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Optimised procedures for the cryopreservation of different species of *Heterorhabditis*

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Summary – It is demonstrated that the protocols developed for the cryopreservation of *Heterorhabditis bacteriophora* could be successfully applied to strains of the *H. bacteriophora* species complex and to members of the North West European species of *Heterorhabditis*. However the currently available protocols were not suitable for cryopreserving the Irish or the tropical *Heterorhabditis* species. Therefore, the postcryopreservation viabilities of *Heterorhabditis* species in response to preincubation in a range of cryoprotectants at varying concentrations and incubation times were determined. Species and strain specific differences in cryopreservation response were observed among *Heterorhabditis* isolates and optimal conditions were developed for the cryopreservation of Irish, NW European and tropical isolates of *Heterorhabditis*. These optimum conditions are described in this paper.

Résumé – ***Techniques optimisées pour la cryoconservation de différentes espèce d'*Heterorhabditis** – Il est démontré que les techniques mises au point pour la cryoconservation d'*Heterorhabditis bacteriophora* pouvaient être appliquées avec succès à différentes souches du complexe d'espèces *H. bacteriophora* et aux espèces d'*Heterorhabditis* présentes dans le Nord-Ouest de l'Europe. Par contre, ces techniques ne conviennent pas pour la cryoconservation des espèces irlandaises ou tropicales d'*Heterorhabditis*. A donc été déterminée la survie après cryoconservation de différentes espèces d'*Heterorhabditis* en fonction de la nature du traitement préalable à l'application du cryoconservateur (méthanol), de son intensité et de sa durée. Des différences inter- et intraspécifiques sont observées parmi les souches d'*Heterorhabditis* et les conditions optimales pour la cryoconservation des souches d'Irlande, d'Europe du Nord-Ouest et tropicales ont été déterminées et sont décrites dans le présent article.

Key-words : cryopreservation, *Heterorhabditis*, glycerol, dimethyl sulfoxide, desiccation, nematodes.

As part of a research project aimed at isolating cold active strains of the insect parasitic nematode *Heterorhabditis* for use in biological control, a large number of entomopathogenic nematode isolates have been collected from Ireland, Britain and NW Europe (Griffin *et al.*, 1994; unpubl.). Routine subculturing of heterorhabditids for stock maintenance is costly in terms of labour and space and in addition strains are vulnerable to accidental loss, to contamination and to changes in genotype resulting from genetic drift and from adaptive selection in response to laboratory culturing. Cryopreservation offers the possibility of preserving such isolates free from contamination by other strains and of maintaining intact their original genetic constitution.

Infective juveniles (IJs) of *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* have been successfully cryopreserved by Popied and Vasquez (1991) by preincubation in 22 and 14 % glycerol, respectively, for 24 h followed by incubation in 70 % methanol for 10 min prior to immersion in liquid nitrogen. This procedure is a modification of the original evaporative desiccation method developed for *Steinernema*, which used exposure to 97 % rh for three days instead of preincubation in glycerol (Popiel *et al.*, 1988). Curran *et al.* (1992) further modified the cryopreservation protocol for *H. bacteriophora* by replacing centrifugation with a filtration step to concentrate and wash the nematodes, by preincubating the IJs in 17 % glycerol for 72 h and by freezing them in liquid suspensions rather than on filter paper strips.

We have found that the modifications of Curran *et al.* (1992), while successful with strains of the *H. bacteriophora* species complex, were not suitable for cryopreserving Irish *Heterorhabditis* isolates. We therefore determined the post-cryopreservation viabilities of *Heterorhabditis* species in response to preincubation in a range of cryoprotectants at varying concentrations and incubation times. Species and strain specific differences in cryopreservation response were observed and optimal conditions were developed for the cryopreservation of Irish, NW European and tropical species of *Heterorhabditis*. These optimum conditions are described in this paper.

