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Preservation of *Meloidogyne hapla* and *M. chitwoodi* in liquid nitrogen : differences in response between populations

Hans J. G. VAN DER BEEK, Wim B. J. VELDHUIS, Carolien ZIJLSTRA and Cor H. VAN SILFHOUT

Research Institute for Plant Protection (IPO-DLO), P.O. Box 9060, 6700 GW Wageningen, The Netherlands.

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Summary – A procedure for long-term preservation of germplasm of *Meloidogyne hapla* and *M. chitwoodi* in liquid nitrogen is described, including a pretreatment with 10 % ethanediol for 2 h at room temperature and 40 % ethanediol for 45 min on ice. Survival rates ranged from 45 to 98 % with an average of 75 %. Comparison of three different populations of *M. hapla,* two populations of *M. chitwoodi* and two populations of *Meloidogyne* n. sp. revealed a significantly higher survival for one *M. hapla* population, while the survival rates of the other six were not significantly different. It was shown that higher lipid reserves in juveniles could possibly explain the high survival of this *M. hapla* population. Juveniles after freezing were able to reproduce on plants, but infectivity was significantly lower than of non-frozen juveniles. It is recommended to multiply juveniles, stored in liquid nitrogen, for one generation before being used as inoculum for experimentation.

Résumé – ***Conservation de* Meloidogyne hapla *et* M. chitwoodi *dans l'azote liquide : différences de réaction en fonction des populations*** – Une procédure est décrite pour la conservation de lignées de *Meloidogyne hapla* et *M. chitwoodi* dans l'azote liquide avec un prétraitement avec de l'éthanediol à 10 % pendant 2 h à la température du laboratoire et avec de l'éthanediol à 40 % pendant 45 min sur la glace. Les taux de survie varient de 45 à 98 % avec une moyenne de 75 %. La comparaison de trois populations de *M. hapla,* de deux populations de *M. chitwoodi* et de deux populations de *Meloidogyne* n. sp. révèle un taux de survie significativement plus élevé pour une des populations de *M. hapla* alors que les taux de survie des six autres populations ne sont pas significativement différents. Il a été démontré que les réserves lipidiques plus importantes chez les juvéniles de cette population de *M. hapla* pourraient expliquer son taux élevé de survie. Les juvéniles sont capables de se reproduire aux dépens des plantes après congélation, mais leur pouvoir infestant est significativement plus faible que celui des juvéniles n'ayant pas été congelés. Il est recommandé de multiplier le nématode à partir des juvéniles conservés dans l'azote liquide pendant une génération avant utilisation comme inoculum pour l'expérimentation.

Key-words : cryopreservation, cryoprotectant, ethanediol, freezing, infectivity, lipid staining, root knot nematode, survival.

Long-term cryopreservation of obligate plant parasitic nematodes would be advantageous over preservation on plants, because the latter is laborious and demands much greenhouse space and care to avoid contamination. Additionally, certain genetic studies require maintenance of the original populations or certain generations in order to preserve genetic variation. In general, cryopreservation seems to be a suitable method for long-term preservation of nematodes without substantial losses in survival and viability (e.g. Sayre & Hwang, 1975; Bridge & Ham, 1985).

Already in 1920, Rahm (1921, 1922) had carried out cryobiological experiments with the nematodes species *Plectus rhizophilus* and *P. parietinus*. In a condition of asphyxia, *Plectus* spp. could survive exposure to – 272 °C for a few hours and to – 192 °C for as long as 5 days. Rahm (1921) found also that, in moist condition, *Plectus* spp. could survive up to 24 h at – 253 °C, but only if the nematodes were first slowly frozen in water. It was hypothesised that cold could be a stimulus to bring the nematodes to an asphyctic condition, which is favourable for successful freezing at extremely low temperatures.

Further experiments with liquid nitrogen, at – 196 °C, were performed by De Coninck (1951) with the free living nematode species *Anguillula silusiae*. He found 5 % survival of third and fourth stage juveniles, using a two-step procedure with – 30 °C and – 196 °C without adding a cryoprotectant.

