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Long-term storage of *Heterodera avenae* cysts

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Summary – Cysts, extracted from soil, of *Heterodera avenae,* pathotype Ha11, were stored at + 20, + 2, and – 18 °C, with or without $CaCl_2$ as a drying agent. Freeze-dried cysts (– 60 °C for 5 h 30 min) were stored at – 18 °C, and unextracted cysts in soil were stored at + 2 °C as controls. After 6, 12, 18, 24, and 60 months, cysts were transferred into hatching conditions at + 12 °C, and the emergence of juveniles was observed for 26 weeks. The best juvenile emergence was obtained from cysts kept at + 2 °C in the soil for 6 months. At longer storage of inocula, the highest relative juvenile emergence was obtained for freeze-dried cysts stored at – 18 °C. In these conditions, a higher proportion of cysts with at least one emerging juvenile was recorded.

Résumé – *Conservation de longue durée des kystes d'* Heterodera avenae – Des kystes d'*Heterodera avenae* pathotype Ha11, extraits du sol, sont stockés à + 20, + 2 et – 18 °C, avec ou sans $CaCl_2$, agent deshydratant. Des kystes congelés à sec (– 60 °C pendant 5 h 30 min) ont été stockés à – 18 °C et, en guise de témoin, des kystes sont maintenus dans le sol à + 2 °C. Après respectivement 6, 12, 18, 24 et 60 mois, les kystes sont mis en condition d'éclosion (+ 12 °C) et la sortie des larves est observée pendant 26 semaines. L'éclosion la plus forte est obtenue à partir de kystes placés à 2 °C dans le sol pendant six mois. Avec des stockages plus longs d'inoculum, la plus forte éclosion moyenne est obtenue chez des kystes congelés à sec et conservés à – 18 °C. Les lots congelés à sec ont également la proportion la plus élevée de kystes dont au moins un juvénile est éclos.

Key-words : cryo-preservation, drying agent, *Heterodera avenae.*

Maintaining cultures of obligate plant parasitic nematodes by propagation on host plants is often labor and space intensive. In addition, it may be difficult to prevent the spread and contamination of cultures. Long-term storage could avoid these problems. Long-term storage which conserves an acceptable level of juvenile emergence would be valuable, e.g., to preserve a particular pathotype or to undertake experiments independently of the season. The aim of this study was to develop a method to preserve infective juveniles within cysts of *Heterodera avenae.*

In the northern hemisphere, *H. avenae* has been found to lose viability after some months of storage, and to be quite sensitive to desiccation (Hesling, 1956; Fushtey & Johnson, 1966; Juhl, 1968). Meagher (1974) successfully stored Australian cysts of *H. avenae* for at least 5.5 years at + 5 °C at 75 % relative humidity and at + 15 °C at 40 % relative humidity. In India, Rao and Dwahan (1988) stored cysts at + 30 °C in dry sandy soil for at least 6 months without decrease in viability and infectivity.

Almost all parasitic nematode species can interrupt their development at an early stage and extend their survival by means of depressed metabolic activity. Several authors have reviewed survival strategies and arrested development (e.g., Van Gundy, 1965; Clark & Perry, 1977; Norton, 1978; Demeure & Freckman, 1981; Evans, 1987; Antoniou, 1989).

Freezing and storing in liquid nitrogen has been used to preserve some mycophagous nematode species and the plant parasitic nematode *Ditylenchus dipsaci* (Hwang, 1970; Sayre & Hwang, 1975). A two-step cooling technique with ethanediol as cryoprotectant, developed for animal parasites, was found by Bridge and Ham (1985) to work also with the rice root-knot nematode *Meloidogyne graminicola.* Dwahan and Rao (1988) stored cysts of *H. avenae,* mixed with dry sandy soil or in 0.3 % agar gel, in vials in liquid nitrogen at – 180 °C for 6 months. From cysts mixed with sand, 26 % of the juveniles emerged, while no emergence occurred from cysts in agar gel.

