Cryopreservation studies on the nematophagous fungus *Drechmeria coniospora*

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SUMMARY

Three preservation techniques and three cryoprotectants were tested for efficacy in treating conidia of the endoparasitic nematophagous fungus *Drechmeria coniospora*, the nematodes *Caenorhabditis elegans* or *Panagrellus redivivus*, or these nematode species previously infected with this fungus. Significant delay in infection of nematodes from conidia adhering to their cuticles occurred following exposure of conidia to freeze-drying, — 80° or — 170° storage. These observations support a previous hypothesis that different mechanisms are operative in the events associated with adhesion of the conidia to the nematode cuticle and activities related to penetration of the nematode cuticle and colonization by the fungus. Nematodes did not survive freeze-drying. Low rates of survival of nematodes alone, the fungus alone and fungus-infected nematodes were achieved in most of the techniques tested, but greater efficacy is required for practical delivery aimed at biological control of plant parasitic nematodes.

RÉSUMÉ

Études sur la cryoconservation du champignon nématophage Drechmeria coniospora

L'efficacité de trois techniques de conservations et de trois produits cryoprotecteurs a été testée envers les conidies du champignon nématophage endoparasite *Drechmeria coniospora*, les nématodes *Caenorhabditis elegans* ou *Panagrellus redivivus*, et envers ces mêmes nématodes infestés par le champignon cité. Après stockage des conidies à — 80° ou — 170°, le champignon montre un retard significatif dans l'infestation du nématode à partir des conidies fixées à la cuticule. Ces observations confirment l'hypothèse émise antérieurement suivant laquelle des mécanismes de natures différentes entrent en jeu lors de l'adhérence des conidies à la cuticule du nématode, d'une part, et lors de la pénétration à travers la cuticule et de la colonisation ultérieure de l'hôte, d'autre part. Les nématodes ne survivent pas aux très basses températures testées. La plupart des techniques essayées ne permettent qu'un faible taux de survie des nématodes, du champignon, ou des nématodes infestés par le champignon, mais une plus grande efficacité doit, et continue à être recherchée pour la manipulation de ces champignons en vue de la lutte biologique contre les nématodes phytoparasites.

Among the problems encountered in using nematophagous fungi to control plant parasitic nematodes are the introduction of the fungus predator into the soil and/or rhizosphere and the maintenance of sufficiently high inoculum levels to ensure efficacy. To this end, Jansson, Jeyaprakash and Zuckerman (1985*a*) obtained control of root-knot on tomato by adding *Panagrellus redivivus* previously infected with the endoparasitic nematophagous fungus *Drechmeria coniospora* (Drechsler, 1941; Gams & Jansson, 1985). These workers suggested that the *P. redivivus* provided a nutrient source for the fungus, thus allowing for production of a heavy spore load in the soil. The current work was designed to carry this effort forward on a practical level by evaluating methods for storage and delivery of the fungus-infected nematodes to the appropriate site in a form amenable to large scale, commercial usage.

Materials and methods

ORGANISMS

D. coniospora was cultured on dilute corn meal agar plates (CMA 1:10, 1.5 % agar) and conidia collected as described by Jansson, Jeyaprakash and Zuckerman (1985*b*). *Caenorhabditis elegans* and *P. redivivus* were grown axenically on a medium containing heme as the growth factor by the method of McClure and Zuckerman (1982).

The organisms were divided into three categories for the different preservation experiments; 1) nematodes alone, 2) *D. coniospora* alone and 3) nematodes infected with *D. coniospora*.

ADHESION AND INFECTION

Adhesion of *D. coniospora* spores to nematodes either

prior to or following the treatments in the current studies proceeded as in previous experiments (Jansson, Jeyaprakash & Zuckerman, 1985*b*). Briefly, *ca* 10^7 conidia in sterile water were spread on 1.5 % water agar plates and several hundred nematodes in water or tris buffer (pH 7.2) added to the plates. Plates were dried aseptically under a Microvoid Air Control Hood to the point where the nematodes were able to move freely over the agar surface. Under these conditions adhesion of conidia to the nematodes occurs within 1 h. Plates containing nematodes were then observed for the onset of infection which varied with the method of cryopreservation. The life cycle of *D. coniospora* is characterized by vegetative stages of the fungus being restricted to the nematode body (colonization) and to spore-bearing conidiophores which emerge from the body. For the current study, the first observation of conidiophores emanating from the nematode body was designated the onset of infection, since this phase could be readily seen. Thus " days to initial infection " (Tab. 1) was the time when one or more nematode on a plate showed external fungal growth.