Gehinio and Luyet (1951) were able to induce a " cold hardening " effect by exposing vinegar eelworms *(Turbatrix aceti)* for 16 h at 95 % relative humidity causing a dehydration of the nematodes, which resulted in a survival of 90 % after freezing at – 77 °C for 1 h. Freezing techniques were improved by the application of cryoprotectants of which the four most common are : methanol, ethanediol (= ethylene glycol), glycerol and dimethyl sulphoxide. Different genera and sometimes

even different species within a genus react differently to these cryoprotectants. Several methods were described which provided various percentages of survival of the following nematode species : 56 % survival of *Aphelenchoides sacchari,* 63 % survival of *Caenorhabditis briggsae,* 87 % survival of *Panagrellus redivivus* and 40 % survival of *Turbatrix aceti* (Hwang, 1970), 23-35 % survival of *Ditylenchus dipsaci* (Sayre & Hwang, 1975), 75 % survival of *Caenorhabditis briggsae* (Haight *et al.*, 1975), 25 % survival of *Meloidogyne graminicola* (Bridge & Ham, 1985), 50-90 % survival of *Meloidogyne* spp. (including *M. hapla*) and *Heterodera* spp. (Triantaphyllou & McCabe, 1989), 30-34 % survival of *Steinernema feltiae* (Smith *et al.,* 1990), 80 % survival of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* (Popiel & Vasquez, 1991), 17 % survival of *Bursaphelenchus* spp. (Riga & Webster, 1991) and 69 % for *Steinernema* spp. and 68 % for *Heterorhabditis* spp. (Curran *et al.,* 1992). For many *Meloidogyne* species ethanediol was found to be effective (Bridge & Ham, 1985; Triantaphyllou & McCabe, 1989). However, freezing techniques for *M. chitwoodi* have not yet been reported.

Besides the choice of a competent cryoprotectant, the duration and temperature at pretreatment, condition (age) of the nematodes, and the speed of freezing and thawing are factors determining the efficiency of the method.

The present study is aimed at verifying and optimizing the procedure described by Triantaphyllou and McCabe (1989), in order to find a quick and effective method for long-term preservation of *M. hapla* and *M. chitwoodi.* Special attention was paid to the effect of storage in liquid nitrogen on the infectivity of these two species and on possible differences between populations of *M. hapla* and *M. chitwoodi.*

Materials and methods

Nematode populations

Three populations of *Meloidogyne hapla :* Hb, Hc and Hl, two of *M. chitwoodi :* Ce and Ci and two of *Meloidogyne* n. sp. (Table 1), were used in this study. The *M. hapla* and *M. chitwoodi* populations were found to be true to species by isozyme electrophoresis, as described by Esbenshade and Triantaphyllou (1990). *Meloidogyne* n. sp. populations Cf and Cg showed isozyme patterns deviating from the described patterns for *M. chitwoodi,* indicating that they belong to the " Baexem " type of *M. chitwoodi,* as mentioned by Van Mechelen *et al.* (1994). This new species is currently under description (Karssen, pers. comm.).

Experiments for optimization of the freezing technique were mainly carried out with 2nd stage juveniles (J2) of *M. hapla* population Hl. All populations were maintained at IPO-DLO on *Lycopersicon esculentum* cv. Moneymaker for several generations. Only J2 of an age of 1-2 days after hatching were used for experimentation.

Table 1. *Origin of the Dutch populations of* Meloidogyne hapla, M. chitwoodi *and* Meloidogyne *n. sp.*

Population	Species	Origin	Provided by
Hb	*M. hapla*	Zwaanshoek	PD*
Hc	*M. hapla*	Bavel	PD
Hl	*M. hapla*	unknown	CPRO**
Ce	*M. chitwoodi*	unknown	PD
Cf	*M.* n. sp.	Baexem	PD
Cg	*M.* n. sp.	Vredepeel	PD
Ci	*M. chitwoodi*	Maasbree	PD

* PD = Plant Protection Service, Wageningen, The Netherlands.
** CPRO = Centre for Plant Breeding and Reproduction Research, Wageningen, The Netherlands.

