Journal of Nematology 27(4):483–485. 1995.

Cryopreservation of *Pratylenchus spp.*

N. J. GALWAY[1] AND J. CURRAN[2]

Abstract: Optimal conditions for cryopreserving of populations of root lesion nematode (*Pratylenchus spp.*) were determined. Nematode survival was achieved using glycerol pre-treatments in the range of 14–17% (w/w). Increasing duration of the incubation in glycerol (up to 5 days) before immersion in liquid nitrogen significantly influenced nematode survival. The highest mean survival for *P. thornei* was 76% after incubation in glycerol for 5 days. Nematodes were able to reproduce and infect carrot disc cultures after cryopreservation. This technique has valuable applications for long-term germplasm storage and maintenance of genetic lines.

Key words: cryopreservation, lesion nematode, *Pratylenchus.*

The benefits of indefinite storage of biological material have been recognized for several decades. Initially, maintaining a specimen or germline for long periods was the major reason for cryopreservation. More recently, it has become a key tool in recording and maintaining genetic diversity in a given population. Cryopreservation has become a reliable and effective means of maintaining lines and is essentially a readily accessible, viable database. It can save time for the continuous culturing of organisms, eliminates the recurring problems of loss of lines through infection or cross-contamination, and ensures the availability and uniformity of material or lines for ongoing research.

Many parasitic and free-living nematodes have been cryopreserved with varying success, using cryoprotective agents (dimethylsulfoxide, ethanediol, glycerol) or partial dehydration approaches that are believed to induce a cryoprotectable state in nematodes (1,2,4,6–8). In the present work, the partial dehydration method that was successfully applied to entomopathogenic nematodes (1,4) was evaluated for cryopreserving the plant-parasitic lesion nematodes (*Pratylenchus spp.*).

MATERIALS AND METHODS

Populations of *Pratylenchus thornei* were maintained on carrot discs following Moody et al. (3). Juvenile and adult nematodes were extracted 3 to 4 months after inoculation by washing the carrot discs with tap water and excess water removed by suction on a Buchner funnel. The method of Popiel and Vasquez (4), first modified by Curran et al. (1), was revised by us to work with the smaller quantities of nematodes usually encountered when working with plant-parasitic nematodes.

We assessed the effects of four glycerol concentrations and five incubation times on nematode survival. Incubations in 14, 15, 16, and 17% glycerol (w/w) were trialed over a period of 1, 2, 3, 4, and 5 days. An equal volume of double-strength glycerol solution, prepared using tap water, was added to the nematode suspension (containing 500–5,000 nematodes per replicate), mixed well, and incubated at room temperature in a 6-cm-d petri-dish.

After the incubation, excess glycerol was removed using a Buchner funnel with a Whatman no. 1 filter paper (Whatman International Limited, Maidstone, UK) to support the nematodes. The filter paper was washed thoroughly with 15 ml of 70% (v/v) methanol (room temperature), and the paper containing the nematodes was placed in a 6-cm petri dish on ice. The filter paper was immersed immediately in 5–7 ml of ice-cold 70% (v/v) methanol. The petri dish was swirled gently to wash the

Received for publication 24 March 1995.

[1] The Cooperative Research Centre for Plant Science, GPO Box 475, Canberra, ACT, 2601, Australia. Email: norag@ento.csiro.au

[2] CSIRO Division of Entomology, P.O. Box 1700, Canberra, ACT, 2601, Australia.

The authors thank Bill Ballard, Greg Lawrence, Graham Crompton, Robin Bedding, and Ursula Grott for reviewing this manuscript.

nematodes off the filter paper and was incubated on ice for 10 minutes. The nematode solution was pipetted into prechilled 1.5 ml microfuge tubes, centrifuged briefly in a cooled microfuge (5 seconds at 10,000 rpm), and 30 µl of the pelleted nematode suspension was transferred to prechilled, 2-ml, round-bottom cryovials (Nunc Inter Med, Kampstrup, Denmark) and immersed in liquid nitrogen.

The sample was thawed after 24 hours cryopreservation by immersing the cryovial in Ringers solution (80% NaCl, 2% $CaCl_2$, 2% KCl_2, 2% $NaHCO_3$) at room temperature. Survival was assessed as nematode mobility 24 hours after thawing. The percentage survival for each treatment was analyzed statistically using ANOVA and LSD following arcsin square-root transformation. Nematode infectivity was assessed by inoculating sterile carrot discs with nematodes 24–48 hours after thawing. Infectivity was also tested after 3 and 12 months storage in liquid nitrogen to determine if nematode infectivity deteriorated after prolonged periods of cryopreservation. Carrot cultures were checked for population growth 1–2 months post-inoculation.

