

# Cryopreservation of *Radopholus similis*, a tropical plant-parasitic nematode

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## Abstract

For obligate plant-parasitic nematodes, cryopreservation has advantages over the usual preservation methods on whole plants or axenic culture systems, because the latter two are laborious and time and space consuming. In addition, cross contamination among different isolates can occur easily. Moreover, specific genetic studies require maintenance of the original population. The nematode under investigation, *Radopholus similis*, is a plant-parasitic nematode from the humid tropics. Therefore, any treatment at low temperatures is likely to add extra stress to the nematode, making the development of a cryopreservation protocol extremely difficult. In this paper, we describe experiments to achieve a successful cryopreservation protocol for the tropical nematode *R. similis* using vitrification solution-based methods based on a well defined mixture of cryoprotectants in combination with ultra-rapid cooling and thawing rates. A two-step treatment was used consisting of an incubation in glycerol followed by the application of a vitrifying mixture of methanol, glycerol and glucose. After cryopreservation, the pathogenicity of the nematodes was not altered, since they could infect and reproduce on carrot discs after recovery in the Ringer solution. The cryopreservation method described can be used for routine cryopreservation of *R. similis* lines from different origins.

**Keywords :** Cryopreservation ; *Radopholus similis* ; Migratory endoparasitic nematode ; Vitrification

## 1 Introduction

Nematodes, the tiny roundworms that make up the phylum Nematoda, are among the most abundant creatures on earth. Most nematodes are free-living and feed on bacteria or other microscopic organisms. Other species are parasites of plants or animals. Plant-parasitic nematodes can devastate a wide range of crop plants, causing billions of dollars in agricultural losses each year [20,29]. All plant-parasitic nematodes are obligate parasites, feeding

exclusively on the cytoplasm of living cells. The maintenance of stock cultures of obligate plant-parasitic nematodes on their respective host plants or on monoxenic culture systems over a long time is often difficult. Preservation on plants or on axenic culture systems, are laborious and time and space consuming. In addition, cross contamination among different isolates and also bacterial or fungal contamination can occur easily and certain genetic studies require maintenance of the original population. Wang and Grewal [34] reported a rapid deterioration of environmental tolerance and reproductive potential of an entomopathogenic nematode during normal laboratory maintenance.

Cryopreservation or preservation of cells, tissues or even whole organisms by cooling to low sub-zero temperatures such as  $-196\text{ }^{\circ}\text{C}$  (the boiling point of liquid nitrogen) can be considered as the ultimate storage method. Liquid nitrogen is easily obtainable, economical, and essentially all life processes are arrested at its boiling point. For the nematode *Steinernema carpocapsae* it was shown that there was no significant difference in post-thaw survival whether they were stored in liquid nitrogen for 24 h or 3 years [7]. Obviously, most biological materials will not survive an immediate submersion in liquid nitrogen. For that reason, they must undergo several preparative steps before storage at  $-196\text{ }^{\circ}\text{C}$ . Generally, successful cryopreservation comprises six essential steps: (1) an appropriate pretreatment to prepare the biological material to survive dehydration, (2) a water content reducing step, (3) freezing with an appropriate (rapid) cooling rate, (4) storage in liquid nitrogen, (5) thawing with an appropriate (rapid) thawing warming rate and (6) recovery.

The reduction of the amount of freezable intracellular water that forms damaging ice-crystals upon lowering the temperature is essential for survival after cryopreservation [22]. These ice-crystals cause irreversible damage to cell membranes and destroy their semi-permeability. The dehydration can be achieved through (i) the application of cryoprotective agents that act osmotically or small molecules that enter cells easily, respectively called non-penetrating and penetrating cryoprotective agents [1,33], (ii) slow freezing resulting in freeze-dehydration [3,15] and (iii) air drying [17]. Prior to this cellular dehydration, the presence of a cryoprotective solution, containing compounds such as glycerol, dimethyl sulphoxide, ethylene glycol and methanol, is usually required to protect cells from injury by the effects of concentrated extra-cellular solutes and physical deformation caused by osmotic shrinkage.

Thus, specimens to be preserved have to be sufficiently dehydrated to avoid intracellular freezing and to achieve vitrification upon rapid cooling in liquid nitrogen. Vitrification may be the only freeze-avoidance mechanism that enables dehydrated cells, tissues and organs to survive at the temperature of liquid nitrogen [28]. Vitrification, a physical process, can be defined as the phase transition of an aqueous solution from liquid into an amorphous glassy solid, or glass, at the glass transition temperature, while avoiding crystallization.

By cooling a highly concentrated cryoprotective solution at a rapid cooling rate, the solution will pass below its freezing temperature (or "supercool") and vitrify at its glass transition temperature ( $T_g$ ). Viscosity increases rapidly near  $T_g$ . The change that happens at  $T_g$  is simply a rapid increase in viscosity, not a change of state [10]. The glass transition takes place over a small temperature range, rather than at a precise temperature. In addition,  $T_g$  depends on the cooling rate. Indeed, a fast cooling rate will result in  $T_g$  at a higher temperature, leading to a solid phase that has a high volume (lower density) and is more amorphous and less viscous. However, the volume continues to decrease and viscosity

continues to increase below  $T_g$ , as the change in temperature is quantitative, not qualitative (in contrast to crystallization). Since cooling occurs from the outside to the inside, rapid cooling creates stress when the warmer core needs to contract more than the cooler surface. This might be the reason for tissue to fracture, or crack into pieces when rapid cooling rates are applied. Fracturing of a nematode, will always result in irreversible damage. However, this problem can be overcome by using small volumes for immersion in liquid nitrogen. Moreover, by plunging small volumes in liquid nitrogen, the solidification of a sample will be more homogeneous.

