Journal of Nematology 36(3):281–284. 2004.

Effect of Entomopathogenic Nematode Concentration on Survival during Cryopreservation in Liquid Nitrogen

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Abstract: Entomopathogenic nematodes are used for biological control of insect pests. A method for improved cryopreservation of infective juvenile stage nematodes has been developed using *Steinernema carpocapsae* and *Heterorhabditis bacteriophora.* Optimum survival for both species was achieved with 12,000 infective juveniles/ml in glycerol and 7,500/ml in Ringer's solution. For *S. carpocapsae,* maximum survival also was observed with 60,000 infective juveniles/ml in glycerol and 25,000/ml in Ringer's solution. These concentrations resulted in 100% post-cryopreservation survival of *S. carpocapsae* and 100% retention of original virulence to *Galleria mellonella* larvae. This is the first report of achieving 100% survival of an entomopathogenic nematode after preservation in liquid nitrogen. Maximum survival of *H. bacteriophora* following cryopreservation was 87%.

Key words: concentration, cryobiology, cryopreservation, *Heterorhabditis bacteriophora, Steinernema carpocapsae,* survival.

Entomopathogenic nematodes (EPN) (genera: *Steinernema* and *Heterorhabditis*) are biopesticides used for control of a wide variety of economically important insect pests (Grewal and Georgis, 1999; Shapiro-Ilan et al., 2002). These nematodes comprise a diverse group of more than 30 species and numerous strains (Adams and Nguyen, 2002; Poinar, 1990); pest control ability among these species and strains can vary greatly (Shapiro-Ilan et al., 2002). Further, with the aim of improving biocontrol efficacy, new strains are being developed through classical or transgenic methods (Burnell, 2002). Repeated subculturing of EPN, however, can lead to loss of beneficial traits and reduction in pest control abilities (Shapiro et al., 1996; Stuart and Gaugler, 1996; Wang and Grewal, 2002). Therefore, improved methods that preserve the array of EPN genetic materials are critical.

Cryopreservation in liquid nitrogen has been demonstrated as a method for long-term storage of EPN (Curran et al., 1992; Nugent et al., 1996; Popiel and Vasquez, 1991). Storage in liquid nitrogen can hinder (though not prevent) change or loss of EPN biocontrol traits that may occur during subculturing (Wang and Grewal, 2002). Cryopreservation of nematodes in liquid nitrogen is accomplished through preincubation in cryoprotectants (which minimize intracellular and (or) intercellular crystal formation), a precisely controlled rate of freezing, and a controlled rate of thawing (Nugent et al., 1996; Triantaphyllou and McCabe, 1989).

Prior studies have indicated variable levels of survival following cryopreservation of entomopathogenic nematodes. Curran et al. (1992) reported a mean survival rate of 58% (ranging from 25% to 97%) for all 167 isolates of EPN tested, 58% (25% to 97%) for *Steinernema* spp., and 51% (25% to 87%) for *Heterorhabditis* spp. Similarly, Nugent et al. (1996) reported 0 to 83% survival when studying cryopreservation of 25 *Heterorhabditis* strains. For use in research or in field application of EPN, improved cryopreservation techniques for EPN are desirable. Previous studies have demonstrated that survival of different species and strains of EPN following cryopreservation is influenced by length of preincubation period, speed of thawing, and the type and concentration of cryoprotectant used (Curran et al., 1992; Nugent et al., 1996). Our objective was to determine the effects of nematode concentration during the cryopreservation process on their subsequent survival.

Materials and Methods

Source and maintenance of nematodes: Nematodes studied were *Steinernema carpocapsae* (mixed strain) and *Heterorhabditis bacteriophora* (mixed strain). These mixed strains were created by combining two isolates of *S. carpocapsae* (Cxrd from Arkansas and NJ1 from New Jersey) and five isolates of *H. bacteriophora* (Hb 2a-2, Hb 4a-1, Hb-VS, and Hb-V1 from Georgia and Hb RU1 from New Jersey). These mixed "foundation" strains were created as part of a larger series of studies to characterize genetic stability in EPN. The isolates were collected in 2002 (unpubl. data) and passed only twice through the greater wax moth, *Galleria mellonella* prior to experimentation. The nematodes were cultured in last instar of *G. mellonella,* and infective juveniles (IJ) were stored in culture flasks at 13 °C according to procedures described by Kaya and Stock (1997). Nematodes were stored up to 45 days before experimentation.