Materials and methods

SOURCE AND MAINTENANCE OF THE NEMATODE ISOLATES AND BACTERIAL ISOLATES

The *Heterorhabditis* isolates used in this study are maintained in the Department of Biology, St. Patricks College, Maynooth. The sources from which the isolates were originally obtained are listed in Table 1. Nema-

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Table 1. *The* Heterorhabditis *isolates used in this study. They have been placed in species groups according to cross breeding data (Dix* et al., *1992) and the patterns obtained from IEF and RFLP analysis (Griffin* et al., *1994; Joyce* et al., *1994* a, b*).*

Isolate	Location	Source
H. BACTERIOPHORA SPECIES COMPLEX		
H. bacteriophora	Brecon, South Australia	Dr. Ray Akhurst[1]
HI82	Italy	Ir. Paula Westerman[2]
HP82	Utah, USA	Ir. Paula Westerman
V16	Victoria, Australia	Ms. Tracey Nelson[3]
H. zealandica	New Zealand	Ir. Paula Westerman
H. MEGIDIS	Ohio, USA	Dr. Ray Akhurst
NW EUROPEAN GROUP		
HL81	The Netherlands	Ir. Paula Westerman
HF85	The Netherlands	Ir. Paula Westerman
NLHB	The Netherlands	Ir. Paula Westerman
HSie	Siedlce, Poland	Dr. P. H. Smits[4]
UK211	Dorset, England	Dr. W. Hominick[5]
TROPICAL GROUP		
D1	Darwin, Australia	Dr. ray Akhurst
P$_2$M	Artemisa, Cuba	Dr. Z. Mracek[6]
IRISH GROUP		
K122	North Slobs, Wexford, Ireland	Dr. C. T. Griffin[7]
M145	Kilmore Quay, Wexford	Dr. C. T. Griffin[7]
M145	Kilmore Quay, Wexford	Dr. C. T. Griffin[7]
M227	Clogherhead, Louth, Ireland	Dr. C. T. Griffin[7]
M288	Ballyhiernan Bay, Donegal	Dr. C. T. Griffin[7]
M170	Rosses Point, Sligo	Dr. C. T. Griffin[7]
S29	Balinkeil, Scotland	Dr. C. T. Griffin[7]
W9	Pendine, Wales	Dr. C. T. Griffin[7]
W24	Freshwater East, Wales	Dr. C. T. Griffin[7]
W26	Freshwater East, Wales	Dr. C. T. Griffin[7]
W50	Llandmadco, Wales	Dr. C. T. Griffin[7]
S159	Fraserburgh, Scotland	Dr. C. T. Griffin[7]
UK462	Norfolk, England	Dr. W. Hominick

(1) CSIRO, Canberra, Australia; (2) Agrarische Hogeschool Friesland, The Netherlands; (3) Canterbury Agricultural and Science Centre, Lincoln, New Zealand; (4) Institute for Plant Protection, Wageningen, The Netherlands; (5) International Institute of Parasitology, St. Albans, England; (6) Institute of Entomology, Bransovska, Czech Republic; (7) St. Patrick's College, Maynooth, Co. Kildare, Ireland.

Table 2. *The post-cryopreservation survival values* for a range of* Heterorhabditis *isolates obtained using the preincubation modifications of Curran* et al., *(1992) i.e. preincubation in 17 % glycerol at 23 °C for 72 h followed by preincubation in ice cold 70 % methanol for ten min. The IJs were then frozen in liquid nitrogen in 30 µl volumes or on filter paper strips.*

Isolate	Survival (%)*	
	Method of freezing	
	In liquid suspension	On filter paper strips
H. BACTERIOPHORA SPECIES COMPLEX		
H. bacteriophora		
BRECON	35.8 ± 6.0	49.9 ± 1.2
HI82	75.2 ± 9.3	80.0 ± 3.2
HP88	43.1 ± 5.0	77.3 ± 2.4
V16	40.3 ± 5.7	63.1 ± 2.2
H. zealandica	0	9.5 ± 0.6
H. MEGIDIS	12.1 ± 7.3	43.5 ± 1.1
NW EUROPEAN GROUP		
HL81	30.4 ± 4.6	80.1 ± 1.2
HF85	31.6 ± 3.2	81.1 ± 2.7
HSie	18.7 ± 2.6	89.8 ± 1.4
NLHB	21.2 ± 5.1	73.0 ± 0.7
UK211	31.9 ± 3.5	60.0 ± 1.1
TROPICAL GROUP		
D1	4.4 ± 0.5	7.9 ± 0.1
P$_2$M	0.2 ± 0.0	6.2 ± 1.2
Irish Group		
K122	2.9 ± 1.1	3.2 ± 0.2
M170	0	0
M145	0	0.1 ± 0.1
M227	0	0
M288	0	0
UK462	0	0
W9	0	0
W24	0	0
W26	0	0
W50	0	0
S159	0	0
S2	0	0

* Each value is the mean ± SE of six replicates.

todes were cultured *in vivo* in *Galleria mellonella* larvae as described in Woodring and Kaya (1988) or on lipid agar plates (Dunphy & Webster, 1989). Symbiotic bacteria *(Photorhabdus luminescens)* were isolated from surface sterilized IJs as described by Akhurst (1980). Primary forms of *P. luminescens* were maintained on NBTA plates (Akhurst, 1986).