Finally, after storage in liquid nitrogen an appropriate thawing rate has to be used. Even if ice crystal formation has been avoided during ultra-rapid freezing, crystallization can easily occur during slow thawing [10]. This phenomenon is called devitrification (or cold crystallization) and will again have a lethal effect on the viability of multicellular structures or organism if it happens intracellularly.

Successful cryopreservation has been reported for various free-living, entomopathogenic, animal- and plant-parasitic nematodes, using different cryoprotective solutions. Despite the fact that cryopreservation proved to be a suitable method for long-term preservation of nematodes, reports on cryopreservation of plant-parasitic nematodes are extremely scarce (Table 1). Moreover, all described protocols deal with nematodes of temperate regions. Some nematodes have developed systems to survive ice crystal formation in their bodies, making them freezing tolerant [35]. In the anhydrobiotic nematode *Aphelenchus avenae* a gene was identified that is upregulated in response to desiccation stress. This gene showed similarity with a gene coding for a late embryogenesis abundant protein induced in many plants when they are deprived of water [4]. Such natural protection might have had an effect on the survival after cryopreservation. The nematode under investigation in our study, *Radopholus similis*, is a nematode from the (sub)humid tropics. As such, it is never exposed to low temperatures in nature and there is little or no reproduction temperatures below 21°C [9,11,12]. Thus, it is probable that any treatment below these temperatures (for example incubation on ice) adds an extra stress to the nematodes making the development of a cryopreservation protocol extremely difficult. Moreover, this species does not desiccate and become anhydrobiotic under natural conditions.

In this paper, we describe a sequence of experiments to achieve a successful cryopreservation protocol for the tropical nematode *R. similis* using vitrification solution-based methods since it has been observed that, compared to other protocols like the slow cooling method, this often leads to marked improvement in the levels of survival attained [18].

## 2 Materials and methods

### 2.1 Nematode populations

The *R. similis* populations used in the experiments were monoxenically maintained on carrot discs [24] or on alfalfa callus [8] at 26°C in the dark. The different populations were selected for their differences in pathogenicity and geographical origin [31]. Nematode populations isolated from banana roots in Cuba, Ghana, Uganda and Vietnam were included in our tests. A mixture of vermiform nematodes was used in all experiments.

**Table 1.** Cryopreservation of plant-parasitic nematodes.

Nematode	Pretreatment (in final concentrations)	Freezing rate	Survival (%)	Reference
<i>Aphelenchoides sacchari</i>	10% Dimethyl sulfoxide	Slow	56	Hwang [16]
<i>Ditylenchus dipsaci</i>	7.5% Dimethyl sulfoxide	Slow	23-35	Sayre and Hwang [30]
<i>Meloidogyne graminicola</i>	10% Ethylene glycol (at 37°C) +40% Ethylene glycol (at 5°C)	Rapid	25	Bridge and Ham [1]
<i>Meloidogyne incognita</i>	10% Ethylene glycol (at RT)	Rapid	50-90	Triantaphyllou and Mc Cabe [32]
<i>M. javanica</i>	+40% Ethylene glycol (at 5°C)			
<i>M. hapla</i>				
<i>M. arenaria</i>				
<i>M. microtyla</i>				
<i>M. hispanica</i>				
<i>M. microcephalia</i>				
<i>Heterodera glycines</i>				
<i>H. schachtii</i>				
<i>H. trifolii</i>				
<i>Bursaphelenchus xylophilus</i>	1% Dimethyl sulfoxide OR	Slow	17	Riga and Webster [27]
<i>B. mucronatus</i>	15% glycerol + 35% S <sup>a</sup> -50% M9 <sup>b</sup> buffer OR 40% S-60% M9 buffer			
<i>Bursaphelenchus mucronatus</i>	10% Ethylene glycol (at 27°C)	Rapid	66-86	Irdani et al. [17]
<i>B. eremus</i>	+25% Ethylene glycol (at 0°C)			
<i>B. thailandae</i>	+Drying			
<i>B. xylophilus</i>				
<i>Pratylenchus thornei</i>	14-17% Glycerol + 70% methanol (at RT) + 70% methanol (at 5°C)	Rapid	76	Galway and Curran [13]
<i>Meloidogyne hapla</i>	10% Ethylene glycol (at RT)	Rapid	75	Van der Beek et al. [33]
<i>M. chitwoodi</i>	+40% Ethylene glycol (at 5°C)			

RT, room temperature.

<sup>a</sup> S buffer: 0.1M NaCl and 0.05M potassium phosphate (pH 6.0).

<sup>b</sup> M9 buffer: 6g Na<sub>2</sub>HPO<sub>4</sub>, 3g KH<sub>2</sub>PO<sub>4</sub>, 5g NaCl and 0.25g MgSO<sub>4</sub> · 7 H<sub>2</sub>O per litre.