Materials and methods

A nematode population originating from Ask in the province of Skåne in southernmost Sweden was used. It is classified as pathotype Ha11 of *H. avenae* (Ireholm, 1990) and belongs to the northern ecotype (Rivoal & Ireholm, 1990). The nematodes were reared outdoors in 1987 on oat cv. Selma in large pots filled with 10 dm^3 of naturally infested soil. In autumn (late October), the soil from all pots was carefully mixed. Mature brown cysts were extracted with a modified Seinhorst cyst elutriator and hand-picked from the wet debris. The average content of cysts was 239 eggs with juveniles (mean of ten cysts, S.D. = 51.8). Batches of 25 cysts were kept in glass tubes (30 × 8 mm), closed with cotton. Each of these tubes was stored in a second tube (52 × 10.5 mm), with 0 or 0.3 g $CaCl_2$ as a desiccant, and tightly closed with a rubber stopper.

The tubes with cysts were stored in darkness at three temperatures : + 20, + 2, and – 18 °C, with or without

a drying agent ($CaCl_2$). In addition, one set of cysts was freeze-dried at – 60 °C for 5 h 30 min) and stored at – 18 °C. As a reference, 12 × 500 g samples of infested moist soil were stored in plastic bags at + 2 °C.

After 6, 12, 18, 24, and 60 months of storage, cysts from each storage treatment were exposed to hatching conditions. All cysts were kept at + 20 °C for 5 h before transfer to water. Ten cysts were put into 0.8 ml analytical vials, one cyst per tube (Rivoal, 1978), and 25 cysts were placed in 5 cm-diam. Petri dishes. Distilled water was added to the vials and the Petri dishes, which were kept at + 12 °C. At the same time, the control cysts stored in soil were extracted, hand-picked from the wet debris and placed in distilled water in analysis vials and Petri dishes. Emerged juveniles were recorded at 2 week-intervals and the water was changed at the same time. The emergence was followed for 26 weeks. After this period, the cysts were crushed and the remaining juveniles were counted.

Seedlings of susceptible oats (cv. Selma) were inoculated with juveniles collected from each storage treatment.

Analysis of variance of juvenile emergence was made on angular transformed data by the REGWF test (Anon., 1990). The differences between juvenile emergence from cysts stored without and with a drying agent were tested pair-wise for each storage treatment with Wilcoxon rank sum test, using normal approximation and continuity correction. The differences between cysts freeze-dried or not and stored at – 18 °C were also tested with this ranking test.

Results

After 6 months of storage, the average relative emergence of juveniles from all storage regimes was rather low (0-24 %) with the exception of the cysts stored in soil, from which 70 % of the juveniles emerged (Table 1). With increasing storage time, juvenile emergence either decreased or remained low with two exceptions : *i)* more juveniles emerged from freeze-dried cysts stored at – 18 °C after 5 years than after 6 months; *ii)* no juveniles emerged from cysts kept at + 20 °C with drying agent. Empty eggshells were found in cysts stored in soil for more than 18 months, though no juvenile emergence was observed during the 26 weeks at hatching conditions, indicating a slow juvenile emergence at + 2 °C.

A delay in juvenile emergence of 4 to 6 weeks occurred in all storage treatments except for cysts stored in soil at + 2 °C, where the emergence began within the first 2 weeks and was the highest of all treatments. Emergence continued until the experiment was ended, except for cysts stored in soil, where the main emergence took place within 8 weeks (Fig. 1).

Cysts stored at lower temperatures (– 18 °C) with drying agent and freeze-dried cysts or cysts stored with or without desiccant gave a greater proportion of cysts with at least one emerging juvenile, irrespective of the length of the storage period. At + 20 °C, the proportion of cysts with emerging juveniles was even smaller when the cysts were stored with a drying agent (Fig. 2).