CRYOPRESERVATION PROTOCOL

Freshly harvested IJs were sedimented and the resulting pellet was preincubated (at a concentration of *ca.* 5000 IJs/ml) in varying concentrations of glycerol, or the other cryoprotectants listed in Table 5, at 23 °C for a variety of preincubation periods. After preincubation, excess cryoprotectant was removed from the IJs by filtering them onto a 2.5 cm diameter Whatman No. 1 filter paper disc using a Sartorious funnel (25 mm glass vacuum filter holder with 30 ml funnel Cat. No. SM16315) and a vacuum flask attached to a vacuum pump. The nematodes were then rinsed under suction with 2 ml of 70 % ice cold (i.e. 0 °C) methanol. The filter disc (containing the nematodes) was transferred to a 3 cm diameter Petri dish on ice containing 3 ml of ice cold 70 % methanol. The IJs were resuspended by agi-

Table 3. *The effect of delays in thawing after removal of cryopreserved nematodes from liquid nitrogen. The HI82 strain of* Heterorhabditis *used in this experiment was preincubated in 17 % glycerol for 72 h followed by a ten min methanol preincubation and rapid freezing in liquid in 30 μl volumes or on filter paper strips.*

Time to thawing (s)	Survival (%)*	
	Filter paper strips	Liquid suspension
15	83.4 ± 2.2	44.6 ± 1.5
30	82.5 ± 2.2	44.6 ± 1.5
60	67.7 ± 0.5	22.4 ± 1.6
90	63.6 ± 3.8	0
120	44.7 ± 0.7	0
150	1.0 ± 0.6	0
180	0	0
240	0	0
300	0	0

* Each value is the mean ± SE of 3 replicates.

tation and were incubated on ice for 7 min. They were then transferred to two × 1.5 ml Eppendorf tubes and centrifuged at 4000 rpm for two min. Excess methanol was pipetted off until *ca.* 60 μl of nematode pellet/suspension remained in each tube. The IJs were then transferred in 30 μl volumes to filter paper strips (Whatman No. 1, 2 cm × 0.5 cm) and placed in 2 ml round bottomed cryovials (Nunc Cat. No. 103-043) or they were transferred as 30 μl liquid aliquots to the cryovials. The vials were then capped and plunged immediately into liquid nitrogen.

POST-CRYOPRESERVATION SURVIVAL

The cryovials were held in liquid nitrogen for 24 h after which the samples were thawed rapidly by addition of 2 ml of tap water at room temperature. The vials were vortexed and the contents were immediately poured into 3 cm Petri dishes. Percentage survival was assessed 24 h later by microscopic observation of motility and response to probing. The ability of thawed IJs to develop *in vitro* and *in vivo* was evaluated by transferring thawed IJs onto lipid agar plates (Dunphy & Webster, 1989) and observing their development at 23 °C or by measuring their ability to infect *Galleria mellonella* larvae.

In order to assess the effects of long term storage in liquid nitrogen on survival, IJs were cryopreserved as described above and were held in liquid nitrogen for a variety of time intervals up to ten weeks. They were then thawed as described and post-cryopreservation survival was determined.

EVAPORATIVE DESICCATION OF IJS

A 1 ml suspension of IJs in water (concentration 200 IJs/ml) was vacuum-filtered onto a Whatman No. 1 filter paper disc as described above. Each filter paper disc was placed singly in a 3 cm Petri dish which were then transferred to a 30 cm diameter glass desiccator. The relative humidity in the desiccator (95 %) was controlled using a sulphuric acid solution (Solomon, 1951). After the 4 day desiccation period the Petri dishes were removed, the IJs were transferred to ice cold 70 % methanol and they were cryopreserved as described above. The capacity of the IJs to withstand evaporative desiccation was determined by desiccating the IJs for four days as described above. The IJs were then rehydrated with tap water at 20 °C and their survival was assessed 24 h later.

