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Cryopreservation of the Pinewood Nematode, *Bursaphelenchus* spp.

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Abstract: Populations of three isolates of *Bursaphelenchus xylophilus,* the pinewood nematode, and one of *B. mucronatus* were treated with three cryoprotectants at −70 C for 24 hours followed by deep freezing at −180 C in liquid nitrogen for different periods of time. A solution of 15% glycerol, 35% buffer S, and 50% M9, or 1% aqueous solution of dimethylsulfoxide (DMSO), or a mixture of 60% M9 and 40% S buffer were used as cryoprotectants. A significantly larger number of juveniles than adults survived deep freezing. Significantly more nematodes were motile after cryopreservation in the 15% glycerol–S–M9 solution than in the M9–S buffer solution or the DMSO aqueous solution. When cryopreserved nematodes that had been treated with glycerol solution were plated onto *Botrytis cinerea,* they reproduced rapidly over several generations. Cryopreserved nematodes were as pathogenic as untreated nematodes to Scots pines.

Key words: Bursaphelenchus mucronatus, B. xylophilus, cryopreservation, cryoprotectant, nematode, pinewood nematode, *Pinus sylvestris,* Scots pine.

We have need to maintain cultures of a large number of isolates of *Bursaphelenchus xylophilus, B. mucronatus,* and other closely related species, members of the pinewood nematode species complex (PMNSC)(8), for long periods in order to perform comparative tests between isolates.

Cryopreservation procedures have been explored for the storage of several species of animal-parasitic nematodes and a few plant-parasitic nematodes. *Onchocerca* spp. microfilariae survive deep freezing at −196 C in liquid nitrogen (5), and *Heligmosomoides polygyrus* (= *Nematospiroides dubius*) juveniles are infective to mice after 3.5 years in liquid nitrogen (4). Among the plant-parasitic nematodes, juveniles of *Ditylenchus dipsaci* are infective to alfalfa seedlings after being treated with 7.5% dimethylsulfoxide (DMSO), cooled to −25 C and then stored in liquid nitrogen for 18 months (9). Juveniles of *Meloidogyne graminicola,* preserved in liquid nitrogen with ethanediol as a cryoprotectant, recovered after thawing and reproduced vigorously on rice plants (3).

Thousands of isolates of the soil nematode *Caenorhabditis elegans* are routinely maintained in liquid nitrogen or at −70 C using 15% glycerol as the cryoprotectant (2). The oldest frozen isolates have been maintained for 20 years without loss of viability (D. Riddle, pers. comm.). Nearly 4,000 isolates are maintained in the laboratory collection of Dr. D. L. Baillie in our department at Simon Fraser University. This paper describes the application of *C. elegans* cryopreservation methods for four isolates of the pinewood nematode and demonstrates their subsequent reproductive potential and affect on the host.

Materials and Methods

Three isolates of *B. xylophilus* (St. William, St. John, and Ibaraki) and one isolate of *B. mucronatus* (French) were maintained separately on *B. cinerea* cultured on potato dextrose agar at 27 C. Nematodes, both adults and juveniles, were washed off the culture dishes into a 2.0-ml screw top tube (Simport plastics, T500-2) with 1 ml of one of the three following solutions: 1% aqueous dimethylsulphoxide (DMSO), (BPH Chemicals, code ACS 306); 15% glycerol (Caledon, code 5350-1), 50% M9 buffer, and 35% S buffer as described by Brenner (2); and a 60% M9 and 40% S buffer. Freezing was carried out according to the method of Brenner (2) modified by D. L. Baillie (pers. comm.) as follows: The nematodes

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were stored in tubes for 24 hours at −70 C and then submerged in a 35 VHC liquid nitrogen tank (Union Carbide) at −180 C. This two-step cooling process was preferred to the gradual cooling process in Linde liquid nitrogen refrigerators (2).

Tubes were removed from the liquid nitrogen after 2, 21, 30, and 60 days, the nematodes were thawed at 24 C, and their viability (ability to reproduce and to cause pathogenicity on 2-year-old Scots pine seedlings, *Pinus sylvestris*) was assessed. Those nematodes that were motile after thawing were considered to be viable. Their ability to reproduce was determined by measuring their population increase over 13 days on *B. cinerea* cultured on 1% potato dextrose agar in 9-cm-d petri dishes at 27 C. Nematodes that had been treated with 15% glycerol–S–M9 solution and cryopreserved for 60 days were thawed and transferred from each tube to separate *B. cinerea* cultures, and the numbers of nematodes per culture were recorded 0, 3, 9, and 13 days later. In those cultures where the fungus was destroyed by feeding nematodes, the nematodes were transferred to fresh fungal cultures. There was threefold replication of each experiment and the data were analyzed using the two-sample Mann–Whitney Test (10).