Two-step pretreatment with ethanediol

Ethanediol was used because of its effectiveness in earlier research (Bridge & Ham, 1985; Triantaphyllou & McCabe, 1989). Pretreatment was applied in two steps, as suggested for *Meloidogyne* spp. by Triantaphyllou and McCabe (1989). The J2 were first incubated in approximately 10 % ethanediol at room temperature, i.e., 22-24 °C (first step) then incubated in approximately 40 % ethanediol on ice (second step).

The following general procedure was followed : 100-500 J2 were placed in 100 ml suspension in an Eppendorf vial. For the first step, 900 ml 10 % ethanediol was added. Just before the second step, the vials were centrifuged at 9000 *g* using a top-bench centrifuge for 0.5 min, and the upper layer was removed leaving a volume of 80 ml. 80 ml 70 % ethanediol (0 °C) was added while on ice, which resulted in a final concentration of ethanediol of approximately 40 %.

This general procedure was optimized by studying the best periods of pretreatment in the first and second steps. The duration of the first step of the pretreatment was optimized in three experiments with four or five replications. Twelve different periods were tested : 15, 30, 45, 60, 90 min, 2, 2.5, 3, 4.5, 6, 7.5 and 17 h, followed by a second step of 45 and 90 min. For optimization of the second step, five different periods were applied in an experiment with five replications : 15, 30 45, 60 and 90 min, preceded by a first step of 2 or 17 h.

Three-step pretreatment

To study the effect of 50 % ethanediol on toxicity and protection, an experiment with six replications was done with a three-step pretreatment : from 10 to 30, and finally to 50 % ethanediol. Pretreatment with 30 and 50 % ethanediol was applied at 0 °C. In this experiment, the period for the first step was set at 2 h while the second and the third steps lasted 45 and 45 min, 60 and 30 min and 60 and 45 min, respectively.

Freezing, storage and thawing

For freezing, 100 ml pretreated J2 suspension was quickly put onto 0.8 × 4.0 cm pre-chilled strips of filter paper (Whatman No 3) and placed in 1.8 ml Nunc cryotubes already filled with liquid nitrogen. The tubes remained uncovered during storage and were attached to holders containing a maximum of six tubes. Contact of the filter paper strip with the liquid nitrogen caused an immediate adhesion of the J2 to the filter paper, preventing loss of J2 and possible contamination. For experimentation purposes, juveniles were stored for 2 h in liquid nitrogen.

Juveniles which have been stored in liquid nitrogen are called hereafter " cryo-juveniles ". Thawing of cryo-juveniles was done by quickly transferring the paper strips to Petri dishes containing 15 to 20 ml water at approximately 35 °C.

Survival was estimated 24 h after thawing by the number of motile cryo-juveniles per total cryo-juveniles.

Infectivity of cryo-juveniles

The infectivity of cryo-juveniles, i.e., the ability of cryo-juveniles to cause infection, was estimated in terms of reproduction. This was done in two experiments by inoculating 2 to 3 week-old plants of *Lycopersicon esculentum* cv. Moneymaker, a tomato cultivar susceptible to *Meloidogyne* species. The plants were grown in open-ended plastic tubes of 96 ml filled with moist silver sand, which received a nutrient solution (Boukema *et al.*, 1984). These tubes were placed in a growth chamber with constant temperature of 20 °C and relative humidity of 70 %.

In the first experiment, with *M. hapla* population Hl, fifteen plants were inoculated with 5 ml each of the suspension of cryo-juveniles and five plants with untreated J2 of population Hl with known concentrations varying from 75 to 200 nematodes ml^{-1}. In the second experiment, with *M. hapla* population Hc and *Meloidogyne* n. sp. population Cf, ten plants were inoculated with 5 ml suspension of cryo-juveniles and five with untreated J2 of these two populations containing 20 nematodes ml^{-1}. Eight to 9 weeks after inoculation, infectivity was estimated by the following three reproduction parameters : number of egg masses after one generation per initial number of J2, number of eggs per egg mass and number of hatched J2 after one generation per initial number of J2 *(Pf/Pi)*. Egg masses were counted visually. Eggs and J2 were counted on a Context Vision image analyzer, using a nematode count programme for J2, as described by Been *et al.* (1996), or a modified version for egg counting. In the next generation, 400 hatched juveniles from each of the twenty plants in the first experiment, were used for the inoculation of twenty other plants, in order to study infectivity of J2 that were hatched from egg masses originating from cryo-juveniles.