Effects of nematode concentration on post-cryopreservation survival: General methods for cryopreservation were based on those described by Curran et al. (1992) and Nugent et al. (1996). In all our experiments, 100 ml of IJ were first filtered (Whatman filter paper No. 1) through a vacuum filtration system to remove water. The IJ on the filter paper were immersed in petri dishes (10-cm diam.) containing 18% glycerol solution for *S. carpocapsae* or 13% glycerol solution for *H. bacteriophora.*

Received for publication 7 January 2004.
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The authors thank Kathy Halat and Wanda Evans for technical assistance and Ian Brown and Ed Lewis for reviewing an earlier draft of this manuscript. This research was supported in part by USDA-NRI grant 0201974.
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This paper was edited by S. Patricia Stock.

After 48 hours for *S. carpocapsae,* or 168 hours for *H. bacteriophora,* the IJ were vacuum filtered on a filter paper disc, which was then dipped in pre-chilled 70% methanol (approximately –10 °C) for 10 minutes, and the IJ suspension was filtered again while rinsing with pre-chilled 70% methanol. The filter paper was rolled and placed in pre-chilled cryogenic vials (in NaCl salted ice, about –5 °C), which were held in a pre-chilled cryogenic box and immediately plunged into liquid nitrogen. After 72 hours the vials were removed from liquid nitrogen and thawed by pouring ca. 1.5 ml of Ringer's solution (Kaya and Stock, 1997) (but using $NaHCO_3$ rather than NaH_2CO_3 to make Ringer's solution) into the vial. Nematode survival was determined based on nematode movement response when probed with a dissecting needle (Kaya and Stock, 1997). Incubation periods and cryoprotectant concentrations used in these studies were chosen based on maximum survival observed during preliminary studies (unpubl. data), which used a fixed concentration of IJ (approximately 15,000/ml in glycerol). The observed optimal survival rates in these preliminary studies were mean (±sd) 71 ± 7.0% in *S. carpocapsae* and 43 ± 23.6% in *H. bacteriophora.*

To determine the effects of IJ concentration on survival during cryopreservation treatment, 12 different combinations of concentrations (treatments) of IJ, cryopreserved in glycerol and subsequently thawed in Ringer's solution, were tested (Table 1). Four IJ concentration groups were generated in glycerol solutions for each nematode species and designated as very high (V), high (H), medium (M), or low (L), relative to each other. These groups of IJ concentrations were further subdivided into different concentrations (subgroups) in Ringer's solution and designated high (h), medium (m), and low (l), relative to each other (Table 1). The final volume of Ringer's solution was the same for all treatments (4 ml). All treatments contained three replicates.

The first round of experiments only included the H, M, and L concentration groups (and their subgroups). Results indicated that nematode survival increased with concentration in both glycerol and Ringer's, i.e. the highest concentration (Hh) resulted in the greatest survival. Therefore, to determine the effects of concentrations above Hh, the V group was tested in subsequent experiments (in comparison to the Hh concentration). The percentage of live nematodes (average of four counts of 100 IJ each) was recorded from each vial after thawing in Ringer's solution for 4 hours in the first round of experiments and for 8 hours in the subsequent experiments.

Virulence assays: An additional assay was conducted to determine if the level of IJ virulence was retained in the cryopreservation process. The virulence of *S. carpocapsae* IJ from the concentration that exhibited the highest rate of survival during cryopreservation (Hh) was compared with that of IJ (originating from the same culture flasks) that had been kept at 13 °C. Fifty *G. mellonella* larvae were placed in 150-mm-diam. petri dishes lined with filter paper. Approximately 150 IJ (3 IJ/insect) were applied to each dish in a 3.3-ml water suspension. The experiment contained six replicates per treatment, and the entire experiment was repeated once. Mortality was determined after 24, 48, and 72 hours of incubation at 25 °C.

Statistical analysis: Mean percentage nematode survival following cryopreservation among treatments was compared by ANOVA and Student-Newman-Keuls' test (SAS Institute, Inc, Cary, NC). Mean percentage insect mortality from virulence assays was analyzed with a *t*-test. Percentage data were arcsine-transformed prior to analysis; non-transformed means are presented in tables and figures.

TABLE 1. *Steinernema carpocapsae* (mixed strain) and *Heterorhabditis bacteriophora* (mixed strain) treatment designations (group and subgroup) for infective juvenile concentrations in glycerol prior to immersion, and in Ringer's solution following preservation, in liquid nitrogen.