Nematodes of St. William and French isolates that had been cryopreserved for 60 days in 15% glycerol–S–M9 solution and nematodes of the same isolates that had been reared on fungal cultures at 27 C were tested for their pathogenicity on 2-year-old Scots pine. Cryopreserved nematodes were thawed and mass produced on *B. cinerea*, and an inoculum level of 1,000 nematodes per isolate was introduced into the seedlings (7). Ten trees per treatment along with controls of *B. cinerea* filtrate and distilled water and untreated controls were maintained at 27 C for 4 months. The resulting data were subjected to Chi-square analysis at the 95% confidence level (10).

Results and Discussion

More juvenile than adult nematodes survived after deep freezing at −180 C for 2 days, and more juveniles were successfully cryopreserved in 15% glycerol–buffer solution than in DMSO (Table 1). Similarly, more juveniles of the Ibaraki and French isolates survived cryopreservation in 15% glycerol–S–M9 solution than in buffer. The percentage of motile juveniles after freezing at −180 C for 60 days in 15% glycerol–S–M9 solution was not significantly different from the percentage of juveniles that were cryopreserved at −180 C for 2 days (data not shown).

Table 1. Motility of isolates of *Bursaphelenchus xylophilus* and *B. mucronatus* after storage at −180 C for 2 days in one of three solutions.

Isolates	Treated nematodes (N)	Motility (%) Females	Males	Juveniles
15% glycerol + S–M9 buffers				
St. William	14,780	2.1	2.8	17.6 a
St. John	22,410	2.2	0.1	7.7 a
Ibaraki	20,050	0.6	0.4	4.8 a
French	19,870	0.1	0.1	16.2 a
DMSO				
St. William	20,980	1.9	1.4	3.9 b
St. John	22,480	0.1	0.1	1.0 b
Ibaraki	19,900	0.3	0.1	1.3 b
French	21,280	0.1	0.1	2.0 b
S–M9 buffers				
St. William	21,270	0.3	0.2	13.6 a
St. John	20,360	0.1	0	7.4 a
Ibaraki	19,220	0.1	0	5.1 b
French	17,710	0	0	7.4 b

Values for the same isolate followed by different letters are significantly different from each other (95% confidence level).

The pinewood nematode isolates were cryopreserved best in 15% glycerol–S–M9 solution. Successful cryopreservation in buffer solution alone has been reported for microfilariae of *Dirofilaria immitis* (1) and *D. repens* (6). However, PWNSC cryopreservation in buffer alone appeared to be somewhat erratic. The DMSO treatment did not protect the pinewood nematodes during deep freezing. Similarly, cryopreservation of second-stage juveniles of a *Meloidogyne* spp. with DMSO was unsatisfactory (9). All four pinewood nematode isolates were cryopreserved at −180 C equally well for up to 60 days. Despite the high mortality, juveniles survived better

TABLE 2. Nematodes produced at intervals over 13 days on *Botrytis cinerea* cultures after cryopreservation of *Bursaphelenchus xylophilus* and *B. mucronatus* isolates in 15% glycerol–S–M9 solution in liquid nitrogen for 60 days.

Isolates	0	3	9†	13
St. William	3,030	10,560	10,370	19,850
St. John	1,950	20,160	17,660	27,970
Ibaraki	3,210	29,280	16,500	21,740
French	3,900	27,280	17,930	29,960

Values at day 0 are significantly smaller than all other values (95% confidence level).

† Nematodes were transferred to fresh cultures of *B. cinerea* at day 9.

than adult nematodes; following cryopreservation, juveniles were used to initiate new cultures.

The number of nematodes per culture increased 6.5–14.4-fold in 13 days following cryopreservation and transfer to *B. cinerea* (Table 2). Cryopreserved individuals of St. William and French isolates caused 50% and 30% mortality, respectively, of inoculated pine seedlings. Mortality of pine seedlings inoculated with nematodes that had not been cryopreserved was 60% for the St. William isolate and 30% for the French isolate. The control treatments did not cause seedling death. Therefore, cryopreserved pinewood nematodes did not lose their infectivity.

Some 50–90% of the second-stage juveniles of *Meloidogyne* spp. and *Heterodera glycines* that were pretreated with 10% ethylene glycol were successfully cryopreserved in liquid nitrogen and were used to infect tomato or soybean seedlings, respectively (11). The fact that fewer pinewood nematodes than heteroderids survived cryopreservation may be attributed to the use of a different cryoprotectant.

As a consequence of these results, we use freezing mediated with a 15% glycerol–S–M9 solution for the long-term storage of more than 30 PWNSC isolates. It provides secure, reliable storage of large numbers of nematodes so that they are readily available for use. Following cryopreservation and thawing, sufficient numbers of juveniles of any of the isolates are available to initiate new, vigorously reproducing populations within a few days. This is a more cost-effective and reliable method of maintaining cultures of *B. xylophilus* and *B. mucronatus* isolates than is continual subculturing on fungal cultures. Moreover, long-term cryopreservation is less subject to human error than are numerous, frequent passages on fungal cultures.

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