Effect of storage time on survival

The effect of freezing periods varying from 15 min to 2 weeks on the survival after storage was studied in two experiments with five replications.

Effect of short thawing intervals on survival during and after storage

The influence of a short interruption in the freezing process on the J2 was examined by exposing the paper strips for different periods ranging from 0 to 60 s to room temperature. Also, the necessity of a quick transfer of the paper strips from the liquid nitrogen to the Petri dish with water was verified by exposing the paper strips during different periods ranging from 1 to 60 s to room temperature before this transfer. These two experiments were performed with four replications.

Effect of pretreatment on survival and infectivity

In an experiment with seven replications, the effect of pretreatment only, without additional freezing, on the survival and on the infectivity was examined. As a control, pretreatment was followed by storage in liquid nitrogen. Plants of cv. Moneymaker were inoculated with 5 ml of suspensions containing 20 nematodes ml^{-1}. Infectivity was estimated as described above.

Variation in survival between and within *M. hapla*, *M. chitwoodi* and *Meloidogyne* n. sp.

In three experiments, the seven populations mentioned in Table 1 were tested for variation between the three *Meloidogyne* species and between the different populations. In the first experiment Hc and Cf were tested, in the second : Hb, Hl, Ce, Cg and Ci and in the third : Hb, Hc, Cf and Cg. The experimental design was a complete block with five replications. Survival was estimated after 24 h of cryo-preservation as the percentage motile cryo-juveniles.

Neutral lipid staining of J2

Neutral lipid reserves were estimated for nematode populations Hb, Hc, Cg and Ce by a histochemical lipid staining procedure of individual J2 using Oil-Red-O, as described by Storey (1984) for *Globodera* spp. Relative values for neutral lipid reserves were obtained by measuring the red coloured area inside the nematodes by image analysis, for approximately ten J2 per population.

Statistical analysis

Means were compared by analyses of variance, using the Genstat programme (Payne *et al.*, 1989). Testing differences of means was done with a multiple range test at 5 % confidence interval.

Results

Two-step pretreatment with ethanediol

The two steps in the pretreatment with the cryoprotectant ethanediol were studied in detail for *Meloidogyne hapla* population Hl, in order to find optimum protec-

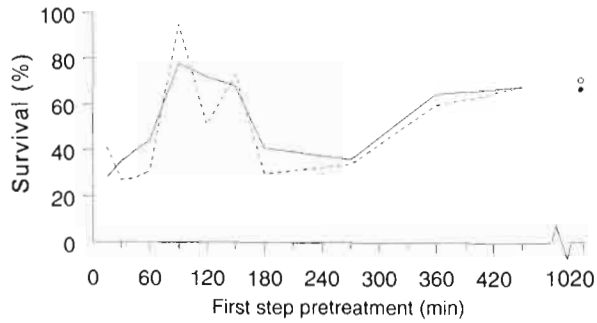

Fig. 1. *Means survival rates after storage in liquid nitrogen of J2 of* Meloidogyne hapla *population Hl, for different periods of the first step of the pretreatment with ethanediol, with a 45 (continuous line, ●) and at 90 min (dotted line, ○) second step.*

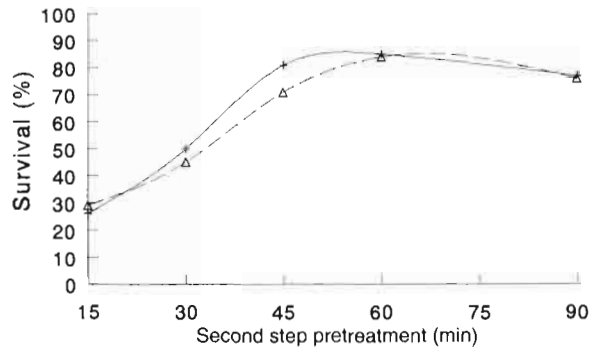

Fig. 2. *Means survival rates after storage in liquid nitrogen of J2* Meloidogyne hapla *population Hl, at different periods of the second step of the pretreatment with ethanediol, with a 2 h (+) and at 17 h* n*) first step.*

tion of the J2. Results for various periods at the first step indicated significant differences between periods. The course of the survival rate at the second step of 90 min was largely below that at 45 min second step but in only one of the three experiments, this tendency was statistically significant (Fig. 1).

