Technique for Long-Term Preservation of Phytopathogenic Fungi in Liquid Nitrogen

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The authors thank M. C. Schilling for skillful technical assistance.
Accepted for publication 29 June 1982.

ABSTRACT

Dahmen, H., Staub, Th., and Schwinn, F. J. 1983. Technique for long-term preservation of phytopathogenic fungi in liquid nitrogen. Phytopathology 73:241-246.

Survival, pathogenicity, and sporulation of 31 phytopathogenic fungi (including species from the Myxomycetes, Oomycetes, Ascomycetes, Basidiomycetes, and Deuteromycetes) were tested after storage in liquid nitrogen for up to 9 yr. The protective solutions used and rates of freezing and thawing were crucial for successful long-term preservation. Some fungi survived as well in water as in a protective solution. Skim milk-glycerol (8.5% + 10%) and DMSO (15%) were good cryoprotectants for all propagules tested except urediospores of rust fungi, which required dry storage. The samples were frozen either rapidly by direct immersion of the vials in liquid nitrogen or slowly (decreasing 1 C/min to −40 C) followed by transfer into liquid nitrogen. The samples were thawed either in air at room temperature or in water at 20 or 40 C. For successful recovery, all Oomycetes and *Colletotrichum lagenarium* required slow freezing. The recovery of all other fungi was equally good after rapid or slow freezing. The rust fungi required thawing at 40 C. All other fungi survived both quick or slow thawing. All but one of the 31 fungal species studied could be preserved for up to 9 yr. We failed to preserve *Erysiphe graminis*, but we could preserve other powdery mildew fungi such as *Podosphaera leucotricha* and *Erysiphe cichoracearum*. This study demonstrates that with suitable protective solutions and proper handling the viability and pathogenicity of a wide range of fungi can be preserved in liquid nitrogen for long periods of time.

Additional key word: cryopreservation.

Phytopathogenic fungi are difficult to maintain in pure culture without loss of pathogenicity, virulence, and the ability to sporulate. The handling and maintenance of obligate plant pathogens can present several problems, especially when large quantities of inocula are frequently required. Several methods for preserving living cultures are known, such as oil-cover slants, soil or sand cultures, drying (6,8), freezing (1,3), or freeze-drying. Freeze-drying is a useful method for preserving many fungi (11,13,25–27). However, alternatives to freeze-drying are needed for fungal species or isolates that do not sporulate and for others whose spores do not survive the freeze-drying process (eg, sporangia of the Oomycetes and conidia of the powdery mildew pathogens [8,14]).

Successful storage of a broad range of living material (semen, blood cells, etc.) in liquid nitrogen at −196 C has led to the adaptation of this method for the preservation of fungal cultures. A number of microorganisms are routinely stored in liquid nitrogen in type culture collections (14,16) and in the brewing and fermentation industry to minimize variability of cultures (12,23,29). The methods for cryogenic storage of rust fungi, which require storage in dry conditions, are already well established (5,7,17,18,25). Methods for the preservation of other fungi, such as the Oomycetes and powdery mildew fungi, are not well defined (2,9,19,24).

Several cryoprotectant solutions have been reported in the literature. Several authors (2,7,9,15,19) have reported DMSO to be an excellent cryoprotective agent for various fungi. Skim milk alone was used as protective solution for freeze-drying bacteria (11) and glycerol alone gave varying results with bacteria (11,22) and fungi (7,8,10,14–16,19). L-Proline was an excellent cryoprotectant for cell cultures of higher plants (30,31). Preliminary experiments with a mixture of skim milk and glycerol in our laboratory showed promising cryoprotective effects (*unpublished*).

The purpose of this study was to examine more systematically the effectiveness of cryogenic (liquid nitrogen) preservation of a wide range of phytopathogenic fungi with DMSO, skim milk-glycerol and, for some species, L-proline-glycerol used as cryoprotectants. For the rust fungi, these methods were compared to the known procedures for preserving dry urediospores in liquid nitrogen.

MATERIALS AND METHODS

Production of fungal material for storage. The spores of the obligate parasites were produced on their host plants under suitable conditions in growth chambers. Spores or mycelia of the facultative parasites were produced on artificial media (Table 1). Spore germination, mycelial growth, and pathogenicity were tested prior to freezing.

Preparation of the fungal material for storage. Freshly produced spores were collected by washing them from infected leaves (obligate parasites) or agar plates (nonobligate parasites) with deionized water, or for dry storage, by brushing them from infected leaves.

To obtain a high spore concentration, spores suspended in the washing solution were centrifuged (about 3,000–5,000 *g* for 10 min) and resuspended in a smaller volume of the protective solution. The final spore density varied from 10^6 to 10^8 spores per milliliter depending on the fungus.