## 2.2 Two-step pretreatment (Fig. 1)

Each step of this protocol was performed under sterile conditions. The nematode suspensions were incubated in 10% glycerol for 4-5 days. To obtain this concentration, an equal volume of double strength glycerol was added to the nematode suspension and incubated at 26°C in the dark (Step 1). Following the glycerol incubation, 400 or 800µl samples (containing an average of 89 living nematodes) were transferred to pre-chilled Eppendorf tubes. Immediately, 1200 or 800µl of pre-chilled cryoprotective solution was added. The final composition of the cryoprotective solutions varied among experiments, but care was taken to select only those solutions that vitrify upon rapid freezing (Step 2). The nematode suspensions were incubated on ice for 10 min. In one experiment (see Table 3) the effect of pre-chilled solutions was compared to the effect of non-chilled solutions. In order to reduce the volume of the nematode suspension that has to be immersed in liquid nitrogen (resulting in a more homogeneous freezing), the Eppendorf tube was centrifuged in a pre-chilled microcentrifuge at 8900g for 30s. Nematodes precipitated at the bottom of the Eppendorf tube. A 200µl sample, including the pellet with the nematodes was then transferred

to a sterile pre-chilled cryovial or a sterile aluminum foil spoon (Fig. 1). Next, the cryovial or the foil spoon were immersed in liquid nitrogen. For storage, the foil spoon was introduced into a cryovial (Fig. 1). The small volume and the thermal conductivity of the aluminium foil spoon will result in ultra-rapid freezing rates.

### **2.3 Storage and thawing**

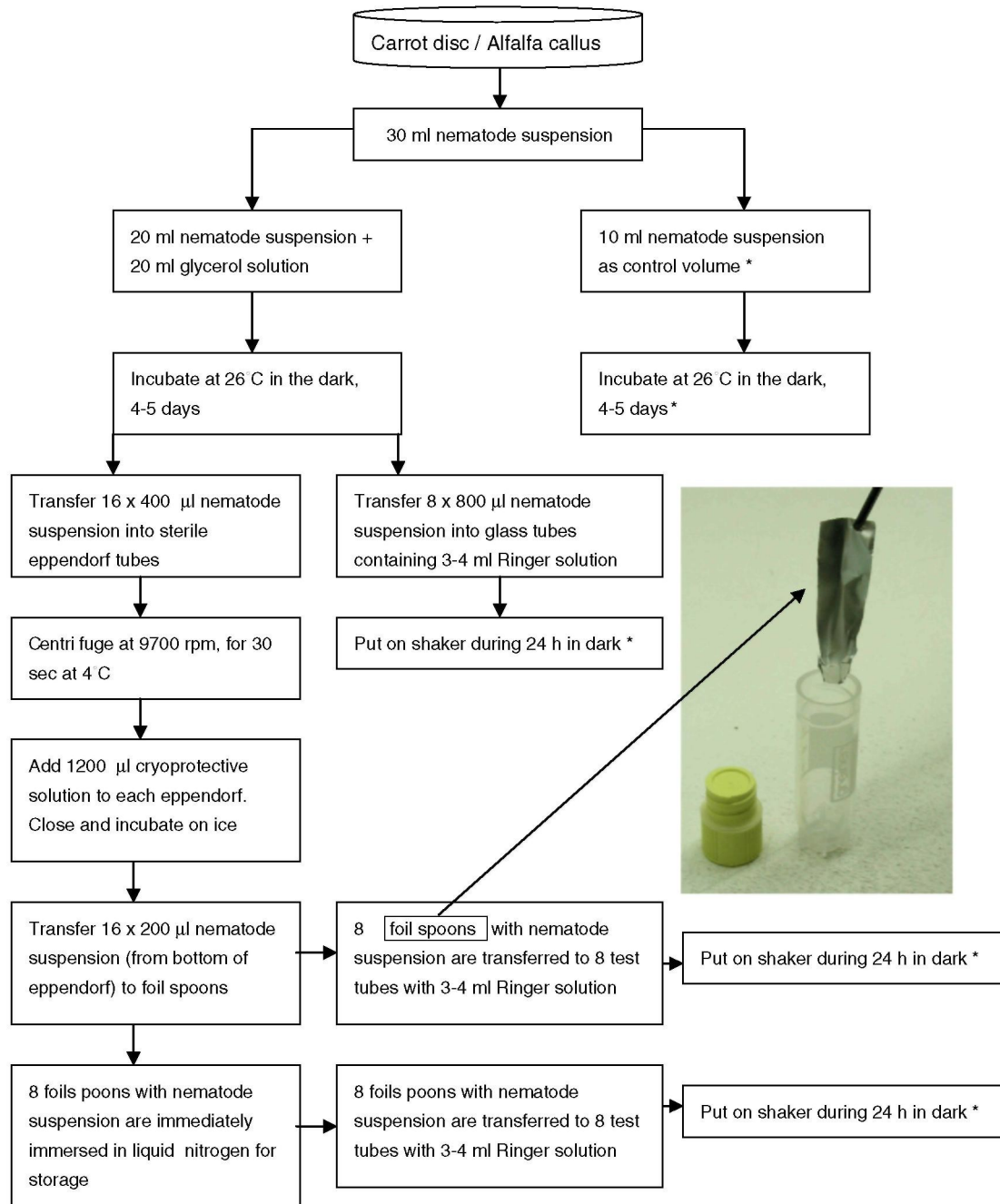
For experimental purposes, nematodes were stored for 24h in liquid nitrogen. Nematodes which have been stored in liquid nitrogen are called hereafter "cryo-nematodes". The samples were thawed by immersing the cryovials in a warm water bath (40°C) for 50-60s and later transferred to 3-4ml Ringer solution (80% NaCl, 2% CaCl<sub>2</sub>, 2% KCl<sub>2</sub>, 2% NaHCO<sub>3</sub>) at room temperature. In case the cryopreservation took place in the aluminum foil spoon, spoons containing the nematodes were directly transferred to 3-4ml Ringer solution at room temperature. After 24h on a shaker and after oxygenation of the suspensions, the viability of the nematodes was estimated by counting the number of motile cryo-nematodes per total cryo-nematodes.