An average survival of 94 % was obtained with a pretreatment consisting of a 90 min first step and a 90 min second step. However, there was more fluctuation in survival with a 90 min rather than a 45 min second step. A more reliable and repetitive result was obtained with a 45 min second step pretreatment. The highest average survival with the 45 min second step was 78 % at 90 min first step. In the 45 min second step, no significant differences between survival rates were found in first steps lasting 90, 120, 150 min, 6, 7.5 and 17 h. Means of survival for these periods fluctuated between 65 and 78 %. In general, the confidence intervals for the means for these periods were smaller than for other periods.

A significantly lower average survival of 28 to 44 % was obtained at periods of the first step shorter than 90 min and of 3 and 4.5 h.

It was demonstrated that both steps of pretreatment were indispensable for high survival rates. A pretreatment of a single step of 17 h resulted in only 2 % survival, and direct application of the second step during 90 min gave 27 % survival.

In Fig. 2 survival rates are shown at various periods of second step pretreatment. No significant difference was obtained for survival between different periods when pretreated during 2 h and 17 h at the first step. Survival rates at short periods of 15 and 30 min second step treatment were significantly lower than those at periods of 45, 60 and 90 min. Optimum survival of approximately 80 % was obtained for these last three periods.

Three-step pretreatment

Survival at a three-step pretreatment with different periods of 30 and 50 % ethanediol were not significantly different from those at a two-step pretreatment.

Infectivity of cryo-juveniles; variation in infectivity between nematode populations

The storage in liquid nitrogen induced a significant decrease in *Pf/Pi*-values and number of egg masses per inoculated J2 (Fig. 3). These diminutions varied between the three populations Hl, Hc and Cf that were used in this experiment. However, the number of eggs per egg mass was less influenced than the two other parameters. Only for population Hl was the number of eggs per egg mass of cryo-juveniles significantly less than that of non-frozen J2. In another experiment with population Hl however, no significant differences (Table 2) were observed between eggs per egg mass of cryo-juveniles and untreated J2.

No significant differences between infectivity of the second generation cryo-juveniles and non-frozen J2 were found (Fig. 3B).

Effect of storage time on survival

No significant differences were found between means of survival for the period of storage tested. The overall means for the two experiments were 72.2, 74.1, 72.1 and 79.1 ($Isd_{0.5} = 11.3$) for 15 min, 2 h, 1 day and 2 weeks respectively.

Effect of short thawing intervals on survival during and after storage

For thawing, it is important to know whether short intervals of temperatures above 0 °C during storage and shortly before thawing have an effect on the survival (Table 3). During storage, when the cryo-juveniles on paper strips were exposed 15 s or longer to room temperature, there was a significant decrease in survival of the juveniles; 1 min exposure caused a decrease in survival of 68 %. A comparable effect was obtained when paper strips were exposed to room temperature directly after storage and just before thawing : also there was a significantly lower survival at 15 s exposure to room temperature, and almost no survival of the J2 after 1 min.

