 (0.9)	26.4 (3.1)	95 (1.1)	95 (0.9)
1.0	97.3 (0.9)	24 (2.9)	95 (1.1)	95 (0.6)

Values are means of three replicates.
† Samples were frozen for 30 minutes.

kit (DMA Inc. Arlington TX). For total glucose determination, 20-µl aliquots of the clear supernatant were incubated with 30 µl of 33 mM phosphate buffer, pH 5.7, and 10 µl of trehalase (Sigma Chemical Company, St. Louis, MO) at 37 C for 30 minutes. The glucose assay was then performed. Trehalose levels were determined by subtracting the free glucose values from those for total glucose.

Free glycerol was determined using an enzyme-reagent mixture comprising 6 ml of each of the following: 40 mM magnesium chloride, 0.5 M sodium phosphate, 3.85 mg/ml ATP, 0.081 mg/ml 4-aminopyrine, 2.12 mg/ml 3,5 dichloro-2-HO-benzene sulphonate, 0.303 mg/ml glycerol kinase, 1.21 mg/ml glycerol phosphate oxidase, 0.406 mg/ml horseradish peroxidase, and deionized water. For the assay, 10 µl sonicate was diluted 20-fold with 0.05 M sodium phosphate buffer, pH 6.7, vortexed and centrifuged at 10,000 *g* for 10 minutes. Reagent (900 µl) was then added to a mixture of 20 µl clear supernatant and 80 µl deionized water. The tubes were vortexed and maintained at room temperature for 60 minutes, and the absorbance was read at 520 nm.

Lipids were extracted according to the method of Folch et al. (4) and quantified gravimetrically. The results are expressed as a percentage of the dry weight, which was determined by lyophilization of subsamples of nematode sonicate.

RESULTS

Survival of *S. carpocapsae* IJ was 91.6% after incubation in 22% glycerol for 24 hours (Table 1). Freezing survival was > 80% in samples frozen both directly after glycerol incubation and after subsequent methanol incubation. The survival of IJ incubated in glycerol for periods longer than 24 hours declined; this was followed by a further decline when the samples were frozen.

When *S. carpocapsae* IJ were frozen for 30 minutes in volumes of 0.5 and 1 ml, freezing survival was only 26.4 and 24% respectively, when samples were frozen directly after glycerol incubation (Table 2), but after subsequent incubation in methanol, freezing survival was 95%. When 1-ml volumes of nematodes were frozen in methanol for long-term maintenance, survival declined to 71% in the first month and levels ranging from 60 to 80% were obtained for the next 8 months (Fig. 1). Pathogenicity of IJ frozen for 8 months was retained (Table 3), and in vitro development of IJ inoculated onto dogfood slants took place, as did growth of the symbiont *Xenorhabdus nematophilus*.

The trehalose content of *S. carpocapsae* IJ incubated in 22% glycerol increased from

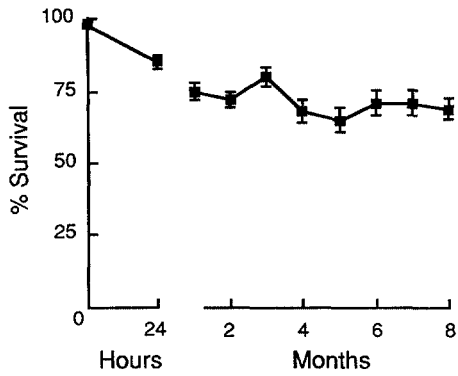

FIG. 1. Survival of infective juveniles of *Steinernema carpocapsae* stored in liquid nitrogen in 1-ml volumes for 8 months. Data are reported as percentage of survival + SD, N = 3.

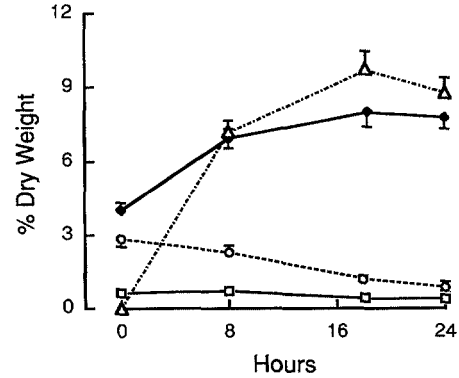

FIG. 2. Changes in trehalose (◆), glucose (□), glycogen (○), and glycerol (△) content in infective juveniles of *Steinernema carpocapsae* incubated in 22% glycerol for up to 24 hours. Data are reported as percentage of dry weight + SD, N = 9.

4 to 6.9% during the first 8 hours of incubation and reached a maximum level of 8% by 18 hours (Fig. 2). Glycogen content declined from 2.8 to 0.9% and glucose content remained relatively constant. Glycerol increased from 0 to 7.1% during the first 8 hours of incubation and was maximal at 9.8% by 18 hours. These trends were reversed during incubation of the thawed IJ in saline (Fig. 3). Trehalose and glycerol contents declined to 1.4 and 0.3%, respectively, and the normal glycogen level was restored.