Results

IJs representative of the known biological species of *Heterorhabditis* (Dix *et al.,* 1992; Griffin *et al.,* 1994; Joyce *et al.,* 1994 *a, b*), were cryopreserved using the preincubation modification described by Curran *et al.* (1992) and the post-cryopreservation survival-values obtained are presented in Table 2. These data show that although published protocols can be used to successfully cryopreserve members of the *H. bacteriophora* group and the NW European group, these protocols were less successful in cryopreserving the tropical isolates and were unsuitable for cryopreserving isolates of the Irish species. Popiel and Vasquez (1991) have observed that while *Steinernema carpocapsae* could be successfully cryopreserved in 0.5 ml volumes of 70 % methanol; post-cryopreservation survival was considerably higher for *H. bacteriophora* when the pretreated IJs were frozen on filter paper strips rather than in liquid suspension. The data presented in Table 2 confirm these findings.

Curran *et al.* (1992) have noted that considerable variation can occur in the post-cryopreservation viabilities recorded for batches of a single nematode isolate frozen on the same day. These authors noted that the speed of thawing was critical, and that even slight delays (more than 15 s) in thawing resulted in a marked drop in survival. Table 3 shows the effect of delays in thawing after removal from liquid nitrogen on the postcryopreservation survival of IJs of *Heterorhabditis* sp. HI82. From this table it can be seen that in order to obtain high rates of survival. IJs must be thawed quickly (30-60 s), however IJs which were frozen in 30 μl liquid suspension were much more sensitive to delays in thawing than were IJs frozen on filter paper strips.

The effects of preincubation in different glycerol concentrations for various time intervals up to 10 days prior to treatments with methanol and freezing in liquid nitrogen are illustrated in Table 4. For members of the Irish species it can be seen that preincubation in 11 or 15 % glycerol for eight days gave significantly better results than preincubation in 17 % glycerol. Better and more consistent post-cryopreservation survival was also recorded for IJs of *H. zealandica* when they were preincubated in 15 % glycerol for 4 to 6 days. The concen tration of glycerol and the length of preincubation was

Table 4. *Survival of* Heterorhabditis *isolates following preincubation in varying concentrations of glycerol for different periods of time followed by treatment in ice cold 70 % methanol and freezing in liquid nitrogen on filter paper strips.*