### **2.4 Infectivity of cryo-nematodes**

The infectivity of those nematodes surviving cryopreservation was tested by inoculating all living nematodes on carrot discs and analyzing their reproduction after 7 weeks at 26°C in the dark. To study the reproduction of cryo-nematodes compared to the reproduction of non-cryopreserved nematodes, 25 females of first generation cryo-nematodes and non-cryopreserved nematodes were inoculated on carrot discs and incubated at 26°C, in the dark. The reproduction was studied at different time intervals (1-2-4-8 weeks).

### **2.5 Statistical analysis**

Data that were normally distributed and homogeneous variances were subjected to analysis of variance, using the STATISTICA® programme. Means were separated by the Tukey test ( $P \leq 0.05$ ).



**Fig. 1.** Overview of the cryopreservation protocol for *Radopholus similis*, using a two-step pretreatment, including the different controls (as indicated with an asterisk).

### 3 Results

For each experiment several controls were used in different steps of the protocol (indicated with an asterisk in Fig. 1). Mortality could thus be checked at different stages of the experiment. As such, modifications could be made in order to reduce the mortality probably caused by toxic effects of cryoprotectants in each of the treatments. Depending on the results of the experiments, time-consuming procedures or toxic substances were replaced by other procedures or products.

#### 3.1 Effect of glycerol pretreatment

Initially, different glycerol concentrations and exposure times were tested. A 15% glycerol solution had a toxic effect on the survival of infective vermiforms of *R. similis* after 2 days incubation (Table 2), compared with the nematode population that was active before the glycerol incubation. In addition, the nematodes showed an increase in mortality with the time exposed to 15% glycerol. After 5 days incubation at 26°C, the survival percentage decreased further to only 1%. For the lower concentrations tested, the survival rate of *R. similis* was not influenced by the glycerol concentrations (5-10%), or by the incubation period (2-5 days) tested. More than 50% of the exposed nematodes survived (Table 2).

Based on these results, the first step of the cryopreservation protocol for *R. similis* was fixed at a 4-5-day incubation in 10% glycerol at 26°C in the dark. These conditions should allow a sufficient exchange of internal body fluids with glycerol without being too toxic for the nematode, thus inducing a better protection against freezing injuries. Surviving nematodes showed a sluggish behaviour, probably due to the higher viscosity of glycerol compared to their body fluids.

**Table 2.** Survival of *Radopholus similis* (Ugandan isolate) during 5-day incubation in 5%, 10% and 15% glycerol at 26°C.

	<i>n</i>	% Survival	
<i>5% Glycerol</i>			
Day 2	7	59.5 (±5.3) <sup>a</sup>	a <sup>b</sup>
Day 5	5	53.0 (±12.2)	a
<i>10% Glycerol</i>			
Day 2	6	58.3 (±6.0)	a
Day 5	7	52.9 (±10.5)	a
<i>15% Glycerol</i>			
Day 2	8	15.5 (±5.9)	a
Day 5	8	1.0 (±1.6)	b

<sup>a</sup> Mean (±SD).

<sup>b</sup> Statistical analysis was performed using the Arcsin(x/100) transformed data.

For each glycerol concentration, means followed by a different letter differ significantly ( $P \leq 0.05$ ) according to the Tukey test.

### **3.2 Two-step pretreatment with glycerol and glycerol-methanol**

For *R. similis*, the three-step procedure as proposed by Galway and Curran [13] consisting of a glycerol treatment followed by a methanol treatment at room temperature and a methanol treatment at 0°C, was not successful (data not shown). The vitrifying solution (70% methanol) proved to be too toxic for our nematodes. In order to obtain a suitable cryoprotective solution, a balance between the toxicity of the incubation (composition of the nematode suspension and conditions of the incubation) and the vitrifying properties of the solution needs to be found.

