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# Freezing and Storing *Meloidogyne* spp. in Liquid Nitrogen

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**Summary:** Carneiro, R.M.D.G, I. Martins, A.C.O. Teixeira & F.C. Mota. 2005. Freezing and storing *Meloidogyne* spp. in liquid nitrogen.

Second–stage juveniles of 23 populations of *Meloidogyne* spp. were successfully preserved in liquid nitrogen using a simple two-step cooling technique with ethylene glycol as a cryoprotectant. Thawing was carried out by very quickly dropping a paper strip into beakers containing 30 ml of warm water at 35 °C, which gave both a rapid thaw and diluted the cryoprotectant. The survival rates (S) after freezing and thawing varied from 33.3 to 71.1% (average 53%) and the reproduction factors (RF) following thawing from 1.5 to 54.4. Considering the values of S and FR, most of the species obtained satisfactory results, although the values differed considerably among populations of the same or different species. These variations are probably due to the great importance of speed in the cryopreservation process. No influence of storage time (2–24 months) or nematode species was observed in the rates of survival (S).

**Keywords:** cryopreservation, liquid nitrogen, ethanodiol, root-knot nematodes

**Resumo:** Carneiro, R.M.D.G, I. Martins, A.C.O. Teixeira & F.C. Mota. 2005. Congelamento e armazenamento de *Meloidogyne* spp. em nitogênio líquido.

Juvenis de segundo-estádio de 23 populações de *Meloidogyne* spp. foram preservados, eficientemente, em nitrogênio líquido, usando uma técnica simples de congelamento em duas etapas que utiliza o etileno glicol como crioprotetor. O descongelando foi realizado submergindo, rapidamente, as tiras de papel em béqueres contendo 30 ml de água aquecida a 35 °C, de maneira a prover um descongelamento muito rápido e diluir o crioprotetor. As porcentagens de sobrevivência (S), após o congelamento e descongelamento, variaram de 33,3 a 71,1% (média de 53%) para as diferentes espécies de *Meloidogyne* e o fator de reprodução em tomateiros (FR) variou de 1,5 a 54,4. Considerando os valores de S e FR para a maioria das espécies, obtiveram -se resultados satisfatórios, embora, os valores tenham variado, consideravelmente, entre populações das mesmas espécies ou de espécies diferentes. Essas variações são devidas, provavelmente, à grande importância da rapidez nos processos de criopreservação. Nenhuma influência do tempo de armazenamento (2-24 meses) ou das espécies de *Meloidogyne* foram observadas na porcentagem de sobrevivência (S).

**Palavras-chave:** criopreservação, nitrogênio líquido, nematóide de galhas.

## Introduction

The maintenance of reference cultures of microorganisms and invertebrates, prior to identification and characterization studies, is one of the aims of the EMBRAPA - Genetic Resources and Biotecnology projects. The maintenance of pure species of *Meloidogyne* on host plants over a long time is not always a simple procedure, because it requires inoculations and constant purifications of the nematode populations. Temperatures below -130°C (the recrystallization point of ice) are known to assure long–term, and possibly indefinite, preservation of certain biological specimens (White & Warton, 1984, Whittingham, 1980). Cryopreservation through freezing and storing in liquid Nitrogen (-196°C) has been used to preserve some plant-parasitic nematodes, including those of the genus *Meloidogyne* (Bridge & Ham, 1985, Triantaphyllou & McCabe, 1989; Carneiro *et al.*, 2001).

Successful crypreservation, however, almost always

requires: 1) an appropriate pretreatment of the species with cryoprotectants that minimize intracellular and intercellular crystal formation, 2) a precisely controlled cooling rate of the specimens, at least during the early stages of freezing, and 3) a controlled thawing rate.

Several substances have been used with success in cryopreservation. Ethylene glycol (ethanodiol) has been identified as an efficient cryoprotectant since at least 1952 (Luyet *et al.*, 1952) and was the most efficient for root-knot nematodes (Bridge & Ham, 1985, Triantaphyllou & McCabe, 1989). The described techniques have proven moderately effective to effective for root-knot nematode preservation (Bridge & Ham, 1985, Triantaphyllou & McCabe, 1989). Recently, Carneiro *et al.* (2001) suggested another technique mixing the methods described by these authors with some modifications.

With the aim of preserving our populations of *Meloidogyne* spp., we tested the described technique of Carneiro *et al.* (2001) using different *Meloidogyne* species and different storage times. We quantified the survival rates of second-stage juveniles (J2) and the development and reproduction of nematodes following the freezing and thawing processes.