Monilinia fructigena, *Phytophthora citrophthora*, and *P. cactorum*, all nonsporulating strains, were stored by suspending mycelial fragments directly in the protective solution. *Rhizoctonia solani* either was suspended in a protective solution, when present as mycelial mat, or was stored dry, in colonized grains. The resting spores of *Plasmodiophora brassicae* were stored within cabbage root fragments without cryoprotectant solution. Urediospores of rust fungi were air-dried for 24 hr at room temperature prior to storage.

The spore and mycelial suspensions and dry material were dispensed in cryogenic storage vials. For storage of nonsterile material, the vials were polypropylene cryogenic screw-cap serum tubes (A/S Nunc, Roskilde, Denmark) with 2- or 5-ml sample capacity. For sterile storage of nonobligate parasites, polypropylene vials for heat-sealing (Steriline, Teddington, UK)

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with a sample capacity of 1.5 ml were used. All vials with screw-cap closures were stored in the vapor phase of nitrogen and all sealed vials were stored in the liquid phase. The vials were stored in a model CG 140 liquid nitrogen refrigerator (Cryodiffusion, Léry, France).

Cryoprotective solutions. The following cryoprotective solutions were used:

a. Skim milk, 8.5% - glycerol, 10% (SG). Stock solutions of 17% skim milk (Difco) and 20% glycerol (C. Roth KG, Karlsruhe, Germany) were mixed 1:1 after autoclaving.

b. Dimethyl sulfoxide (DMSO) (Merck) 15%. Because DMSO can be toxic to several biological systems (12), it was washed from the fungal samples after they were removed from storage.

c. L-Proline, 5%-glycerol, 5%. Stock solutions of 10% L-proline (Senn Chemicals, Dielsdorf, Switzerland) and 10% glycerol were mixed 1:1 after autoclaving. The solution was washed from the fungal samples after they were removed from storage.

d. Water control.

Freezing and thawing procedures. The samples were frozen either slowly at a controlled rate of 1 C/min from ambient temperature to −40 C followed by quick immersion in liquid nitrogen, or rapidly by direct immersion in liquid nitrogen. The controlled rate of decrease in temperature was achieved with the controlled temperature capabilities of a model L2 Ismatec freeze-dryer (Ismatec, Zürich, Switzerland).

Three rates of thawing were tested:

a. Slow thawing—20–30 min stationary in air at room temperature;

b. Intermediate thawing—the storage vials containing the frozen material were swirled for 5–7 min (depending on vial size) in a 20 C waterbath; and

c. Rapid thawing—the storage vials containing the frozen material were swirled for 2–4 min in a 40 C waterbath.

These methods do not distinguish between the effects of the thawing conditions on viability and on cold-induced dormancy, which is important for the rust fungi (17).

TABLE 1. Media and techniques used to cultivate various phytopathogenic fungi and to increase their inoculum

Fungal species	Media and their ingredients or references	Incubation conditions and special techniques	Incubation time in weeks
Phytophthora infestans	Rye decoction agar medium: 200 g rye grain, 5 g D-glucose, 20 g agar, 1 liter deionized water. The rye is boiled for 1.50 hr, the grains are discarded, the remaining ingredient added; pH 5.6 (C. A. Drandarevski, *personal communication*)	2 weeks at 18 C, 1 week at 12 C, in the dark, Roux-bottles	3
P. cactorum and *P. citrophthora*	Rye decoction broth medium: same ingredients as above without agar	22 C, in the dark, Roux-bottles	2
Pythium ultimum	Carrot medium: 150 g grated carrots in 1 liter of deionized water	18 C, in the dark, Roux-bottles	2
Rhizoctonia solani	Millet medium: 25 g millet suspended in 50 ml deionized water	24 C, in the dark, 300 ml flasks	3
Botrytis cinerea	Pea agar medium: 250 g canned peas are blended, 20 g saccharose, and 15 g agar added, and made up with deionized water to 1 liter; pH 6.5	24 C, cool white fluorescent light[a], Roux-bottles	2
Pyricularia oryzae	Oat decoction agar medium: 30 g rolled oats are boiled for 60 min in 1 liter of deionized water. The rolled oats are discarded, 20 g saccharose and 18 g agar added and made up with water to 1 liter.	24-26 C, 1 week in the dark, 1 week under cool white fluorescent light[a], petri plates	2
Cercospora arachidicola	Czapek-Dox V-8 medium (4)	24 C, cool white fluorescent light[a], circular cellulose filter[b] on the agar, petri plates	2
Alternaria solani	Potato-carrot-agar medium (20)	(28)	(28)
Helminthosporium gramineum and *H. oryzae*	Lactose-casein-hydrolysate medium (28)	(28)	(28)
Septoria nodorum	Czapek-Dox V-8 medium (4)		
Colletotrichum lagenarium	V-8 agar medium (28)	24 C, in the dark, in Roux-bottles	2
Fusarium oxysporum f. sp. *lycopersici*	V-8 agar medium (28)	24 C, in the dark, in Roux-bottles	2
Gloeosporium musarum	V-8 agar medium (28)	24 C, in the dark, in Roux-bottles	2
Penicillium digitatum	V-8 agar medium (28)	24 C, in the dark, in Roux-bottles	2
Verticillium albo-atrum	V-8 agar medium (28)	24 C, in the dark, in Roux-bottles	2