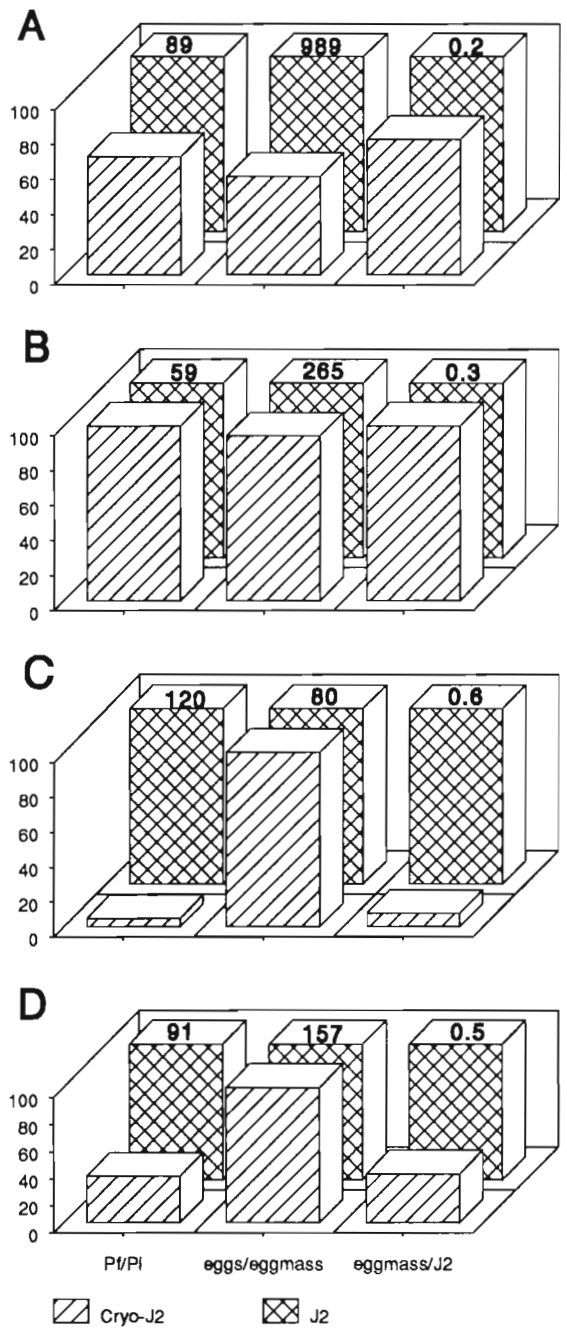

Fig. 3. *Effect of storage of J2 in liquid nitrogen on three parameters :* Pf/Pi, *number of produced eggs per egg mass and number of produced egg masses per inoculated J2, expressed as percentages of the value of these parameters for non-frozen, untreated J2 (Y-axis : percentages).* A : M. hapla *population Hl;* B : M. hapla *population Hl, after one generation of multiplication of the frozen and non-frozen J2.* C : M. hapla *population Hc;* D : Meloidogyne *n. sp. population Cf. (The numbers on top of the double-shaded bars correspond to the mean values for the parameters as estimated for the non-frozen, untreated J2).*

Table 2. *Effect of pretreatment with and without storage in liquid nitrogen on survival and infectivity of J2 of* Meloidogyne hapla *population Hl.*

Variable	J2*	Pretr.**	Pretr./N_2***
Survival (%)	88 *b*	95 *b*	64 *a*
Egg masses/inoculated J2	0.4 *b*	0.5 *b*	0.2 *a*
Eggs/egg mass	114 *a*	169 *b*	104 *a*
Produced J2/inoculated J2	88 *b*	98 *b*	32 *a*

* = J2 without pretreatment (control).
** = Only pretreatment, as described in text.
*** = Pretreatment followed by storage in liquid nitrogen.
For each variable, data followed by the same letter are not significantly different at $P < 0.05$.

Table 3. *Effect of short time periods at room temperature on the survival of frozen J2 of* Meloidogyne hapla *population Hl during and after storage in liquid nitrogen.*

Time period(s)	Survival (%)	
	during*	after**
0	94 *c*	–
1	–	94 *d*
5	86 *c*	–
15	64 *b*	74 *c*
30	63 *b*	18 *b*
60	32 *a*	4 *a*

* = Paper strips were taken out of the liquid nitrogen during storage.
** = Paper strips were taken out after storage, prior to thawing.
In each column, data followed by the same letter are not significantly different at $P < 0.05$.

Effect of pretreatment on survival and infectivity

Comparison of survival of pretreated J2 with cryo-juveniles that were pretreated and exposed to liquid nitrogen for storage, resulted in significant differences for all four parameters studied (Table 2). Furthermore, no significant differences occurred between the pretreated J2 and non-pretreated J2 (control), except for a higher number of eggs per egg mass for the pretreated J2, indicating that the applied pretreatment with ethanediol had no toxic effect on J2.

Variation in survival between and within *M. hapla*, *M. chitwoodi* and *Meloidogyne* n. sp.