## Material and Methods

*Meloidogyne* spp. J2 were obtained from infected roots of tomatoes, using the Hussey & Barker (1973) methodology with NaOH at 0.5 % and modified Baermann funnel technique (Whitehead & Hemming, 1965). The nematode suspension was concentrated in 50 ml Falcon tubes by centrifuging at 3000 rev/min for 5 minutes. The excess of water was removed to provide about 32000 J2/800 µl. A small quantity of 10% (v/v) ethylene glycol (about 10 times the volume of J2) was added to the vial at room temperature and then the falcon tubes were maintained at 27 °C for 2 hours. The vial was then transferred to a bath of crushed ice and an equal quantity of cold (4°C) 70 % ethylene glycol was added to the vial, so that the final concentration of ethylene glycol was about 35 % . After forty-five minutes about 50 µl of J2 suspension was drawn from the vial with a micropipet and delivered onto a strip of Whatman 3 mm chromatography paper (about 4.5 ×0.7 mm) held at one end by a pair of fine point forceps. The strip of paper was quickly submerged into liquid nitrogen contained in a open styrofoam container. After a few seconds, when bubbling of the liquid nitrogen ceased around the forceps, the paper strip was lowered into a 3.6 ml cryogenic vial which had been placed in the liquid nitrogen earlier. Two paper strips supporting frozen J2 can be accommodated in the same cryogenic vial and the caps were kept loosely screwed on the vials allowing free exchange of liquid or nitrogen vapors between the interior and the exterior of the vial during storage. Afterward, the vial was transfered to a holder of a liquid-nitrogen freezer for long-term storage. The transfer was done very quickly to ensure that the paper strips inside the small cryogenic vial were constantly immersed in the rapidly evaporating liquid nitrogen (Carneiro *et al.*, 2001).

Thawing was achieved by very quickly transferring a paper strip from the liquid nitrogen into a beaker containing 30 ml of water (15ml/strip) and maintained in a water bath at 35°C, which caused a rapid thaw and diluted the cryoprotectant. Unless the paper strip was submerged in water within 10 seconds after being removed from the liquid nitrogen, a slow thaw occurred and the J2 died. The beakers were kept in an oven at 25°C, for 2 hours, and the survival rate was evaluated by counting the moving and immobile J2 in Peters slides (Carneiro *et al.*, 2001)

Treated *Meloidogyne* spp. J2 were inoculated on tomato plants after different periods of frozen storage (2–24 months). The pots were arranged in a randomized complete block design with eight replications. Three months after the nematode inoculation, the treatments were evaluated by extracting the eggs from the entire root system (Hussey & Barker, 1973), using a mixer instead of manual agitation. The number of J2 in the soil were also evaluated by Jenkins (1964) extraction method. The reproduction factor (RF) was calculated by dividing the final population (n° of eggs + J2) by the initial population.

## Results and Discussion

The survival rates (S) after freezing and thawing varied from 33.3 to 71.1% (average 53%) for *Meloidogyne* spp. and the reproduction factors (RF) from 1.5 to 54.4. (Table 1). Considering the values of S and FR, most of the species obtained satisfactory results, although the values have varied considerably (Table 1) among populations of the same species and of different species. For *M. javanica,* for example, the values of S varied from 35.5 to 62.8 and the RF values from 11.5 to 54.4%. These differences are probably due to the variability of time lost in the cryopreservation process. Speed of freezing and thawing was very important and was related to the ability of the technicians involved in the manipulation of samples (Carneiro *et al.*, 2001). These results agree with the observation made by Triantaphyllou & McCabe (1989), that

Table 1. Species of *Meloidogyne* spp. after freezing and thawing process: showing esterase phenotypes (EST), origin, months of storage, survival rates (S) and reproduction factors (RF)