[a] Fluorescent light: Philips TLMF 40W/33 RS tubes.
[b] Cellulose filter: manufactured by Macherey-Nagel, Düren, West Germany.

Viability tests. Spore germination (direct via germtubes or indirect via zoospores) on agar was used as the indicator for viability. Spore germination was determined with four counts of 100 spores each. For nonsporulating fungi, a mycelial growth test on agar was chosen to demonstrate survival. For most fungi, disease development after inoculation of four to 10 appropriate host plants was observed to determine the effects of the storage conditions on pathogenicity. In each recovery test, the viability of the stored material was compared with that of a control sample of fresh material. *Rhizoctonia solani*, *Pythium ultimum*, or *Plasmodiophora*

TABLE 2. The influence of cryoprotectants on the viability of phytopathogenic fungi after storage in liquid nitrogen[a]

Class and species (strain no.)	Germination (%)[b]						Disease (%)				
		Frozen sample						Frozen samples[c]			
	Fresh samples	Water	Skim milk-glycerol (SG)	DMSO	L-proline-glycerol (PG)	Dry	Fresh samples	Skim milk-glycerol (SG)	DMSO	L-Proline-glycerol (PG)	Dry
Oomycetes											
Phytophthora infestans ATCC 44725	74	0	42	22	···	···	100	100	90	···	···
Plasmopara viticola	79	0	41	41	33	···	100	100	100	100	···
Bremia lactucae	···	0	···	···	···	···	100	80	80	···	···
Phytophthora cactorum[c] (119)	100	···	100	100	···	···	100	98	52	···	···
Ascomycetes											
Erysiphe cichoracearum	···	···	···	···	···	···	100	27	100	100	0
Erysiphe graminis f. sp. *hordei*	···	···	···	···	···	···	100	0	0	0	0
Podosphaera leucotricha	···	···	···	···	···	···	100	87	···	···	67
Basidiomycetes											
Rhizoctonia solani (160)[c]	100	100	100	···	···	100	100	100	···	···	100
Uromyces phaseoli	47	0	0	···	···	54	100	20	···	···	80
Puccinia dispersa	43	0	0	···	···	48	100	0	···	···	100
Puccinia graminis f. sp. *tritici*[d]	93	0	0	0	···	93	100	0	0	···	100
Deuteromycetes											
Colletotrichum lagenarium (4)	76	2	74	40	10	···	100	86	85	0	···
Cercospora arachidicola (59)	94	50	91	80	17	···	100	90	90	0	···
Fusarium oxysporum f.sp. *lycopersici* (198)	100	86	99	99	···	···	100	100	85	···	···
Pyricularia oryzae (283)	85	59	55	30	···	···	100	100	80	···	···
Septoria apii (103)	100	100	100	60	93	···	100	100	60	100	···

[a] All fungi were slowly frozen and rapidly thawed (40 C waterbath), with the exception of *Phytophthora cactorum*, which was thawed in a waterbath at 20 C.
[b] The maximal standard error for germination was ±4.5.
[c] Estimated vigor of mycelial growth in comparison to fresh material.
[d] Spore germination on leaves.

TABLE 3. The influence of freezing rate on spore germination and pathogenicity after storage in liquid nitrogen