Strain	Length of glycerol preincubation (days)	Survival (%)* Glycerol concentration 11 %	15 %	17 %
HI82	2	63.1 ± 3.3	70.1 ± 9.3	72.3 ± 9.4
	3	58.7 ± 4.7	69.6 ± 5.3	80.0 ± 3.2
	4	76.3 ± 7.4	88.3 ± 5.9	75.9 ± 7.6
	6	42.3 ± 3.1	63.2 ± 3.4	63.2 ± 5.6
	8	30.9 ± 1.4	63.3 ± 5.0	41.9 ± 2.5
	10	26.7 ± 5.0	35.2 ± 3.3	24.7 ± 6.1
H. zealandica	2	8.6 ± 1.4	18.5 ± 5.4	8.9 ± 3.6
	3	12.4 ± 2.5	22.5 ± 4.1	9.5 ± 0.6
	4	15.5 ± 7.7	28.9 ± 5.1	20.6 ± 11.6
	6	26.2 ± 5.3	20.4 ± 6.0	8.2 ± 1.7
	8	12.9 ± 3.3	2.6 ± 1.4	3.6 ± 1.2
	10	0	3.0 ± 1.1	1.4 ± 0.7
H. megidis	2	73.5 ± 1.1	75.5 ± 9.4	43.5 ± 6.5
	3	71.4 ± 3.5	68.7 ± 6.2	43.5 ± 1.1
	4	75.2 ± 0.9	65.9 ± 7.2	41.3 ± 9.5
	6	50.6 ± 6.9	48.2 ± 5.4	35.9 ± 5.6
	8	10.8 ± 3.4	8.6 ± 0.9	13.5 ± 2.0
	10	16.4 ± 7.2	3.1 ± 2.4	1.9 ± 0.2
HL81	2	79.2 ± 6.1	75.8 ± 6.6	78.1 ± 15.3
	3	75.4 ± 4.3	71.5 ± 4.9	80.1 ± 1.2
	4	79.3 ± 2.9	75.9 ± 7.7	43.6 ± 6.6
	6	71.1 ± 4.9	59.1 ± 3.1	45.1 ± 8.9
	8	60.7 ± 7.7	62.8 ± 5.8	47.5 ± 2.4
	10	53.4 ± 3.5	45.9 ± 2.5	31.9 ± 1.1
D1	2	10.7 ± 2.9	35.9 ± 3.0	33.1 ± 6.5
	3	16.5 ± 2.5	27.4 ± 1.7	7.9 ± 0.1
	4	43.6 ± 3.5	28.9 ± 0.7	39.9 ± 4.9
	639.5 ± 0.7	61.4 ± 1.6	43.2 ± 10.5	
	8	24.6 ± 4.9	57.8 ± 3.5	31.7 ± 5.5
	10	15.9 ± 3.0	31.2 ± 2.9	23.2 ± 2.3
K122	2	0	5.1 ± 0.6	0
	3	0	9.5 ± 3.6	3.2 ± 0.8
	4	0	18.3 ± 2.9	23.9 ± 10.5
	6	15.5 ± 1.9	28.2 ± 7.6	6.1 ± 3.8
	8	27.9 ± 6.5	32.5 ± 3.1	6.8 ± 0.5
	10	20.9 ± 9.4	10.6 ± 0.6	0.0 ± 0.0
M170	2	0.5 ± 0.0	8.6 ± 3.3	6.5 ± 3.1
	3	0	3.1 ± 0.6	0.0 ± 0.0
	4	0	8.3 ± 2.6	4.5 ± 3.7
	6	8.4 ± 1.4	15.1 ± 0.6	3.1 ± 2.1
	8	29.4 ± 7.9	25.2 ± 5.7	0
	10	17.9 ± 1.6	9.5 ± 1.6	0

* Each value is the mean ± SE of six replicates.

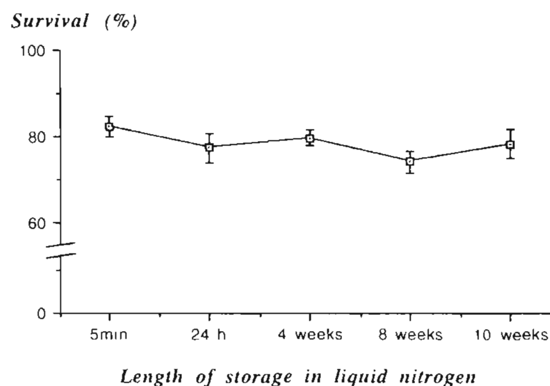

Fig. 1. *Survival of* Heterorhabditis *HI82 infective juveniles during a ten week storage period in liquid nitrogen (n = 6).*

much less critical for HI82 (*H. bacteriophora* species complex) and for HL81 (NW European group), both of which were much more amenable to cryopreservation than any of the other biological species of *Heterorhabditis*. Post-cryopreservation survival values were routinely determined following cryopreservation in liquid nitrogen for 24 h however from Fig. 1 it can be seen that postcryopreservation survival values remain constant over a 10 week storage period in liquid nitrogen.

Post-cryopreservation survival following preincubation in a range of other cryoprotectants prior to incubation in methanol and freezing in liquid nitrogen was also assessed (see Table 5). Of these, dimethyl sul-

Table 5. *Post-cryopreservation survival values of* Heterorhabditis *sp. HI82 upon preincubation at 23 °C for 3 days in various cryoprotectants (or at 95 % rh for 4 days) followed by incubation in 70 % ice cold methanol for 10 min prior to freezing in liquid nitrogen on filter paper strips.*

Cryoprotectant	Postcryopreservation Survival (%)*
8 % Ethylene glycol	13.0 ± 1.6
10 % Ethylene glycol	6.4 ± 0.9
17 % Ethylene glycol	4.4 ± 0.5
25 % Ethylene glycol	3.5 ± 0.9
10 % Polyethylene glycol	0
17 % Polyethylene glycol	0
8 % Dimethyl sulfoxide	81.2 ± 5.0
1 × Artifical sea water	35.0 ± 5.9
1 % Sodium chloride	30.0 ± 4.8
0.5 % Sodium chloride	31.1 ± 4.4
95 % rh for four days at 20°C	56.9 ± 2.5

* Each value is the mean ± SE of three replicates.