Groups	Subgroups	Concentration in diluted glycerol solution	Concentration in Ringer's solution
Very High (V)	Vh	60,000	75,000
	Vm	60,000	50,000
	Vl	60,000	25,000
High (H)	Hh	12,000	7,500
	Hm	12,000	5,000
	Hl	12,000	2,500
Medium (M)	Mh	1,200	750
	Mm	1,200	500
	Ml	1,200	250
Low (L)	Lh	120	75
	Lm	120	50
	Ll	120	25

RESULTS

Effects of nematode concentration: Significant differences in *S. carpocapsae* survival were detected among the treatments within and among the high (H), medium (M), and low (L) concentration groups ($F = 285.1$, df = 8,18, $P < 0.0001$) (Fig. 1A). The range of survival (22% to 100%) varied with the concentration of IJ in glycerol and in Ringer's solution. Survival of *H. bacteriophora* similarly increased with increasing IJ concentration within and among the H, M, and L groups ($F = 626.0$, df = 8,18, $P < 0.0001$) (Fig. 1B).

When comparing the H concentration group to the V group, reduced survival in two of the V treatments (Vm and Vh) for *S. carpocapsae* was observed compared with the Hh and VI treatments ($F = 390.6$, df = 3,8, $P < 0.0001$) (Fig. 2A). All three V group treatments of *H. bacteriophora* resulted in lower survival than the Hh treatment ($F = 200.2$, df = 3,8, $P < 0.0001$) (Fig. 2B).

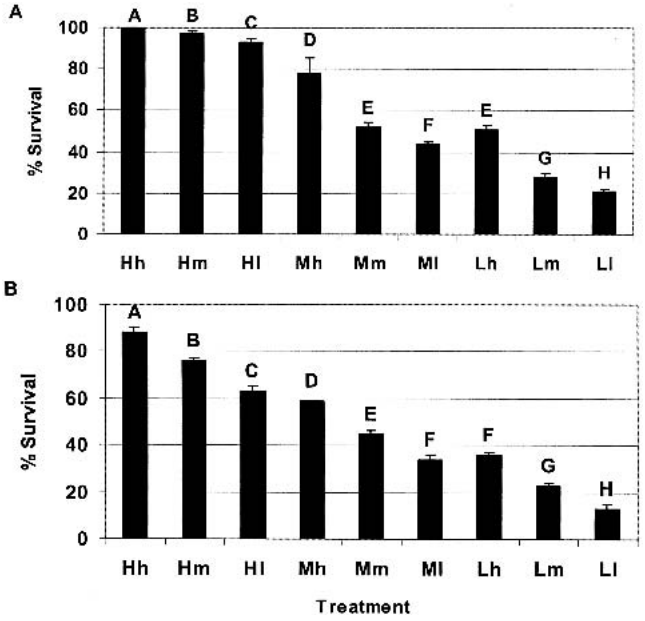

FIG. 1. Effect of nematode concentration on mean survival (±se) of *Steinernema carpocapsae* (mixed strain) (A) and *Heterorhabditis bacteriophora* (mixed strain) (B) following preservation in liquid nitrogen. Infective juvenile nematodes were thawed (25 °C) in Ringer's solution for 4 hours prior to determination of survival. See Table 1 for treatment concentrations. Bars with the same letter above are not significantly different (ANOVA and Student-Newman-Keuls' test, $\propto$ = 0.05).

Overall, the highest *S. carpocapsae* survival was 100%, which was observed in the Hh and VI treatments (Figs. 1A; 2A). Survival for Hh treatment remained 100% to 99.5% for *S. carpocapsae* from 1.5 to 24 hours after thawed IJ were held at 25 °C. The highest level of *H. bacteriophora* survival (87%) was observed in the Hh treatment (Fig. 1B).

Virulence assays: The surviving IJ from the Hh-subgroup retained their original virulence against *G. mellonella* larvae (Table 2). No significant differences in *S. carpocapsae* virulence were detected between nematodes subjected to cryopreservation from Hh subgroup and those from the controls (kept at 13 °C) (T = –0.25, df = 10, P = 0.81 for 24 hours; T = –0.63, df = 10, P = 0.55 for 48 hours; T = –0.42, df = 10, P = 0.68 for 72 h) (Table 2).

Discussion

The survival rate for IJ after storage in liquid nitrogen not only relies on glycerol concentration but is also highly dependent on IJ concentration in glycerol before storage and in Ringer's solution during thawing. Our results further indicate that nematode survival increases with increasing IJ concentration up to an optimum level (e.g., the Hh treatment) and then decreases at higher concentrations. Using an optimum concentration of IJ, we observed no *S. carpocapsae* mortality after liquid nitrogen storage. This level of survival in cryopreservation has not been reported previously for *Steinernema* spp. The maximum observed survival for *H. bacteriorphora* (87%) was somewhat less than for *S. carpocapsae*. We hypothesize that the cause of increased survival post-cryopreservation in some of the higher IJ concentrations (e.g., H group) may be due to an increased overall concentration of natural cryoprotectants (e.g., lipids, trehalose, or glycerol) in the vial; IJ produce trehalose and glycerol as protectants in response to thermo or other environmental stresses (Jagdale and Grewal, 2003; Qiu and Bedding, 2002). However, IJ concentrations that are too high (e.g., the Vh and Vm group in *S. carpocapsae*) reduce survival perhaps due to hypoxia.

