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Routine Cryopreservation of Isolates of *Steinernema* and *Heterorhabditis* spp.

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Abstract: Infective-stage juveniles of *Steinernema* and *Heterorhabditis* spp. were cryopreserved using two-stage incubation in glycerol and 70% methanol before storage in cryotubes in liquid nitrogen. Optimal glycerol concentrations and incubation times for survival were determined for different species, but acceptable survival of all species and isolates of entomopathogenic nematodes can be obtained using 15% (w/w) glycerol and incubation for 48 hours. Mean survival was 69% for isolates of *Steinernema* and 68% for isolates of *Heterorhabditis* (n = 84). The maximum survival recorded was 97% for *S. feltiae* K254 stored in liquid nitrogen for 12 months.

Key words: cryopreservation, *Heterorhabditis*, nematode, *Steinernema*.

Popiel and Vasquez (2) reported a protocol for the cryopreservation of isolates of *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*, using a two-stage procedure of incubation of infective-stage juveniles in a glycerol solution for 24 hours and then incubation in 70% methanol at 0–1 C for 10 minutes before immersion in liquid nitrogen. Their protocol is a modification of the original evaporative desiccation method (1), which used exposure to 97% RH for 3 days instead of incubation in a glycerol solution prior to the methanol step. The procedure described below is a modification of their protocol and employs lower glycerol concentrations with longer incubation periods, replaces centrifugation with a filtration step to concentrate and to wash the nematodes, and replaces the use of glass slivers or filter paper for storage with more easily handled, commercially available cryotubes. To retain genetic diversity within a nematode isolate, the minimum standard for successful cryopreservation was set at a survival of 25% of the nematodes 24 hours after thawing and a minimum population size of 500 viable infective-stage juveniles.

Materials and Methods

A series of glycerol concentrations and incubation times was tested for representatives of four species, *S. carpocapsae, S. feltiae*, *S. glaseri*, and *H. bacteriophora*. Although not all modifications were tested for all isolates, no change in protocol was adopted if a decrease in survivability was noted for any isolate of any species. Glycerol solutions were prepared in distilled water at 2× final concentration, and equal weights of nematode suspension and glycerol solution were mixed by stirring on a magnetic stirrer to obtain the final concentration. The final weight of samples ranged from 10–100 g. The suspensions were poured into petri-dishes and incubated for 24, 48, or 72 hours at 23 C. After incubation, excess glycerol solution was removed by suction filtration of the nematodes onto a Whatman #42 filter paper (Whatman International Limited, Maidstone, UK) supported in a Buchner funnel. Suction was applied until the surface of the nematode mass no longer appeared glossy. The nematodes were rinsed under suction with approximately 15 ml 70% (v/v) methanol in water (23 C) and immediately washed off the filter paper with cool (5C) 70% methanol into tapered 15-ml glass centrifuge tubes on ice. The nematodes were incubated for 10 minutes on ice, resuspended by shaking after 5 minutes, and allowed to sediment. Aliquots (30 µl) of the concentrated nematode pellet were transferred to cool (5 C), 2-ml, round-bottomed polypropylene cryotubes with silicone rubber seals (Nunc Inter Med, Kampstrup, Denmark). The cryotubes were transferred to storage canes and immediately plunged into liquid nitrogen.

After 24 hours, the frozen nematodes

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were thawed by immersing the opened cryotube in 15–30 ml of Ringer's solution (8 g NaCl, 0.2 g $CaCl_2$, 0.2 g KCl, and 0.2 g $NaHCO_3$ per liter of distilled water) at 23 C. Removal from liquid nitrogen and thawing of the nematodes was completed as rapidly as possible (within 15 seconds) to maximize survival. Survival was measured by determining the percentage of motile nematodes after incubation of the thawed nematodes for 24 hours in Ringer's solution in a petri dish at 23 C.

Five samples of each isolate were frozen in liquid nitrogen (−196 C). The percentage nematode survival of one sample was measured, and the four remaining samples stored. One of the four was designated the canonical sample and stored in a separate liquid nitrogen storage canister.

Results and Discussion

The following optimum glycerol concentrations and incubation times were established for each species: *S. carpocapsae,* 18% for 24 hours; *S. feltiae* and *S. glaseri,* 13.8% for 72 hours; and *H. bacteriophora,* 17% for 72 hours. Representative isolates from all species were able to infect and reproduce in *Galleria.*

With adoption of the glycerol protocol, we have obtained mean survival (range in parentheses) of 69% (31–97) for isolates of *Steinernema* and 68% (30–87) for isolates of *Heterorhabditis* frozen during the last 12 months (n = 84). There was no marked decline in survival associated with long-term storage, and survival was comparable using either the evaporative desiccation or the glycerol incubation procedures. The first isolate cryopreserved in our laboratory, *S. carpocapsae* All, had a survival of 63% after 3 years' storage, compared to 66% survival 24 hours after initial freezing (this isolate was processed using the original evaporative desiccation method). The maximum survival recorded for a glycerol incubated isolate is 97% for *S. feltiae* K254 stored under liquid nitrogen for 12 months (compared to 97% survival 24 hours after initial freezing).

The mean survival after storage for 24 hours for all 167 isolates of entomopathogenic nematode was 57% (25–97); these values were 58% (25–97) and 51% (25–87) for species of *Steinernema* and *Heterorhabditis,* respectively. It was observed that larger nematode species such as *S. glaseri, Steinernema* sp. (NC513), and *Steinernema* sp. (CWL05) consistently had lower survival after thawing; for example *S. glaseri,* 41% (25–67). Within a species there was considerable variation in survival of isolates. This may reflect isolate differences, but even for a single isolate there was considerable variation between batches of nematodes frozen on the same day (e.g., *H. bacteriophora* Cl, 16%, 31%, 56%, and 71%).

Experiments on a range of isolates and species indicated that the thoroughness of the methanol wash after incubation in glycerol and the speed of thawing the nematodes are the two most critical steps. Even slight delays during thawing resulted in a marked drop in survivability. Nematode survival was greatest when the nematode concentration was kept below 50,000 ml, when there was a high surface area to volume ratio during incubation, and when the plates were kept away from direct sunlight. A higher survival rate also was noted if round-bottomed rather than conical-bottomed cryotubes were used and if aliquots of <50 µl were frozen.

Glycerol concentrations and incubation times can be altered to optimize the survival of different species of entomopathogenic nematodes. However, acceptable survival of all species and isolates of entomopathogenic nematodes can be obtained using 15% (w/w) glycerol and incubation for 48 hours, and these conditions are now part of the standard cryopreservation protocol used in our laboratory. One hundred and sixty seven isolates representing all species of entomopathogenic nematodes held in the CSIRO Division of Entomology's culture collection have now been successfully cryopreserved.

Literature Cited

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