Results and Discussion

There was no significant difference in survival of *P. thornei* within the range 14–17% glycerol (data not shown, ANOVA $P > 0.05$, 95% LSD test). As a consequence, glycerol percentages for each concentration were pooled for the analysis of incubation times.

The number of days of incubation in glycerol had a significant effect on nematode survival (ANOVA $F_{4,55} = 45.3$, $P < 0.01$, 95% LSD intervals). Original data are presented in Fig. 1. The percentage survival of *P. thornei* increased with incubation time from 1 to 4 days. There was no apparent difference in percentage survival for incubations for 4 and 5 days. All nematodes tested 24 hours post-cryopreservation were able to initiate carrot disc cultures as were nematodes cryopreserved for 3 and 12 months. Further experiments will monitor the effects longer-term storage in liquid nitrogen has on nematode viability.

This procedure was tested on three other species of lesion nematode. After 5 days incubation in glycerol, *P. jordanensus*, *P. zeae*, and *P. neglectus* had a mean survival of 50.6% (n = 3), 62% (n = 3), and 67% (n = 2), respectively. The effect of glycerol on percentage survival over the incubation period was consistent for the four species (data not shown).

Observations suggested that better survival was obtained if freshly extracted, healthy juveniles and adults were used. Furthermore, it appeared that more juve-

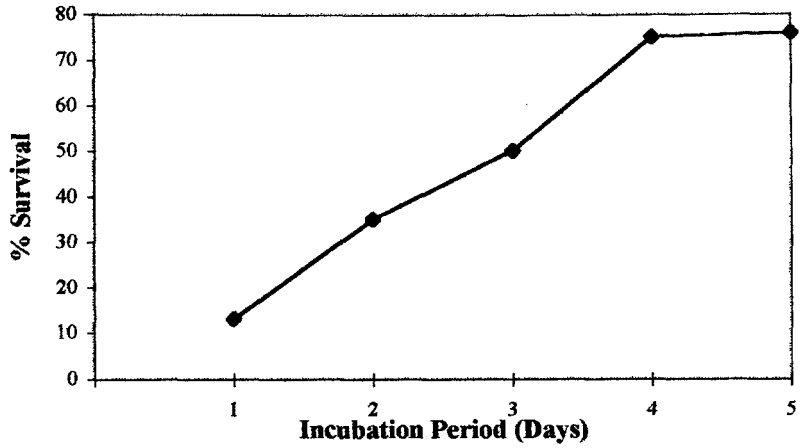

FIG. 1. The percentage survival of *P. thornei* after cryopreservation, as influenced by incubation in glycerol. The viability was assessed by scoring nematode mobility. Data points at day 1, 2, 3, and 4 are significantly different ($P < 0.01$), but there is no significant difference between day 4 and 5 as assessed statistically by ANOVA and LSD following arcsin square-root transformation.

niles than adults survived cryopreservations, as has previously been reported by Riga (5). Nematodes extracted from older carrot cultures or that had been stored at 4 C had lower survival rates than if fresh material was cryopreserved.

This two-step procedure is simple and requires minimal handling steps or equipment. These results establish the potential of this partial dehydration-based cryopreservation protocol for the long-term storage of *Pratylenchus spp.* and illustrates, for the first time, its use for the maintenance of genetic stocks of plant-parasitic nematodes.

Literature Cited

1. Curran, J., C. Gilbert, and K. Butler. 1992. Routine Cryopreservation of isolates of *Steinernema* and *Heterorhabditis spp.* Journal of Nematology 24:269–270.

2. Hwang, S.-W. 1970. Freezing and storage of nematodes in liquid nitrogen. Journal of Nematology 16:305–308.

3. Moody, E. H., B. F. Lownsbery, and J. M. Ahmed. 1973. Culture of the root-lesion nematode *Pratylenchus vulnus* on carrot disks. Journal of Nematology 5:225–226.

4. Popiel, I., and E. M. Vasquez. 1991. Cryopreservation of *Steinernema carpocapase* and *bacteriophora.* Journal of Nematology 23:432–437.

5. Riga, E., and J. M. Webster. 1991. Cryopreservation of the pinewood nematode, *Bursaphelenchus spp.* Journal of Nematology 23:438–440.

6. Sayre, R. M., and S.-W. Hwang. 1985. Freezing and storing *Ditylenchus dipsaci* in liquid nitrogen. Journal of Nematology 7:199–202.

7. Triantaphyllou, A. C., and E. McCabe. 1989. Efficient preservation of root-knot and cyst nematodes in liquid nitrogen. Journal of Nematology 21:423–426.

8. Zuckerman, B. M., M. B. Dicklow, G. C. Coles, and B. Jansson. 1988. Cryopreservation studies on the nematophagous fungus *Drechmeria coniospora.* Revue de Nématologie 11:327–331.