Table 6. *Post-hydration survival of IJs of five different biological species of* Heterorhabditis *desiccated at 95 % rh for 4 days.*

Isolate	Survival (%)*
HP88	74.5 ± 3.9
HL81	92.3 ± 2.3
H. zealandica	38.5 ± 7.5
P_2M	21.3 ± 4.7
K122	35.2 ± 5.1

* Each value is the mean ± SE of three replicates.

foxide (DMSO) was the only cryoprotectant which yielded post-cryopreservation survival values comparable to those obtained upon preincubation with glycerol, however it can also be seen from Table 5 that evaporative desiccation or preincubation in either artificial sea water or in NaCl solution also provided moderate levels of post-cryopreservation survival. Indeed the capacity of the IJs of different *Heterorhabditis* strains to withstand evaporative desiccation appears to be strongly correlated with their amenability to cryopreservation (Table 6). The effect of substituting the glycerol preincubation step by preincubation in various concentrations of DMSO for different time intervals is illustrated in Table 7. Preincubation of HI82, HL81 and *H. megidis* in 8 % DMSO for three days yielded results comparable to those obtained when isolates were preincubated in 15 % glycerol, the maximal survival values ranging from 37.9 *(H. megidis)* to 81.2 % (HI82). Preincubation in DMSO did not permit the successful cryopreservation of *H. zealandica* or the tropical isolate D1 but surprisingly, the post-cryopreservation survival of the Irish isolates K122 and M170 was greatly enhanced by a long (6 to 8 day) preincubation in DMSO.

Discussion

The experiments of Popiel *et al.* (1988) demonstrate that evaporative desiccation followed by immersion in methanol permits the cryopreservation of *S. carpocapsae* IJs. Popiel and Vasquez (1991) subsequently showed that successful cryopreservation of *S. carpocapsae* and *H. bacteriophora* IJs could be achieved by substituting the evaporative desiccation step by a preincubation in glycerol. These authors have also shown that during the preincubation period there is an increase in the glycerol and trehalose content of the IJs which is coupled with a decrease in IJ glycogen levels. These biochemical changes are similar to those which have been observed upon evaporative desiccation in *Aphelenchus avenae* by Madin and Crowe (1975). Trehalose stabilises the lipid bilayer and prevents phase transitions during desiccation and there is much evidence which indicates that this stabilising effect is the result of a direct interaction between the

Table 7. *Survival of* Heterorhabditis *isolates following preincubation in varying concentrations of DMSO for different periods of time followed by treatment in ice cold 70 % methanol and freezing liquid nitrogen on filter paper strips.*