Table 1. *Relative hatch for cysts of* Heterodera avenae, *kept individually in analysis tubes, presented as emerged juveniles as a proportion of the total number of juveniles (i.e. total hatch + unemerged juveniles at 26 weeks).*

Storage regime	Time (months)				
	6	12	18	24	60
WITHOUT DRYING AGENT					
+ 20°	0.12 *bc*	0.01 *cd*	0.03 *bcd*	0.02 *d*	0 *d*
+ 2°	0.14 *bc*	0.08 *abcd*	0.15 *ab*	0.14 *abc*	0.08 *cd*
- 18°	0.07 *b*	0.12 *abc*	< 0.01 *d*	< 0.01 *d*	< 0.01 *cd*
- 18° freeze-dried	0.23 *bc*	0.12 *ab*	0.13 *abcd*	0.26 *a*	0.44 *a*
WITH DRYING AGENT					
+ 20°	0 *c*	0 *d*	0 *d*	0 *d*	0.1 *d*
+ 2°	0.11 *bc*	0.04 *bcd*	0.02 *cd*	0 *d*	0.07 *cd*
- 18°	0.24 *b*	0.23 *a*	0.11 *abcd*	0.07 *bcd*	0.16 *bc*
- 18° freeze-dried	0.15 *bc*	0.10 *ab*	0.05 *bcd*	0.19 *ab*	0.29 *ab*
IN SOIL, + 2 °C	0.70 *a*	0.17 *ab*	0.22 *a*	0.04 *cd**	0.10 *cd**

Means of ten individual cysts. * Cyst with empty eggshells.
Within a column, there are no statistically significant differences between means followed by the same letters ($P \leq 0.05$, REGWF test).

Some cysts have been excluded, since they collapsed during the 26 weeks in water, which probably could be explained by microbiological decay. Even if juveniles emerged before the cysts collapsed, no data from these cysts have been included in the tables and the figures. The number of such excluded cysts for each storage treatment is given in Figure 2.

The results from the pair-wise comparisons by Wilcoxon's two-sample test are that the impact of the drying agent was more evident for cysts stored at + 20 °C and – 18 °C than for cysts stored at + 2 °C and for freeze-dried cysts stored at – 18 °C. The differences between cysts freeze-dried or not and stored at – 18 °C, without or with drying agent, were also tested. When the drying agent was used during storage, the differences were less distinct in juvenile emergence from freeze-dried and not freeze-dried cysts than when the cysts were stored without a drying agent (Table 2).

Cysts from the soil and the roots of the plants inoculated with juveniles from cysts stored 6 and 12 months were extracted and counted. The largest numbers of cysts were recorded for plants inoculated with juveniles from the control cysts, stored in soil at + 2 °C for 6 months. These plants were inoculated with 240, 414 and 526 juveniles, which produced 42, 43 and 284 cysts. Most of the other plants were inoculated with less than 100 juveniles, which produced fewer cysts. Due to