Therefore, the 'less toxic' cryoprotectant, glycerol, was added to replace part of the methanol. Different mixtures of glycerol/methanol were prepared and tested for their vitrification ability and also for the absence of devitrification. For this, 1ml of the mixture was enclosed in a 2ml cryotube and plunged in liquid nitrogen. If the content of the tube remained transparent (glass-like), we could conclude that no crystallization took place. Also the presence/ absence of devitrification (crystallization upon thawing from a previously vitrified solution) was screened. For this the LN (liquid nitrogen) frozen tubes were stirred in a glass beaker containing hot water (40°C). Devitrification had occurred if, during the thawing process, the solution became snow-white, indicating the presence of ice crystals. The mixtures with the lowest concentrations that retained their capacity to vitrify were: 10% glycerol-40% methanol, 15% glycerol-35% methanol and 20% glycerol-30% methanol. Subsequently, the toxicity of these three solutions after a glycerol incubation was tested both at room temperature and at pre-chilled conditions (0°C) (Table 3). At room temperature, *R. similis* survived the 10% glycerol-40% methanol incubation better than in the other solutions. Using pre-chilled conditions during the second incubation, encouraging results were obtained. On average for the three treatments, 82% of the nematodes that survived the 10% glycerol incubation, survived the pre-chilled glycerol-methanol incubation. Therefore, for subsequent experiments, the second incubation of the protocol was done under pre-chilled conditions. However, none of these pretreatments resulted in post-thaw survival when the nematodes were enclosed in 2ml cryovials (results not shown). Either the freezing or the thawing procedures were causing the death of all frozen nematodes. In order to reach more rapid freezing and thawing rates, respectively leading to a greater chance of vitrification and lower chance for devitrification, alternative methods for the liquid nitrogen immersion were investigated.

### **3.3 Two-step pretreatment with glycerol and glycerol-methanol in combination with ultra-rapid freezing**

Ultra-rapid freezing and thawing rates were obtained by applying the foil spoon technique. This method was originally developed for plant tissue cryopreservation and uses a strip of aluminium on which shoot-tips are placed and subsequently plunged into liquid nitrogen [25].

When the ultra-rapid (aluminium foil) freezing and thawing method was applied to *R. similis*, for the first time successful cryopreservation of this tropical nematode was observed (Table 4). Cryotube-frozen material did not survive. However, in contradiction to the previous series of experiments (Table 3), a significant toxic effect of the glycerol/methanol ratio was



observed in the non-frozen material. Toxicity increased with higher glycerol concentrations. The cryopreserved material showed no significant difference. The variation in the response of the biological material from one experiment to another emphasizes the need to include in each experiment sufficient control treatments. Generally, four different types of nematodes can be distinguished after cryopreservation (Fig. 2): (i) living (and moving) nematodes, (ii) normal shaped nematode, with an intact content but lacking any movement, (iii) cut nematodes, broken normal shaped nematodes probably due to cracking, (iv) dead nematodes (with an irregular content). Ultra-rapid freezing results in a lower number of 'cracked' nematodes compared to the cryotube-frozen nematodes.

**Table 3.** Effect of the temperature of the cryoprotective solution on the optimization of the cryoprotective solution for the cryopreservation of *Radopholus similis* (Ugandan isolate).

	At room temperature		At 0°C	
	<i>n</i>	% Survival	<i>n</i>	% Survival
After 4 days glycerol incubation (at 26°C)	8	70.1 (±2.1) <sup>a</sup>	8	68.5 (±3.1)
After 10 min incubation in 10% glycerol- 40% methanol	7	53.4 (±12.0)	8	55.0 (±5.5)
15% Glycerol-35% methanol	8	7.3 (±10.9)	8	55.3 (±4.3)
20% Glycerol-30% methanol	8	0.0 (±0.0)	8	57.2 (±10.9)

<sup>a</sup> Mean (±SD).

<sup>b</sup> Statistical analysis was performed using the Arcsin(x/100) transformed data. For each temperature, means followed by a different letter differ significantly ( $P \leq 0.05$ ) according to the Tukey test.

**Table 4.** Optimization of the cryoprotective solution and the application of ultra-rapid freezing for the cryopreservation of *Radopholus similis* (Cuban isolate).

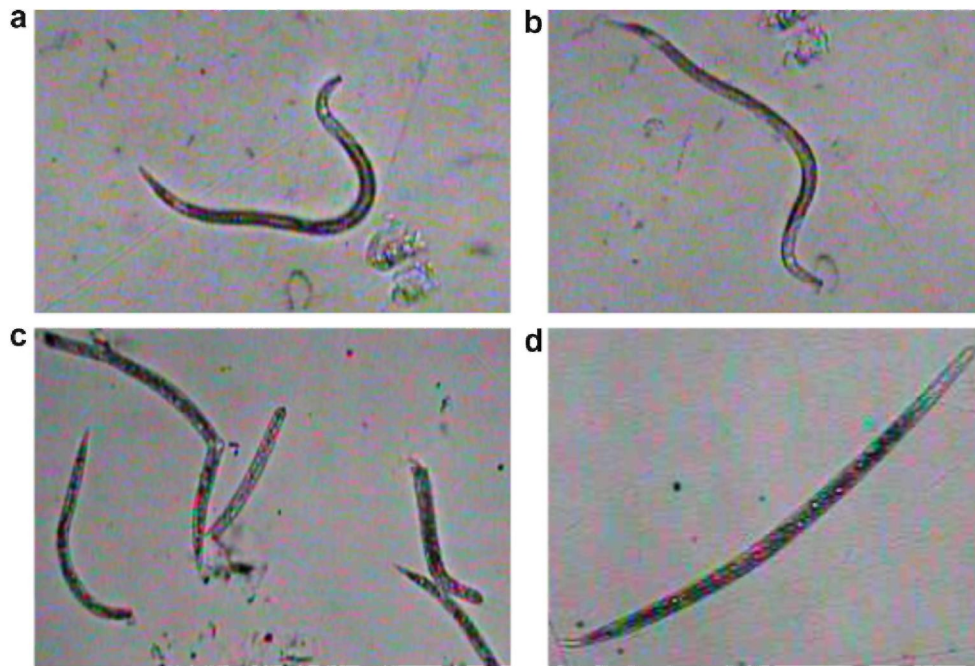
	<i>n</i>	% Survival		<i>n</i>	% Survival
After 4 days glycerol incubation (at 26°C)	8	45.7 (±3.6) <sup>a</sup>	a <sup>b</sup>		
After 10 min in 15% glycerol-35% methanol (0°C)	7	31.1 (±4.3)	b		
20% Glycerol-30% methanol (0°C)	6	16.5 (±16.8)	c		
				Foil spoon	Cryotube
After freezing in 15% glycerol-35% methanol	8	0.8 (±1.1)	d	8	0.0 (±0.0)
20% Glycerol-30% methanol	7	0.5 (±0.7)	d	8	0.0 (±0.0)