| *Meloidogyne* species | EST | Origin of isolates | Months | S (%) ± standard error | RF ± standard error |
|---|---|---|---|---|---|
| *M.incognita* Race 1 | I1 | Londrina, PR, beans | 11 | 33.3 ± 5.4 | 34.3 ± 2.3 |
| *M. incognita* Race 4 | I2 | Londrina, PR. watermelon | 12 | 40.7 ± 6.9 | 23.1 ± 3.5 |
| *M. incognita* Race 4 | I2 | Londrina, PR, watermelon | 18 | 68.2 ± 5.2 | 40.9 ± 4.4 |
| *M. incognita* Race 2 | I2 | Brasília, DF, tomato | 3 | 64.1 ± 3.3 | 10.7 ±2.8 |
| *M. javanica* Race 2 | J2 | Londrina, PR, corn | 7 | 47.4 ± 8.1 | 54.4 ± 4.5 |
| *M. javanica* Race 1 | J3 | Londrina , PR, soybean | 11 | 62.8 ± 7.4 | 15.3 ± 2.7 |
| *M. javanica* Race 1. | J3 | Londrina,, PR, soybean | 2 | 35.5 ± 3.7 | 11.5 ± 2.7 |
| *M. arenaria* Race 2 | A2 | Pelotas, RS, lettuce | 09 | 55.9 ± 5.6 | 16.3 ± 3.1 |
| *M. arenaria* Race 2 | A2 | Pelotas, RS, lettuce | 21 | 61.3 ± 3.9 | 24.9 ± 5.4 |
| *M. arenaria* Race 1 | A2 | Florida, USA, peanut | 03 | 49.3 ± 3.5 | 16.4 ± 4.1 |
| *M. arenaria* | A2 | El Salvador, coffee | 12 | 59.8 ± 3.0 | 35.2 ± 5.3 |
| *M.graminicola* | G1 | Pelotas, RS, rice | 10 | 47.4 ± 9.6 | 1.5 ± 2,2 |
| *M. mayaguensis* | M2 | Petrolina, PE, guava | 08 | 37.4 ± 10.34 | 33.7 ±4.7 |
| *M. paranaensis* | P1 | Londrina, PR, coffee | 03 | 66.2 ± 5.3 | 6.6 ± 2.3 |
| *M. paranaensis* | P1 | Londrina, PR, coffee | 13 | 68.3 ± 2.1 | 6.9 ± 2.2 |
| *M. arabicida* | AR2 | Costa Rica coffee | 24 | 53.7 ± 10.2 | 46.2 ± 3.5 |
| *M. hapla* | H1 | Caxias do Sul, RS kiwi | 08 | 51.6 ± 6.4 | 13.8 ± 8.4 |
| *M. ethiopica* | E3 | Lagoa Vermelha, RS, kiwi | 10 | 65.3 ± 5.3 | 15.2 ± 3.7 |
| *M. exigua* | E1 | Lavras, MG, coffee, tomato | 02 | 70.9 ± 4.5 | 3.6 ± 2.4 |
| *M. exigua* | E1 | Lavras, MG, coffee, tomato | 11 | 70.01 ± 2.2 | 8.9 ± 2.6 |
| *Meloidogyne* sp. | S4 | El Salvador, coffee | 13 | 71.1 ± 2.9 | 8,7 ± 4.1 |
| *Meloidogyne* sp. | Y3 | Capão Bonito,SP yakon | 24 | 35.3 ± 5.3 | 8.6 ± 3.2 |
| *Meloidogyne* sp. | S2 | Dindorama, GO, soybean | 13 | 71.1 ± 7.7 | 8.7 ± 2.1 |

some 50-90 % of 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 tomatoes or soybean seedlings, respectively. Although the survival rates were high for some species, the reproduction factors were low. *Meloidogyne graminicola* (S=47.4%, RF=1.48), *M. exigua* (S=70.9%, RF=3.62) and *M. paranaensis* (S=66.3%, RF=6.6). The low reproduction factors can be explained by the fact that these three species have rice and coffee as preferential hosts, respectively, and they reproduce badly in the tomato plants compared with other *Meloidogyne* species (data not included). The survival rate (S) and reproductive factors (RF) were submitted to analysis of variance. The calculated CV for this analysis was high (32.3 %), showing there is insufficient precision to detect significant parameters. This high CV is probably related to the variability of the previously explained cryopreservation process. Considering now only *M. paranaensis* (storage time varying from 3 to 13 months), and *M. exigua* (storage time varying from 2 to 11 months), it is possible to observe the same survival rates and no influence of the time. *M. incognita* race 4 survived better

over 18 months than over 12 months and *M. arenaria* better over 21 months than over three or nine months (Table 1). Probably, no influence of storage time (2–24 months) or nematode species was observed in the rates of survival (S). This analysis is validated only for the period of 2-24 months. Other experiments should be done using longer storage periods. Based on other reports (White & Warton, 1984 and Whittingham, 1980) and the principle that there is no biological activity at – 196 °C, the temperature of the liquid nitrogen, it can be predicted that freezing for at least 10 or possibly 100 years or more may not substantially reduce J2 survival and infectivity (Triantaphyllou & McCabe, 1989).

Long-term storage in liquid nitrogen can have many benefits over the normal maintenance on plant cultures. It obviates the labour and space requirements and the maintenance of controlled environmental conditions, all of which are costly. The likelihood of cross-infections and mutation or selection of unwanted biotypes is lessened (Bridge & Ham, 1985).

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