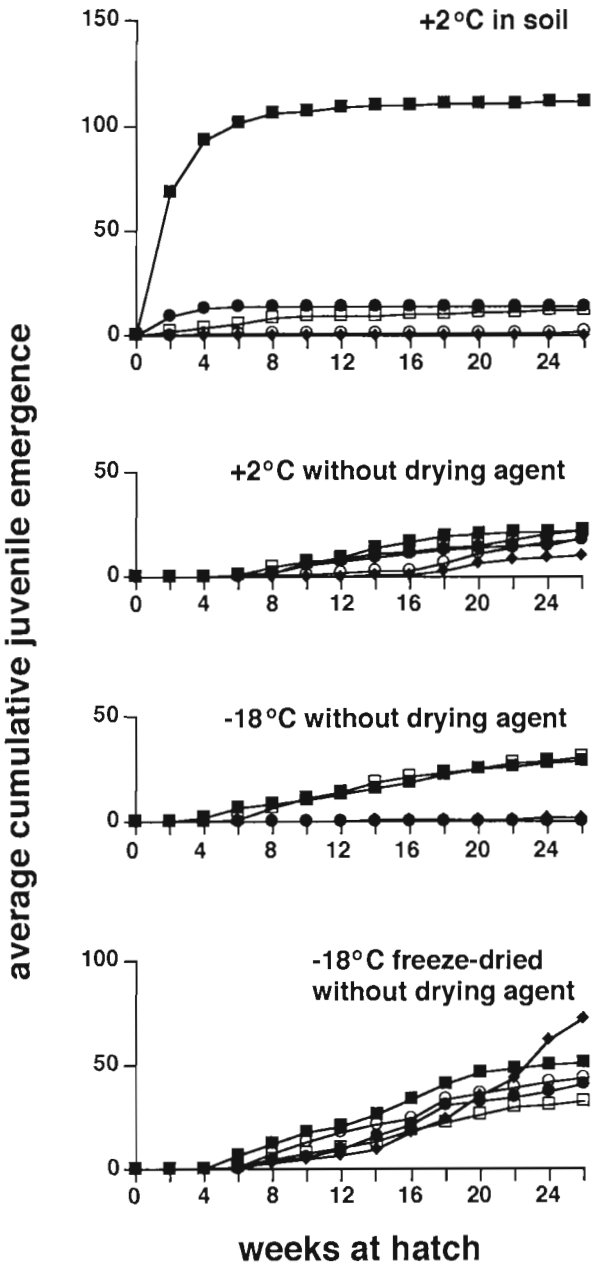

Fig. 1. *Average cumulative emergence from cysts of* Heterodera avenae *stored at four different regimes for five periods of time when exposed to hatching conditions in analysis tubes as well as in Petri dishes (■ 6 months, □ 12 months, ● 18 months, ○ 24 months, ♦ 60 months).*

the differences in the amount of juveniles available for inoculation and in numbers of inoculated plants for the different treatments, a proper comparison seemed impossible. The viability and the infectivity capacity of the juveniles emerging from cysts stored more than

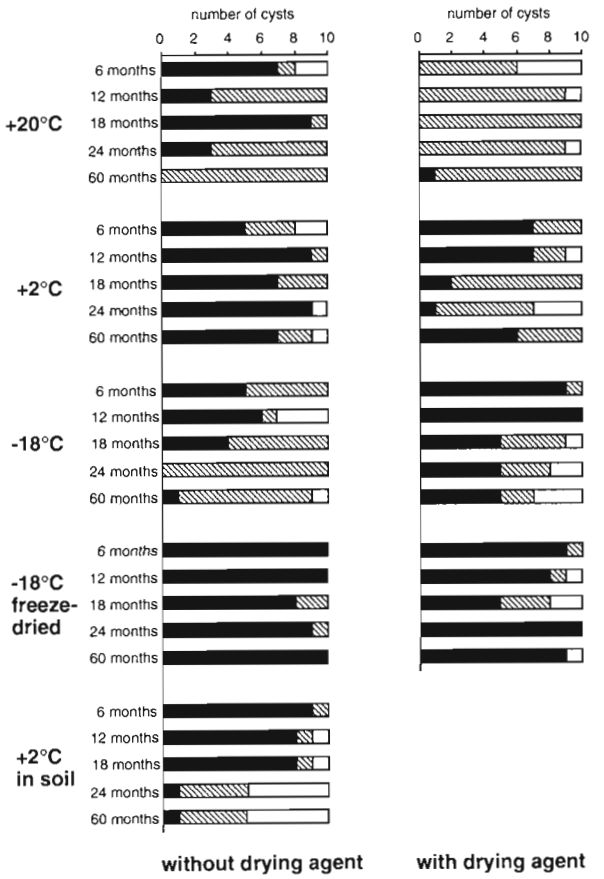

Fig. 2. *Proportion of cysts of* Heterodera avenae *with at least one emerging juvenile in different temperatures and time regimes (black area : cysts with at least one emerging juvenile, striped area : cysts without emerging juveniles, white area : discarded cysts).*

12 months were registered only if females developed. If juveniles emerged from the cysts, females developed regardless of the treatment in which the parent cysts had been stored.