Variation in survival between populations of *M. hapla*, *M. chitwoodi* and *Meloidogyne* n. sp. was studied in three different experiments in which no interaction effects were observed between populations and frozen or non-frozen J2, but only differences in level of survival. Results of the three experiments with populations Hb,

Hc, Hl, Ce, Cf, Cg and Ci showed no significant differences in survival between the three species. However, differences between populations were demonstrated : survival was significantly higher for population Hc than for the other six populations (Fig. 4). No significant differences were found between the remaining six populations.

NEUTRAL LIPID STAINING OF J2

Analysis for lipid reserves showed that percentages area of lipid reserves of individual J2 were significantly higher for *M. hapla* population Hc than for *M. chitwoodi* population Ce and *Meloidogyne* n. sp. population Cg, but not significantly different from population Hb (Table 4).

Discussion

A two-step procedure using ethanediol as cryoprotectant has been described by several authors (Ham *et al.*, 1981; James, 1981; Bridge & Ham, 1985; Triantaphyllou & McCabe, 1989). This procedure has been proven to be successful in overcoming problems in penetration and toxicity of the cryoprotectant, which are related to temperature (Ham *et al.*, 1981).

Periods for the first and the second step of the pretreatment with ethanediol were optimized in this study for cryopreservation of J2 of *Meloidogyne hapla, M. chitwoodi* and *Meloidogyne* n. sp. Results from experiments with *M. hapla* population Hl showed, in the second step, a curve with a maximum after at 45 min, indicating 45 min to be the optimum period (Fig. 2). Periods for the first step treatment shorter than 90 min resulted in a significantly lower survival rate than at 90-150 min, indicating that penetration of ethanediol in the J2 during periods less than 90 min was probably insufficient to provide optimum protection for the nematodes.

Unexpectedly, however, results for the first step treatment were not unambiguously clear : with a 3 and 4.5 h first step, survival was significantly lower than at longer or shorter periods, resulting in a depression in the optimum curve (Fig. 1). This temporal pattern of survival was observed in both experiments with the second step treatment. Ethanediol can cause toxic effects (James, 1981) and apparently this is the case with a first step of more than 2.5 h, resulting in a low survival after freezing. Regaining a higher survival rate after pretreatment of 6 h or longer is harder to explain. To our best knowledge, this phenomenon during first step pretreatment has not been reported in the literature : most authors describe experiments with pretreatments of 4 h maximum. Triantaphyllou and McCabe (1989) found that at 3 and 4 h first step pretreatment of J2 of *Meloidogyne incognita,* survival was not significantly less than at 1 and 2 h. Most likely, the difference between their result and ours is due to physiological differences between nematode species and populations. Based on the present study, it is recommended to apply a two-step pretreatment with incubation periods of 2 h (± 30 min) for the first and 45 min for the second step. These periods of pretreatment were used for the other experiments described in this paper. Survival rates ranged at the recommended pretreatment from 45 to 98 % with an average of 75 % in ten experiments with *M. hapla* population Hl, which is comparable with the results of Triantaphyllou and McCabe (1989) for *M. incognita.*

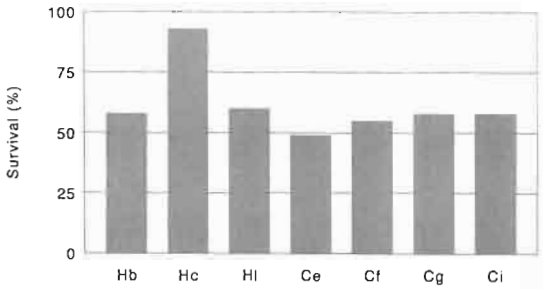

Fig. 4. *Means survival rates after storage in liquid nitrogen of J2 of three populations of* Meloidogyne hapla *(Hb, Hc and Hl) two of* M. chitwoodi *(Ce and Ci) and two of* Meloidogyne *n. sp. (Cf and Cg).*

Table 4. *Relative values for neutral lipid reserves and values for survival after storage in liquid nitrogen of two* Meloidogyne hapla *one* M. chitwoodi *and one* Meloidogyne *n. sp. populations.*

Population	Rel. lipid area (%)	Survival (%)
Hb	67 *a*	58 *b*
Hc	58 *a*	93 *a*
Cg	45 *b*	58 *b*
Ce	29 *c*	49 *b*

In each column, data followed by the same letter are not significantly different at $P < 0.05$.