Strain	Length of DMSO Préincubation (days)	Survival (%)*		
		DMSO concentration		
		6 %	8 %	10 %
HL82	2	19.5 ± 2.5	79.3 ± 9.6	44.6 ± 3.5
	3	11.5 ± 3.1	81.2 ± 5.0	56.0 ± 4.6
	4	30.3 ± 5.4	75.5 ± 11.2	55.5 ± 6.8
	6	8.4 ± 6.4	48.9 ± 3.3	60.3 ± 3.9
	8	19.4 ± 2.4	27.8 ± 1.1	31.4 ± 2.7
	10	13.0 ± 4.2	16.0 ± 1.3	22.0 ± 3.0
H. zealandica	2	2.6 ± 0.9	3.4 ± 1.1	0
	3	1.5 ± 0.3	0	0
	4	0.1 ± 0.0	0	0
	6	0	0	0
	8	0	0	0
	10	0	0	0
H. megidis	2	35.1 ± 1.1	38.9 ± 4.0	40.3 ± 1.8
	3	24.7 ± 5.3	37.9 ± 3.7	39.6 ± 5.8
	4	23.6 ± 3.0	46.6 ± 11.9	36.3 ± 4.6
	6	30.5 ± 4.9	32.6 ± 3.5	63.3 ± 14.4
	8	7.3 ± 1.2	23.5 ± 4.9	32.5 ± 6.7
	10	0.8 ± 0.6	14.2 ± 0.7	21.3 ± 5.0
HL81	2	23.4 ± 3.4	44.6 ± 6.0	56.6 ± 5.9
	3	26.6 ± 1.4	63.1 ± 3.7	43.2 ± 2.1
	4	19.2 ± 2.9	50.9 ± 3.9	52.5 ± 1.1
	6	13.5 ± 7.1	46.1 ± 4.2	39.1 ± 6.2
	8	15.9 ± 4.8	25.9 ± 4.6	28.4 ± 3.6
	10	6.0 ± 1.6	10.4 ± 2.4	17.6 ± 4.9
D1	2	0.1 ± 0.0	0.5 ± 0.2	0
	3	0	0	0.8 ± 0.0
	4	0	0	0
	6	0	0	0
	8	0.8 ± 0.3	0	0
	10	0	0	0
K122	2	0.8 ± 0.3	3.2 ± 0.6	20.2 ± 1.7
	3	10.3 ± 4.1	21.6 ± 3.3	24.0 ± 6.0
	4	23.1 ± 7.2	42.2 ± 2.9	22.9 ± 3.4
	6	41.6 ± 5.2	56.6 ± 1.6	54.1 ± 4.0
	8	53.1 ± 6.5	72.8 ± 7.2	55.8 ± 4.0
	10	23.1 ± 3.1	59.3 ± 3.4	21.1 ± 3.2
M170	2	2.7 ± 0.5	9.7 ± 4.9	0
	3	5.9 ± 1.1	10.4 ± 2.6	6.3 ± 2.8
	4	25.1 ± 2.2	18.6 ± 4.7	9.5 ± 6.2
	6	45.2 ± 3.6	47.1 ± 4.9	26.8 ± 10.9
	8	23.7 ± 1.6	35.1 ± 3.4	18.9 ± 5.4
	10	15.6 ± 2.9	14.7 ± 3.5	12.5 ± 2.3

* Each value is the mean ± SE of six replicates.

– OH groups on the trehalose and the phosphatidyl head groups of the membrane phospholipids (reviewed by Crowe *et al.*, 1992). We have found (data not presented) that when old IJs with reduced food reserves are cryopreserved, their post-cryopreservation survival is poor.

The data presented in Table 5 show that *Heterorhabditis* sp. HI82 can be successfully cryopreserved when an evaporative (95 % rh) or osmotic (incubation in NaCl or sea water) desiccation step preceeds the methanol preincubation step. Popiel and Vasquez (1991) postulate that in their protocol, methanol functions as an internal cryoprotectant and we have confirmed their observation that *Heterorhabditis* IJs do not survive freezing when the methanol preincubation step is omitted from the cryopreservation protocol. In addition to inducing the biosynthesis of glycerol and trehalose the evaporative/osmotic desiccation step presumably removes unbound cellular water, thus minimising intracellular ice formation upon cryopreservation. The data presented here indicate that the ease with which *Heterorhabditis* isolates may be cryopreserved is strongly correlated with their desiccation tolerance.

According to Popiel and Vasquez (1991) the increased survival values obtained when *H. bacteriophora* are frozen on filter paper strips indicate that the freezing rate must be rapid to avoid ice crystal formation. The data obtained in our study show that the speed of thawing also greatly influences post-cryopreservation survival. Rapid warming rates are particularly critical for samples frozen in a vitrified state. Above the devitrification point (which ranges from – 120 to – 70 °C) ice crystals which can cause cellular damage, form spontaneously (reviewed by James, 1985). Although equal volumes of nematode suspension were used when the IJs were frozen either as a liquid suspension or on filter paper strips, the greatly enlarged surface to volume ratio provided by the filter paper strips resulted in a more rapid rate of thawing, with consequent improvements in post-cryopreservation survival. The potential for rapid thawing provided by filter paper strips means that the experimenter can actually delay the time to addition of water during the thawing protocol and still record higher post-cryopreservation viabilities than those obtained when IJs are frozen as a liquid suspension.

Popiel and Vasquez (1991) postulated that the strategy of sequential incubation in glycerol and methanol could be optimised for the cryopreservation of all steinernematid and heterorhabditid nematodes. In this report we show that their expectation has been realised for the heterorhabditids and we further suggest that the approach of Popiel and Vasquez could be successfully modified for the cryopreservation of many other genera of nematodes.

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