Table 1

Effects of freeze-drying and cryopreservation on survival and infectivity of *Drechmeria coniospora* conidia to *Panagrellus redivivus*

Treatment	*Days to initial infection*	*Days to 100 % infection*
Freeze-drying	8.0 ± 1.0 *a*	15.3 ± 1.5 *a*
30 % glycerol/— 80°	3.4 ± 0.6 *b*	7.7 ± 0.6 *b*
30 % glycerol/— 170°	3.7 ± 0.6 *b*	8.0 ± 0.4 *b*
Untreated	2.0 ± 0.1 *c*	3.0 ± 0.1 *c*

Means in columns followed by different letters are significantly different at the 1 % level. Data represents the mean of 3 trials ± standard error.

Cryoprotectants

Three cryoprotectants were evaluated; ethylene glycol, dimethylsulfoxide (DMSO) and glycerol. The procedures for ethylene glycol followed the two-step method (10 to 70 %) used by James (1981). DMSO was tested at 5, 10, and 15 % as described by Hwang (1970) and Haight *et al.* (1975). Glycerol (30 %) was used as described for the cryopreservation of *C. elegans* (Brenner, 1974). Glycerol has been used as a nematode preservative by many workers, including studies on plant parasites by Zuckerman (1963).

Preservation procedures

Three preservation procedures were evaluated : 1) Freeze-drying; 2) Cryopreservation in a Revco freezer with a cooling rate of *ca* 1°/minute down to — 80°; and 3) Cryopreservation under liquid nitrogen (— 170°). Storage under liquid nitrogen was in cryopreservation vials. Each experiment was repeated three times. Approaches to cryoprotection followed guidelines discussed by Mazur (1970).

Freeze-drying

a. *D. coniospora* conidia : Conidia were collected in sterile distilled water, centrifuged for 10 min at 10 000 × g, and resuspended in 0.5 ml sterile, distilled water. The spore suspension was freeze-dried for 3-4 hr until it appeared as a fine, white powder. The freeze-dried conidia were immediately rehydrated in sterile, distilled water and added to 1.5 % water agar plates containing *P. redivivus.* Observations were taken on adhesion of spores to the nematodes and the development of infection.

b. Freeze-drying of *P. redivivus* infected with *D. coniospora :* Infection plates were prepared as described under ADHESION AND INFECTION. Infection was allowed to proceed 2-3 hr. Nematodes were then collected in sterile distilled water, centrifuged briefly to concentrate them, and resuspended in a small amount of sterile, distilled water. Nematodes with attached spores were freeze-dried, rehydrated in sterile, distilled water, and plated on 1.5 % water agar. Observations were made on nematode survival and the development of infection.

Cryopreservation — 80°

a. *D. coniospora* conidia : Conidia were collected in M 9 buffer (Brenner, 1974) and added to an equal volume of 30 % glycerol in M 9 buffer. The vials were placed in a Revco freezer (— 80°) and removed at three day intervals up to 30 days. Conidia were thawed in a 37° water bath and added to 1.5 % water agar plates containing *P. redivivus.* Observations were made on adhesion of spores and development of infection.

b. *C. elegans* in 30 % glycerol : Second-stage larvae of *C. elegans* were collected in M 9 buffer, added to an equal volume of 30 % glycerol, and held at — 80° for 24 hr. Nematodes were thawed in a 37° water bath and plated on 1.5 % water agar. Plates were observed for nematode viability. To provide starved second-stage larvae, eggs of *C. elegans* were placed on 1.5 % water agar, allowed to develop for 36-48 h and treated as above.

c. *C. elegans* in 5, 10, and 15 % DMSO (V/V) were prepared in incomplete medium (without heme). As superior results were obtained with *C. elegans* in a concentration of 10 % DMSO, only those results are reported. *C. elegans* were added to cold DMSO solutions and incubated at 0° in an ice bath for 10 min. The vials were transferred to a — 12° freezer for 10 min. The

supercooled solutions were then seeded with an ice crystal and incubated again for 10 min at — 12°. The frozen samples were then stored at — 80° for 24 h. Samples were thawed in a 37° water bath, centrifuged, and washed in sterile distilled water to remove DMSO, and added to complete medium. Nematode survival was determined.