Prior studies on cryopreservation of EPN did not address effects of nematode concentration on survival following cryopreservation (Curran et al., 1992; Nugent et al., 1996; Popiel and Vasquez, 1991). Popiel and Vasquez (1991) and Nugent et al. (1996) used concentrations of 5,000 and 2.5 × 10^6 IJ/ml. The concentration used by Curran et al. (1992) was not provided. Concentrations used during thawing in all three of these studies were not clear. Therefore, it is difficult to compare their survival rates with those observed in our study. However, we speculate that some of the variation in survival observed in these earlier studies may have been due to variation in concentration of the cryopreserved IJ.

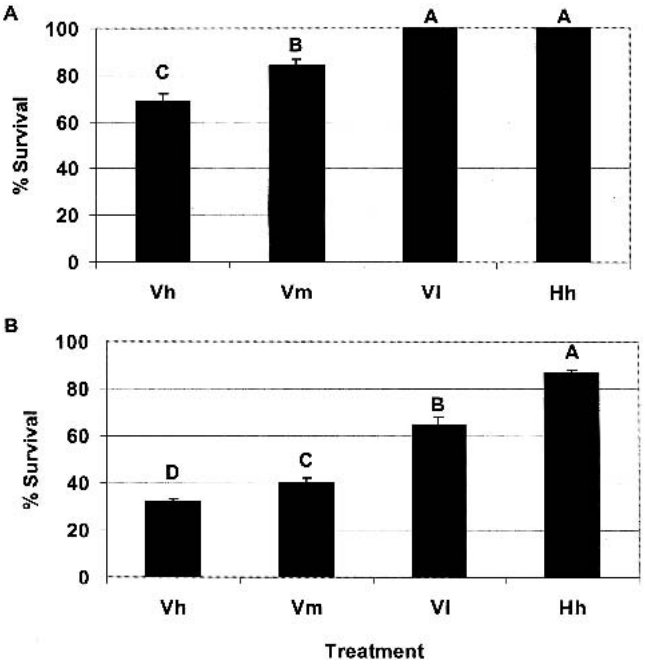

FIG. 2. Effect of very high nematode concentration on mean survival (±se) of *Steinernema carpocapsae* (mixed strain) (A) and *Heterorhabditis bacteriophora* (mixed strain) (B) following preservation in liquid nitrogen. Infective juvenile nematodes were thawed (25 °C) in Ringer's solution for 8 hours prior to determination of survival. See Table 1 for treatment concentrations. Bars with the same letter above are not significantly different (ANOVA and Student-Newman-Keuls' test, $\propto$ = 0.05).

TABLE 2. Nematode-induced mortality of *Galleria mellonella* larvae after exposure to *Steinernema carpocapsae* infective juveniles following cryopreservation in liquid nitrogen.

Treatment[a]	Numbers of nematodes applied per insect	% Mortality (mean ± se)[b] after: 24 hours	48 hours	72 hours
Liquid nitrogen	3	14.3 ± 2.3 C	71.3 ± 2.8 B	80.0 ± 2.7 A
Control	3	13.3 ± 1.1 C	74.0 ± 3.5 B	79.0 ± 1.2 A

[a] Infective juveniles (12,000/ml glycerol solution) were either frozen in liquid nitrogen for 72 hours or kept in culture flasks at 13 °C (control). The nematodes from liquid nitrogen were thawed in Ringer's solution (7,500 IJ/ml) for 4 hours prior to bioassay.
[b] Average of six replicates. Means (±se) followed by the same letter are not significantly different (*t*-test, $\propto = 0.05$).

Cryopreservation of EPN is an established technique to preserve genetic material in both research and commercial practice. We have demonstrated that the concentration of IJ of EPN during the cryopreservation process can have a critical impact on their subsequent survival. Similarly, the initial bacterial concentration (Major et al., 1955; Palmfeldt et al., 2003) and monomeric isocitrate dehydrogenase concentration (from bacteria) (Bai et al., 1999), were positively correlated with the survival or recovery in cold storage at –20 °C and –79 °C, respectively. Our investigation of the effect of organism concentration on survival in liquid nitrogen may have application in improving cryopreservation techniques for other small organisms.

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