Discussion

Studies on several species of cyst nematodes show that the nematodes pass through a diapause (Evans, 1987). Obligate diapause in nematodes is assumed to be initiated by endogenous factors and broken by environmental conditions, which are in effect for a certain period. Facultative diapause is initiated by environmental factors and ended by spontaneous endogenous factors, acting after a minimum amount of time. Temperature is the most important factor known to break diapause.

The cereal cyst nematode, *Heterodera avenae,* exhibits a strong adaptation to climatic conditions and to its host's life cycle, expressed through different tempera-

Table 2. *P-values from Wilcoxon rank seem test for pair-wise comparisons of differences between juvenile emergence from cysts. The cysts hatched individually in analyse tubes. FD = freeze dried, NFD = not freeze dried.*

Comparison	Time of storage (months)				
	6	12	18	24	60
+ 20° without *vs* with $CaCl_2$	< 0.01	0.09	< 0.001	0.09	0.37
+ 2° without *vs* with $CaCl_2$°	0.79	0.99	0.01	< 0.001	0.62
- 18° without *vs* with $CaCl_2$	0.02	0.11	0.25	< 0.01	0.01
- 18° FD without *vs* with $CaCl_2$	0.79	0.41	0.18	0.23	0.13
- 18° without $CaCl_2$ FD *vs* NFD	< 0.01	0.11	0.01	< 0.001	< 0.001
- 18° with $CaCl_2$ FD *vs* NFD	0.65	0.07	0.92	< 0.07	0.24

ture requirements for initiating or breaking the diapause. In North European populations, the facultative diapause, initiated by high temperatures (approx. 20 °C) in the summer is broken by a period of low temperature followed by an increase to 10-15 °C (Rivoal, 1979, 1982, 1983). Mediterranean populations have an obligate diapause, acting when hot and dry conditions prevail, and which is broken by a temperature decrease (chilling response). Hatching occurs when soil temperatures are below 10 °C and stops when the temperature exceeds 10 °C (Rivoal, 1979). Australian populations also show hibernal activity (e.g., Banyer & Fisher, 1971). In the plains of northern India, the cysts can be exposed to temperatures of 35-46 °C in the soil for 5-6 months, and juveniles will still be able to hatch and invade roots when temperatures fall (Rao & Dwahan, 1988).

These differences in climatic adaptations are important in relation to how populations could be stored. Especially important are when and how the diapause is induced and broken. In this experiment, at least one juvenile hatched from some cysts stored at + 20 °C (Fig. 2). The cysts were extracted in late October and might have had a period of temperature low enough to make juvenile emergence possible. Nonetheless some hatching occurs in the autumn from eggs within newly-formed cysts (Rivoal, 1983; Rivoal & Ireholm, 1990), before diapause is induced in all individuals.

The hatching limits for *H. avenae* seem to have a large range : Fushtey and Johnson (1966) suggested from just above 0° C to above + 25 °C but below + 30 °C. *H. avenae* has been described as being quite sensitive to drying but this seems to be the case only once the hatching processes have started (Meagher, 1982).

The present investigation shows that extracted cysts of *H. avenae* could be stored for at least 5 years at - 18 °C, either with a drying agent or after freeze-drying. The described sensitivity to desiccation (Hesling, 1956; Fushtey & Johnsson, 1966) seems to be avoided by freezing or by freeze-drying, which had a positive effect on preserving viability, affecting both the relative number of juveniles emerging as well as the proportion of cysts from which at least one juvenile emerged.

When cysts were stored in soil for longer than about 18 months, juvenile emergence was considerably more reduced than in earlier assessments. Empty eggshells were found in the cysts, indicating a slow emergence even at the relatively low temperature of + 2 °C. The cysts stored in soil also were very fragile, collapsing when they were extracted or transferred into analysis tubes or Petri dishes for the hatching test or when they had been in water for some weeks.

The results of this experiment emphasize that the diapause of this very adaptable nematode can be exploited for storing extracted cysts at low temperatures, especially when combined with an additional drying.

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