The survival rate of *H. bacteriophora* strain HP88 after incubation in 22% glycerol for 8 hours was 89.2% and declined to 28.6% after 24 hours (Table 4). Subsequent incubation in methanol resulted in a further decline in survival. However, it allowed for a low level of freezing survival ranging from 5.5 to 13% compared to 0% when IJ were frozen directly after glycerol incubation.

TABLE 3. Infectivity of *Steinernema carpocapsae* infective juveniles to *Gallaria mellonella* larvae after storage of 0.5-ml volumes in liquid nitrogen.

Storage time in liquid nitrogen (months)	LT_{50} (hours)	Confidence limits (hours)
0	41.3	39.8–43.7
4	40.5	39.2–41.6
0 control	41.5	40.4–42.9
8	41.0	38.8–43.0
0 control	42.0	40.6–43.7

Incubation for 24 hours in lower concentrations of glycerol resulted in improved nematode survival after both incubation steps and after freezing (Table 5). Again the nematodes did not survive when frozen directly after the glycerol incubation. Survival was maximal (54–56%) in samples initially incubated in glycerol concentrations of 14 and 15%. However, subsequent replications of a protocol using incubation in 14% glycerol as the first step, resulted in freezing survival rates varying from 15 to 65% (Table 6). When 20-μl volumes of *H. bacteriophora* IJ were frozen on filter paper rather than on glass, survival was dramatically and reliably increased to > 70% (Table 6).

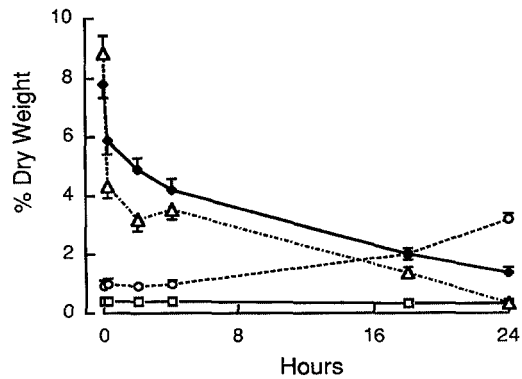

FIG. 3. Changes in trehalose (◆), glucose (□), glycogen (○), and glycerol (△) during maintenance of thawed infective juveniles of *Steinernema carpocapsae* in 0.85% saline for 24 hours. Data are reported as percentage of dry weight + SD, N = 9.

TABLE 4. Survival (in percent) of *Heterorhabditis bacteriophora* infective juveniles after incubation in 25 C 22% glycerol for 0–48 hours and freezing of 20-µl volumes in liquid nitrogen either directly or after subsequent incubation in 70% methanol at 1 C for 10 minutes.

Glycerol incubation time (hours)	Survival (standard deviation)			
	After glycerol incubation	After glycerol incubation plus freezing†	After glycerol plus methanol incubation	After glycerol plus methanol incubation plus freezing†
0	93.4 (2.8)	Not done	Not done	Not done
8	89.2 (3.4)	0	45 (8.0)	13.1 (2.0)
24	28.6 (8.8)	0	15.6 (6.3)	5.5 (1.3)
48	5.8 (1.1)	Not done	Not done	Not done

Values are means of three replicates.
† Samples were frozen for 1 minute.

TABLE 5. Survival (in percent) *Heterorhabditis bacteriophora* infective juveniles after incubation in six concentrations of glycerol at 25 C for 24 hours and freezing of 20-µl volumes in liquid nitrogen either directly or after subsequent incubation in 70% methanol at 1 C for 10 minutes.

Glycerol concentration (%)	Survival (standard deviation)			
	After glycerol incubation	After glycerol incubation plus freezing†	After glycerol plus methanol incubation	After glycerol plus methanol incubation plus freezing†
10	90 (5.0)	0	88 (3.3)	44 (7.2)
14	86 (4.8)	0	85.1 (2.9)	56 (5.8)
15	87.1 (3.9)	0	80 (3.8)	54 (7.7)
16	96.5 (2.1)	0	73.4 (5.4)	49 (6.3)
17	95.4 (2.0)	0	65.5 (7.6)	45 (10.0)
18	79 (5.7)	0	70.4 (8.8)	34 (14.4)

Values are means of three replicates.
† Samples were frozen for 1 minute.

DISCUSSION

In this study we have developed a reliable method for the cryopreservation of infective juveniles of *S. carpocapsae* and *H. bacteriophora* using sequential incubation in glycerol and methanol as cryoprotectants.

Based on the observation that evaporatively desiccated IJ of *S. carpocapsae* could be cryopreserved following incubation in methanol (James and Popiel, unpubl.), our intention was to achieve cryoprotection from the external desiccating effects of glycerol. The increase in internal glycerol content that we measured would also be cryoprotective. We do not know if this glycerol was derived from the external solution or if it was synthesized by the nematodes as it apparently is in response to evaporative desiccation (11). It is interesting to note that the biochemical responses during glycerol incubation and during rehydration are very similar to the biochemical responses to evaporative desiccation. The relevance of this desiccation-related biochemical response to freezing survival remains speculative.