<sup>a</sup> Mean (±SD).

<sup>b</sup> Statistical analysis was performed using the Arcsin(x/100) transformed data. Means followed by a different letter differ significantly ( $P \leq 0.05$ ) according to the Tukey test.

### 3.4 Two-step pretreatment with glycerol and glycerol-methanol-glucose in combination with ultra-rapid freezing

To distinguish between those nematodes that died naturally and those that died because of the toxicity of the glycerol incubation, we decided to include an additional control, consisting of a nematode suspension in distilled water placed in an incubator for the same period as the incubation in 10% glycerol. The results showed that the glycerol incubation was not toxic, since equal proportions of the nematode population survived both treatments (Table 5).



**Fig. 2.** *Radopholus similis* nematodes after cryopreservation. (a) Living nematode, (b) good shape nematode, (c) cut nematode due to cracking, (d) dead nematode (length of a nematode is about 700 $\mu$ m).

Different glucose concentrations were tested in combination with the ultra-rapid freezing and thawing technique (Table 5). Although some variation in toxicity after the second incubation (with pre-chilled glycerol-methanol-glucose solutions) and after subsequent cryopreservation was observed, none of these differences was found to be significant. Thus, the addition of glucose to the cryoprotective solution did not have any toxic effect during the 10min incubation, nor did it have a significant effect on the protection during freezing and thawing.

Based on these results, we considered the following protocol as optimal for the cryopreservation of the tropical nematode, *R. similis*: step 1: 4-5 days incubation in 10% glycerol at room temperature; step 2: 10min incubation in 15% glycerol-35% methanol-0.5M glucose at 0°C; step 3: ultra-rapid freezing and thawing using an aluminium foil spoon. We applied this protocol subsequently to other *R. similis* populations (Table 6) and proved that all tested populations could be cryopreserved successfully. Nevertheless, a high variation in survival of the initial population (ranging between 41.3% and 80.3%) as well as a high variation in survival after cryopreservation (ranging between 0.7% and 11.5%) was observed. Therefore, we decided to investigate the influence of the nematode population itself in a set of experiments studying the effect of the culture conditions and the age of the nematode population.

### 3.5 Influence of culture condition on the viability after cryopreservation

Two sets of experiments were carried out: a first set testing the difference between nematodes cultured on alfalfa callus and nematodes cultured on carrot discs, and a second set testing different ages of nematode populations on carrot discs. For each set of experiments, two independent repetitions were executed. Fig. 3 shows the combined data for the two sets of experiments.

Results from the first set of experiments demonstrated that the culture conditions had a significant ( $p < 0.05$ ) effect on the viability of the nematode population. Except for the initial viability, more living nematodes were observed in the nematode population extracted from alfalfa callus compared to the nematode population extracted from carrot disc (Fig. 3a). In the second set of experiments, *R. similis* was extracted from carrot disc cultures 20, 30 and 40 days after inoculation. However, no significant ( $p > 0.05$ ) effect of age was observed during the different steps of the cryopreservation protocol (Fig. 3).

### 3.6 Infectivity of cryo-nematodes

The infectivity and pathogenicity of *R. similis* was not altered due to cryopreservation (Fig. 4). No differences in penetration or in reproduction were observed during the 8 weeks. All vermiform stages were collected from 2 weeks after inoculation.

**Table 5.** Effect of glucose on the optimization of the cryoprotective solution for the cryopreservation of *Radopholus similis* (Ugandan isolate).