Cryopreservation — 170°

a. Conidia in 30 % glycerol : Conidia of *D. coniospora* were collected in M 9 buffer, added to an equal volume of 30 % glycerol in M 9 buffer and immersed in liquid nitrogen for 24 h. Thawed conidia were treated as described for cryopreservation — 80° (a).

b. *C. elegans* in 30 % glycerol : Vials of nematodes treated as described above for cryopreservation — 80° (b) were removed from — 80° and immediately immersed in liquid nitrogen. Nematode viability was observed.

c. *C. elegans* infected with *D. coniospora* in 30 % glycerol : *C. elegans* were infected with *D. coniospora* as previously described. Infected nematodes were collected in M 9 buffer then added to 30 % glycerol in M 9 buffer (V/V). Vials were placed at — 80° overnight, then plunged into liquid nitrogen for 1-2 h. Samples were thawed in a 37° water bath and plated on 1.5 % water agar. Observations were made on nematode viability and the development of fungal infection.

d. *C. elegans* in ethylene glycol : *C. elegans* were incubated in 10 % ethylene glycol in distilled water (V/V) for 15 min at room temperature. An equal volume of cold 70 % ethylene glycol was added to the vials and the vials incubated at 0° for 30 min. Vials were then immersed in liquid nitrogen for 1-2 h. Nematodes were thawed in a 37° water bath, centrifuged and washed in sterile distilled water to dilute the ethylene glycol, and plated on 1.5 % water agar. Nematode survival was determined. Nematodes were infected with *D. coniospora* as previously described. Infection was allowed to proceed for 24 h prior to cryopreservation. After 24 h the infected nematodes were treated as above.

e. *P. redivivus* infected with *D. coniospora* in ethylene glycol : *P. redivivus* were infected with *D. coniospora* as previously described. The infected nematodes were treated as described for *C. elegans* under section cryopreservation — 80° (d).

f. *C. elegans* in DMSO : Vials of *C. elegans* treated as described for cryopreservation — 80° (e) were removed from — 80° and plunged into liquid nitrogen. They were thawed in 37° water bath, centrifuged and washed with sterile distilled water to dilute the DMSO, and added to complete media. Nematode survival was determined.

Results

FREEZE-DRYING

a. Freeze-dried *D. coniospora* conidia : Adhesion of treated and untreated conidia occurred to 100 % of the nematodes within 1 h. Onset of infection of *P. redivivus* was significantly delayed, only exceeding 10 % of the nematodes exposed to the fungus after nine days as opposed to two days in untreated conidia (Fig. 1). Infection also developed more slowly with freeze-dried conidia as the inoculum. An average of 15.3 days was required to reach 100 % infection, whereas in control plates 100 % of the nematodes were infected by three days (Tab. 1).

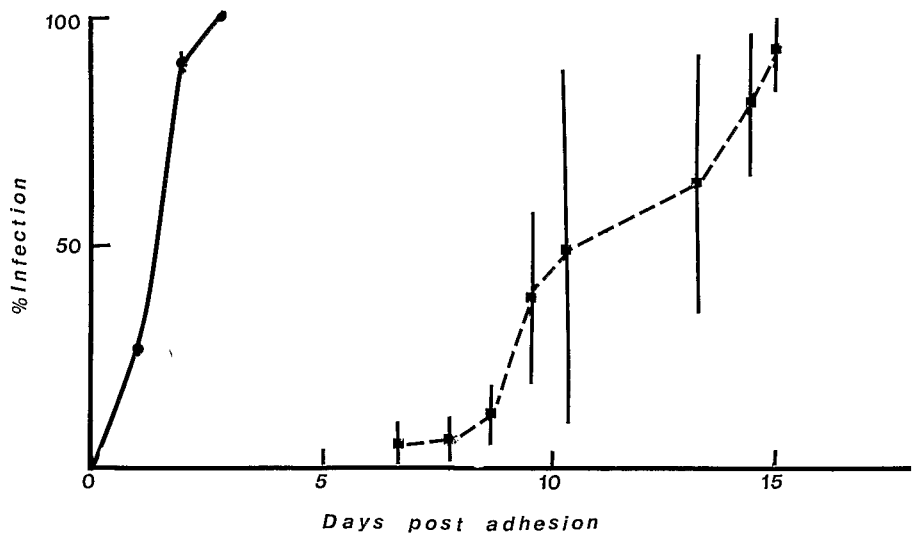

Fig. 1. The infection pattern of *Drechmeria coniospora* to *Panagrellus redivivus* for freeze-dried conidia (-----) compared to untreated conidia (——)

b. Freeze-dried *P. redivivus* infected with *D. coniospora* : Live nematodes were not recovered after freeze-drying. The fungus did not develop within the nematode cadavers.

CRYOPRESERVATION (— 80°)

a. *D. coniospora* conidia in 30 % glycerol : Onset of infection was delayed to a mean of 3.4 days as compared to 2.0 for the control conidia. 100 % infection was achieved by 7.7 days as opposed to three days for the controls (Tab. 1). Varying the length of time spent at — 80° had no effect on the onset or the development of infection, indicating that long-term storage of *D. coniospora* spores is feasible.

b. *C. elegans* in 30 % glycerol : Survival of *C. elegans* was low, averaging only 1.3 % (Tab. 2). Starvation resulted in an increase in nematode survival to an average of 45.3 % (Tab. 2).

c. *C. elegans* in DMSO : Survival rates averaged 15.9 %. Variability among replicates was lower using DMSO than with other methods (Tab. 2). Survival rates probably may have been enhanced if a controlled cooling rate apparatus were available, allowing the slow cooling rate of 0.2°/min to be achieved.