In another study with single cryoprotectants (8), incubation in 20% glycerol for 24 hours was adequate for the cryopreservation of *S. feltiae* (syn. *S. carpocapsae*) frozen in 250-µl plastic straw tubes. In this study, glycerol incubation alone was sufficient for rapid freezing of small volumes of nematodes, but a second incubation in a high concentration of methanol enhanced the survival of nematodes frozen in 0.5-ml volumes. We presume that the methanol penetrated the nematodes, reduced the cooling rate required for vitrification, and, thus, protected them from the slower cooling rates during freezing of larger volumes. The freezing survival of nematode volumes of up to 1 ml indicates that this procedure could be used for storing nematode inocula for in vitro culture. The growth of *Xenorhabdus nematophilus* following the inoculation of thawed nematodes onto dog-

TABLE 6. Survival (in percent) of *Heterorhabditis bacteriophora* infective juveniles after incubation in 14% glycerol for 24 hours at 25 C, 70% methanol for 10 minutes at 1 C, then freezing of 20-µl volumes in liquid nitrogen on glass and filter paper.

Experiment	Survival (standard deviation)			
	After glycerol plus incubation	After glycerol plus methanol incubation	After glycerol plus methanol incubation plus freezing†	
			Glass	Paper
1	85.1 (2.9)	83.8 (5.8)	32.5 (10.3)	73.5 (5.5)
2	92.2 (1.1)	81.4 (7.9)	15.8 (7.7)	91.0 (3.1)
3	82.1 (6.3)	74.7 (8.4)	58.6 (5.4)	83.6 (6.9)

Values are means of three replicates.
† Samples were frozen for 1 minute.

food agar slants indicates that the bacteria carried by the nematodes also survived cryopreservation.

Infective juveniles of *H. bacteriophora* were more sensitive to glycerol incubation than *S. carpocapsae* and survived well only when incubated in lower concentrations. This may be related to the poor desiccation of heterorhabditids (unpubl.) or to a greater susceptibility to the toxic effect of glycerol. Subsequent methanol incubation gave rise to a small reduction in viability but was necessary for the rapid freezing of even small volumes of IJ suspension. The improved survival of *H. bacteriorphora* obtained when IJ were frozen on filter paper suggests that at the level of cryopreservation achieved, the freezing rate must be rapid to avoid ice crystal formation.

Exsheathment is required for successful cryopreservation of most nematodes whose free-living third-stage larvae retain the second-stage cuticle as a sheath (7). It was proposed that artificial exsheathment removes a barrier to water movement, allowing the worms to desiccate during cooling. Exsheathment is not required for survival in the cryopreservation process described herein, possibly because the slower desiccation induced by incubation in glycerol is unaffected by the presence of a sheath.

We have successfully applied this method for the cryopreservation of other geographical isolates of *S. carpocapsae.* In addition, we have modified it for other *Heterorhabditis* species as well as many isolates of *S. feltiae.* Thus, it is likely that the strategy of sequential incubation in glycerol and methanol can be optimized for the cryopreservation of all steinernematid and heterorhabditid nematodes.

Literature Cited

1. Buecher, E. J., and I. Popiel. 1989. Liquid culture of the entomogenous nematode *Steinernema feltiae* with its bacterial symbiont. Journal of Nematology 21:500–504.

2. Dimler, R. J., W. C. Schaeffer, C. S. Wise, and C. E. Rist. 1952. Quantitative paper chromatography of d-glucose and its oligosaccharides. Analytical Chemistry 24:1411–1414.

3. Finney, D. J. 1971. Probit analysis, 3rd ed. London: Cambridge University Press.

4. Folch, J., M. Lees, and G. H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissue. Journal of Biological Chemistry 226:497–509.

5. Friedman, M. J., S. L. Langston, and S. Pollit. 1989. Mass production in liquid culture of insect-killing nematodes. International Patent Publication No. WO 89/04602, U.S. Patent Offices, Washington DC.

6. James, E. R. 1981. *Schistosoma mansoni*: Cryopreservation of schistosomula by two-step addition of ethanediol and rapid cooling. Experimental Parasitology 52:105-116.

7. James, E. R. 1985. Cryopreservation of helminths. Parasitology Today 1:134–139.

8. Ohba, K., T. Ishiguro, and Y. Hayasahi. 1989. Freeze-thaw survival of the entomogenous nematodes *Steinernema feltiae* and *S. glaseri* in brief comparison with survival of *Caenorhabditis elegans* and *Aphelenchus avenae.* Japanese Journal of Nematology 18:30–35.

9. Pace, G. W., W. Grote, D. E. Pitt, and J. M. Pitt. 1986. Liquid culture of nematodes. International Patent Publication No. 86/01074, U.S. Patent Offices, Washington DC.

10. Popiel, I. 1987. Nematodes parasitic in insects. Pp. 379–393 *in* A. E. R. Taylor and J. R. Baker, eds. In vitro methods for parasite cultivation. New York: Academic Press.

11. Popiel, I., I. Glazer, and E. M. Vasquez. 1989. Desiccation of *Steinernema feltiae* infective juveniles. Journal of Nematology 21:580 (Abstr.).