	<i>n</i>	% Survival	
Before glycerol incubation	8	86.6(±1.9) <sup>a</sup>	a <sup>b</sup>
After 4 days water incubation	8	74.1 (±6.0)	b
After 4 days 10% glycerol incubation	8	67.2 (±2.4)	b
After 10 min in 15% glycerol-35% methanol (0°C)	8	33.3 (±14.5)	c,d
20% Glycerol-30% methanol (0°C)	8	20.5 (±8.6)	d
15% Glycerol-35% methanol-0.25M glucose (0°C)	8	17.5 (±12.1)	d,e
15% Glycerol-35% methanol-0.5M glucose (0°C)	7	16.4 (±8.4)	d,e
20% Glycerol-30% methanol-0.25M glucose (0°C)	8	26.7 (±10.1)	c,d
20% Glycerol-30% methanol-0.5M glucose (0°C)	8	7.9 (±3.8)	d,e,f
After freezing in foil spoon in 15% glycerol-35% methanol	8	2.9 (±2.1)	f
20% Glycerol-30% methanol	7	3.7 (±2.9)	e,f
15% Glycerol-35% methanol-0.25M glucose	8	5.1 (±5.1)	e,f
15% Glycerol-35% methanol-0.5M glucose	8	2.2 (±2.2)	f
20% Glycerol-30% methanol-0.25M glucose	7	3.8 (±2.7)	e,f
20% Glycerol-30% methanol-0.5M glucose	8	3.6 (±3.4)	f

<sup>a</sup> Mean (±SD).

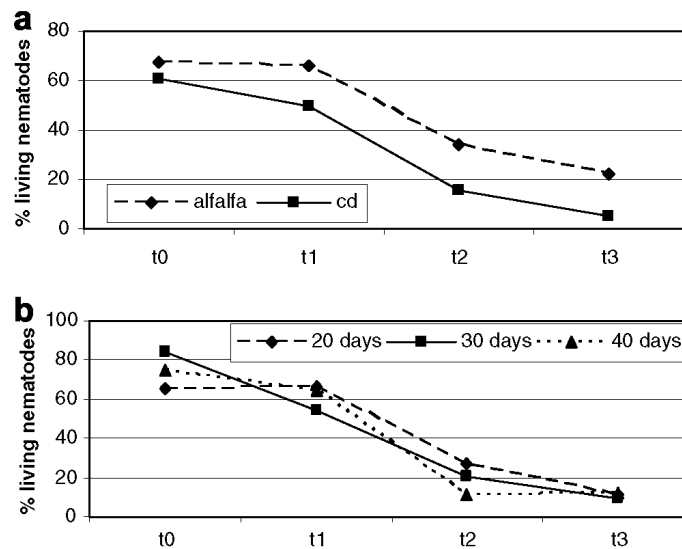
<sup>b</sup> Statistical analysis was performed using the Arcsin( $x/100$ ) transformed data. Means followed by a different letter differ significantly ( $P \leq 0.05$ ) according to the Tukey test.

**Table 6.** Effect of different isolates of *Radopholus similis* on their survival during and after cryopreservation ( $n = 8$ ).

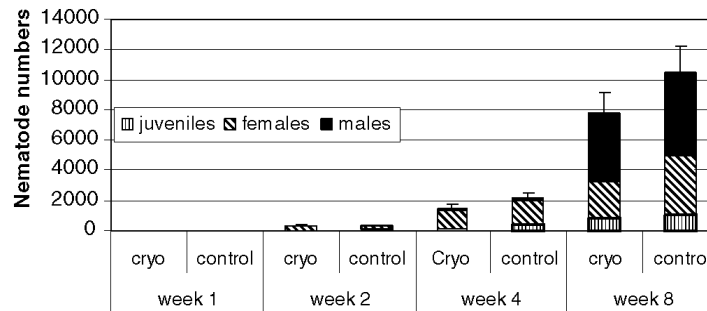
	% Survival			
	Cuban isolate	Ugandan isolate	Ghanean isolate	Vietnamese isolate
Before glycerol incubation	41.3 ( $\pm 3.5$ ) a	78.7 ( $\pm 2.9$ ) a	80.3 ( $\pm 4.5$ ) a	65.1 ( $\pm 1.9$ ) a
After 5 days water incubation	26.3 ( $\pm 3.0$ ) b	75.4 ( $\pm 3.3$ ) a,b	71.5 ( $\pm 8.5$ ) a	63.7 ( $\pm 2.5$ ) a
After 5 days 10% glycerol incubation	28.3 ( $\pm 7.3$ ) b	67.7 ( $\pm 10.4$ ) b	72.8 ( $\pm 8.0$ ) a	41.8 ( $\pm 9.6$ ) b
After 10 min incubation in 15% glycerol-35% methanol-0.5M glucose	0.8 ( $\pm 0.6$ ) c	31.3 ( $\pm 11.8$ ) c	26.3 ( $\pm 26.5$ ) b	2.1 ( $\pm 1.7$ ) c
After freezing in foil spoon in 15% glycerol-35% methanol-0.5M glucose	0.7 ( $\pm 1.1$ ) c	1.8 ( $\pm 5.1$ ) d	11.5 ( $\pm 14.0$ ) c	3.1 ( $\pm 2.7$ ) c

<sup>a</sup>Mean ( $\pm$ SD).

<sup>b</sup>Statistical analysis was performed using the Arcsin( $x/100$ ) transformed data. For each nematode isolate, means followed by a different letter differ significantly ( $P \leq 0.05$ ) according to the Tukey test.