Table 2

Survival of *Caenorhabditis elegans* after cryopreservation treatment

Treatment	*Temperature*	
	— 80°	*— 170°*
30 % glycerol	1.3 ± 1.1*	0
30 % glycerol + starvation	45.3 ± 20.2	5.5 ± 6.4
Ethylene glycol	—	10.9 ± 14.6
10 % DMSO	15.9 ± 11.1	13.7 ± 12.5

* The data are given as percent and represents the mean ± standard error of three trials.

CRYOPRESERVATION — 170°

a. *D. coniospora* conidia in 30 % glycerol : Results were similar to cryopreservation at — 80°; a significant delay in initial infection to 3.7 days and a slowing of infection development, requiring eight days to reach 100 % (Tab. 1).

b. *C. elegans* in 30 % glycerol : Storage under liquid nitrogen sharply reduced the recovery of living nematodes. Without starvation no viable nematodes were observed. Starved *C. elegans* fared better under liquid nitrogen; 5.5 % were recovered alive (Tab. 2).

c. *C. elegans* infected with *D. coniospora* in 30 % glycerol : A substantial number of infected nematodes survived cryopreservation by this method (12.1 %) although only a very few subsequently became infected (0.6 %) (Tab. 3). Initial infection occurred by three days.

d. *C. elegans* in ethylene glycol : For three replicate trials, the mean nematode survival was 10.9 % (Tab. 2). This method had the advantage of ease of execution. After infection for 24 h by *D. coniospora*, a small number of *C. elegans* survived (2.5 %) and a portion of this population developed fungal infection (18.6 %) (Tab. 3).

e. *P. redivivus* infected with *D. coniospora* in ethylene glycol : One percent of the *P. redivivus* survived this cryopreservation procedure and a slightly larger number (3.2 %) developed infection. Initial infection occurred after 4.5 days (Tab. 3).

Table 3

Effects of cryopreservation and freeze-drying on survival and infection of nematodes exposed to *Drechmeria coniospora*

Nematode species	*Treatment*	*Nematode survival (%)*	*Nematode infection (%)**	*Days to sporulation*
P. redivivus	Freeze-drying	0	0	—
P. redivivus	ethylene glycol — 170°	1.0 ± 1.7**	3.2 ± 3.0	4.5
C. elegans	ethylene glycol — 170°	2.5 ± 4.4	18.6 ± 9.0	4.0
C. elegans	30 % glycerol — 80° then — 170°	12.1 ± 13.0	0.6 ± 0.9	3.0
C. elegans	Untreated	—	100.0	2.0

* Number of nematodes that became infected after desiccation treatment.
** Data are given as the mean of three replicate trials ± standard error.

f. *C. elegans* in 10 % DMSO : Storage at — 170° resulted in a slight decrease in nematode survival (13.7 %) as compared to — 80° storage (Tab. 2).

Discussion

Freeze dried conidia adhered to nematodes as readily as did untreated conidia, but infection was significantly delayed. Results strongly suggest that the molecular moieties responsible for adhesion of the spores to the nematode (Jansson & Nordbring-Hertz, 1984 : Zuckerman & Jansson, 1984) remained operative, whereas freeze drying induced a profound effect on the mechan-

isms involved in the infection process. These findings indicate that adhesion and infection by *Drechmeria* spores are separate events, governed by different operational systems, thus adding support for the two stage model for adhesion and infection previously proposed for *Drechmeria* (Jansson, Jeyaprakash & Zuckerman, 1985*b*). Cryopreservation at — 80° and — 170° caused the same delay of infection by *D. coniospora*, as did freeze drying but lag time was shorter.

Freeze drying of fungus-infected nematodes resulted in neither nematode survival nor development of the fungus within the nematode cadaver.

The several combinations of cryopreservants, the two temperature regimes and the added factor of starvation, all resulted in survival of some nematodes. Starvation of second-stage larvae enhanced the recovery rate. While these results may be encouraging to the archivist wishing to perserve cultures, they are unsatisfactory for biocontrol purposes. The relatively high survival of nematodes in the 30 % glycerol : starvation : — 80° treatment indicates that higher survival rates could probably be achieved.

Finally, survival of the fungus within the nematode carrier occurred in all except the freeze dried tests. Further research is required to reproducibly enhance fungus survival in the several systems tested.

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