Short periods of exposure of frozen J2 to room temperature during and immediately after the storage phase can be fatal for the nematodes. Based on the present study, it is recommended not to exceed a 10 s exposure of frozen J2 to room temperature. To minimize the risk of substancial losses, it is suggested to use storage units containing only a few paper strips which can be quickly taken out from and replaced in the storage container.

Survival did not change significantly with different storage periods. Although the tested periods were relatively short (from 15 s to 2 weeks), it is concluded that no marked decrease in survival is to be expected after long periods of storage in liquid nitrogen. Curran *et al.* (1992) found no significant difference between 24 h and 3 years storage in liquid nitrogen of *Steinernema carpocapsae.*

The recommended two-step pretreatment with ethanediol had no toxic effect on J2, as measured by their survival and infectivity (Table 2). Consequently, the decrease in survival and infectivity of cryo-juveniles is en-

tirely due to the freezing, storage and thawing procedure. If required, improvement of the survival and infectivity could only be obtained by refining the freezing, storage and thawing phase. Pretreatment of J2 resulted in an unexpected, significant increase in the eggs per egg mass ratio compared to the non-pretreated J2 (Table 2). Apparently, ethanediol stimulates egg mass filling. This conclusion needs to be verified in further experiments.

The recommended two-step pretreatment with ethanediol for long-term storage in liquid nitrogen has been applied to three populations of *M. hapla,* two of *M. chitwoodi* and two of *Meloidogyne* n. sp., indicating no significant difference in survival between the three species but a significant difference between population Hc and the other populations (Fig. 4). It is unlikely that this was due to the variation in survival, resulting in the range of 45 to 98 % as described above, because two unrelated experiments showed similar high survival rates for Hc. Also Curran *et al.* (1992) found considerable variation in survival of isolates of *Steinernema* spp. Analysis of neutral lipid reserves indicated that higher lipid reserves in individuals of population Hc could explain the significantly higher survival (Table 4). For certain nematode species it is known that higher concentration of trehalose, lipid, and/or glycogen occur when periods of desiccation have to be overcome (Womersley *et al.*, 1982). The authors concluded that the main purpose of lipid in free-living and plant-parasitic nematodes is to provide food reserves which can either be used during periods of environmental stress or at the induction of, and revival from, the anhydrobiotic state. Storey (1984) found a correlation between neutral lipid reserves and mortality (at 20 °C), mobility and infectivity of J2 of *Globodera* spp. In order to explain the high survival rate of population Hc after freezing, as found in this study, it was hypothesized that the survival after freezing could be associated with the neutral lipid reserves of nematode populations. However, satisfactory evidence for this hypothesis was not obtained because of the high value for lipid reserves of population Hb as well. More populations and more individuals per population need to be tested for neutral lipid reserves to provide full evidence for this hypothesis.

Variation was demonstrated not only for survival, but also for infectivity, which was studied in three populations : Hc, Hl and Cf (Fig. 3). The decrease in *Pf/Pi* and number of egg masses per inoculated J2 was very high for Hc (approximately 95 %) and considerably lower for Hl and Cf (30 and 60 %, respectively). In spite of the relatively high survival rate of population Hc (Fig. 4), low values for reproduction parameters were found for this population. It is concluded that survival is not positively correlated with number of produced egg masses and number of produced J2 per inoculated J2. Because of the large variation in the decrease of these parameters, it is recommended to store large quantities of J2 and to use J2 from a second generation of cryo-juveniles as inoculum for tests of virulence.

It is concluded that the described procedure for cryopreservation provides a suitable method for long-term preservation of *M. hapla, M. chitwoodi* and *Meloidogyne* n. sp. germplasm, which can be applied to maintain large numbers of populations for collection purposes, to preserve genetic variation for genetic studies, to provide possibilities for testing different generations simultaneously, and to minimize contamination which is a considerable risk in cultures on plants.

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