**Fig. 3.** Influence of the culturing conditions on the survival of the nematode, *Radopholus similis* (Ugandan isolate) during cryopreservation. (a) Two types of monoxenic culture, alfalfa callus and carrot disc (cd); (b) different ages of carrot disc cultures, 20-30-40 days old.  $t_0$  = before glycerol incubation,  $t_1$  = after 5 days 10% glycerol,  $t_2$  = after 10 min 15% glycerol-35% methanol-0.5M glucose,  $t_4$  = after cryopreservation.



**Fig. 4.** Reproduction of *Radopholus similis* on carrot discs, at 26°C, in the dark, after cryopreservation in comparison with untreated nematodes. Error bars represent the standard errors of total nematode numbers.

## 4 Discussion

Successful cryopreservation has been reported for various free-living, entomopathogenic, animal- and a few plant-parasitic nematodes. Free-living and mycophagous nematodes of the genera *Panagrellus*, *Turbatrix* and *Caenorhabditis* were successfully frozen in liquid nitrogen using dimethyl sulphoxide as sole cryoprotectant combined with slow, controlled freezing [16]. Dimethyl sulfoxide (DMSO) also protected animal-parasitic nematodes, like *Haemonchus* spp., *Trichostrongylus* sp., *Ostertagia* sp. and *Cooperia* sp. during storage in liquid nitrogen [14,19]. Infective juveniles of the entomopathogenic nematodes, *Steinernema* and *Heterorhabditis* spp. were cryopreserved using a two-step protocol with glycerol and methanol before submersion in liquid nitrogen [7]. The survival ranged from 58% up to 95% [26].

For plant-parasitic nematodes, successful cryopreservation has been reported using different cryoprotectants (ethylene glycol, DMSO and glycerol in combination with methanol) (Table 1). For many *Meloidogyne* species, a protocol based on the application of ethylene glycol was found to be effective [1,32,33]. This protocol consisted of a two-step procedure (i.e. incubation in 10% ethylene glycol followed by incubation in 40% ethylene glycol). Different incubation temperatures and durations were tested but the highest survival rate was observed when both rapid freezing and rapid thawing were applied. Treatment with dimethyl sulphoxide in combination with slow cooling (or freeze-dehydration) was used for the cryopreservation of different migratory plant-parasitic nematodes [27,30]. Compared to the root-knot nematodes, post-thaw survival rates were much lower, ranging from 17% to 35%. The successful cryopreservation of *Pratylenchus* spp. was reported by Galway and Curran [13]. Nematode survival was achieved after a three-step procedure. For a period ranging from 1 to 5 days the nematodes were incubated in 14-17% glycerol. Next, they were rinsed with 70% methanol at room temperature and finally they were incubated in 70% methanol on ice and rapidly frozen. The pathogenicity (i.e. their ability to infect plant tissues) of these cryopreserved migratory nematodes was not altered by cryopreservation, since they could infect and reproduce on carrot discs after recovery in Ringer solution. Very recently, an efficient cryopreservation protocol for *Bursaphelenchus eremus* was reported [17]. It combines a non vitrifying concentration of ethylene glycol (25%) with evaporative drying.

Until now most of these cryopreservation procedures were empirically designed. Anhydrobiotic nematodes can be cryopreserved relatively easily since their water content can be reduced to 2-5% [23]. Charwat and co-workers [5] compared the osmotic adaptation of four different nematode species and showed that they all behaved differently. This observation has important consequences as dehydration survival is needed for successful cryopreservation. *Panagrolaimus davidi*, an antarctic nematode, can survive intrabody ice formation in cavities inside the pseudocoel [35]. Also James [18] made the observation that, in general, cryopreservation viability of arctic species is considerably higher than that of temperate and tropical isolates. Acclimation to low temperature can increase freezing tolerance indicating metabolic mechanisms to deal with stress. This acclimation is however species dependent [2] and thus probably completely absent in a tropical nematode like *R. similis*.

In this work we developed a cryopreservation protocol based on the application of a well defined mixture of cryo-protectants in combination with ultra-rapid cooling and thawing rates. In contradiction to the work with *Pratylenchus* spp., we observed that *R. similis* is not resistant to high methanol concentrations (70%) needed for vitrification [13]. In order to overcome toxicity problems a mixture of cryoprotective agents can be used, each able to contribute to a portion of the overall protection by a different mechanism [18]. We opted for a mixture of methanol, glycerol and glucose. Methanol is commonly used as a fast penetrating cryoprotective agent, thus contributing to the vitrification properties of the intracellular cell solution. However, it can also be detrimental to some organisms. Glycerol is also a powerful cryoprotectant. Besides its vitrification inducing properties, it is known to induce tolerance towards dehydration as well as towards freezing [3,5]. The addition of sugars helps in the dehydration of cells and tissues. Kuleshova et al. [21] observed that added sugars contribute to a solution's overall vitrification property. Besides their osmotic and colligative effects, which reduces the amount of intracellular freezable water, it is known that sugars can maintain the liquid crystalline state of the membrane bilayers and stabilize proteins under frozen conditions [6].

We demonstrated that the cryopreservation method described can be used for routine cryopreservation of *R. similis* lines from different origins. Since tropical nematodes could be considered as 'difficult' to cryopreserve, we believe that the vitrification techniques described in this paper can also benefit other 'recalcitrant' organisms.

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