Class and species (strain no.)	Thawing rate[a]	Cryoprotectant[b]	Germination (%)[c]			Disease (%)		
			Fresh material	Freezing rate		Fresh material	Freezing rate	
				Slow	Rapid		Slow	Rapid
Myxomycetes								
Plasmodiophora brassicae	SI	none	···	···	···	100	100	50
Oomycetes								
Phytophthora infestans ATCC 44725	R	SG	···	···	···	100	100	25
Plasmopara viticola	R	SG	···	···	···	100	100	15
Pythium ultimum (71)	R	SG	100	100	0	100	100	0
Ascomycetes								
Erysiphe graminis f. sp. *hordei*			···	···	···	100	0	0
Podosphaera leucotricha	R	SG	···	···	···	100	87	73
Monilinia fructigena (179)[d]	SI or R	SG	100	100	100	100	100	100
Venturia inaequalis (182)	R	SG	64	62	37	80	90	90
Basidiomycetes								
Puccinia dispersa	R	none	43	48	38	100	100	80
Puccinia graminis f.sp. *tritici*	R	none	80	93	89	100	100	100
Uromyces phaseoli	R	none	47	54	38	100	86	96
Deuteromycetes								
Cercospora arachidicola (59)	R	SG	94	91	82	100	90	95
Colletotrichum lagenarium (4)	SI	SG	76	74	0	100	86	0
Fusarium oxysporum f. sp. *lycopersici* (198)	SI or R	SG	100	100	100	100	100	100
Gloeosporium musarum (101)	SI	SG	92	87	77	71	90	98
Penicillium digitatum (155)	SI	SG	98	98	98	100	100	90
Pyricularia oryzae (283)	R	SG	85	55	35	100	100	100
Septoria nodorum (135)	SI	SG	100	100	78	80	100	95

[a] SI = slow-intermediate thawing procedure (20 C air or 20 C waterbath); R = rapid thawing procedure (40 C waterbath).
[b] SG = cryoprotectant skim milk-glycerol; none = dry storage.
[c] The maximal standard error for germination was ±4.1.
[d] Estimated vigor of mycelial growth compared with isolates from fresh material.

brassicae were incorporated into the soil in which test hosts were grown.

Fungal material for storage was produced at least three different times. From each production, fungal material was thawed and analyzed on two or three different occasions. The values in Tables 2–4 represent for each fungus the results of one storage experiment. The results of the repeated experiments were similar.

RESULTS

Effects of the cryoprotective solutions. In the water controls the viability and pathogenicity of *R. solani*, *Fusarium oxysporum* f. sp. *lycopersici*, and *Septoria apii* were just as good after storage in liquid nitrogen as they were for the fresh material (Table 2). In water, however, the Oomycetes and *Colletotrichum lagenarium* did not survive storage in liquid nitrogen and the germination of *Cercospora arachidicola* was reduced 30–40%.

DMSO and skim milk-glycerol (SG) offered good protection against freezing and thawing damage of most fungi (Table 2). *Phytophthora infestans* and *Colletotrichum lagenarium* were more effectively protected by SG than by DMSO, which reduced germination up to 50%. No significant difference in disease development was detected when the two protective solutions were compared, except with *Phytophthora cactorum*, in which a 50% reduction in disease development was caused by DMSO. The novel cryoprotectant solution, L-proline-glycerol (PG) protected *Plasmopara viticola*, and *Septoria apii* as well as DMSO and SG, but it was not suitable for *Colletotrichum lagenarium* and *Cercospora arachidicola*. For both fungi, spore germination and disease development were reduced up to 80 and 100%, respectively, after storage in PG. Preliminary results with *Erysiphe graminis* showed 100% disease development after storage in liquid nitrogen when suspended in DMSO or PG whereas when suspended in SG only 27% disease development was found. *Rhizoctonia solani* survived storage as well in SG as in dry conditions.

Effects of freezing procedure. The results in Table 3 show that the slow-freezing method can be used for all fungi. Rapid freezing was not effective with *Pythium ultimum* and *Colletotrichum lagenarium* and a severe reduction in spore germination and/or disease development was observed with *Plasmodiophora brassicae*, *Phytophthora infestans*, *Plasmopara viticola*, *Venturia inaequalis*, *Gloeosporium musarum*, *Pyricularia oryzae*, and *Septoria nodorum*. The recovery of all other fungi was just as good after rapid freezing as after slow freezing.

Effects of the thawing procedure. For most fungi all the thawing procedures tested proved suitable and provided good spore germination, mycelial growth, and disease development (Table 4). Exceptions were *Botrytis cinerea* and *Erysiphe cichoracearum*. *B. cinerea* survival was 100% after an intermediate rate of thawing (20 C waterbath) whereas its germination was reduced by about 70 and 50% after slow and rapid thawing, respectively. Recovery of *Erysiphe cichoracearum* was reduced almost 100% after slow thawing, but intermediate and rapid thawing gave 100% disease development.

Experience with long-term storage of fungal material. Table 5 summarizes our experience with the preservation of phytopathogenic fungi in liquid nitrogen refrigerators. A number of our organisms have been stored in liquid nitrogen for 5–9 yr without a significant loss in viability compared to fresh, nonfrozen material. *Plasmopara viticola*, *Venturia inaequalis*, and *Penicillium*

TABLE 4. The influence of thawing rate on spore germination and pathogenicity after storage in liquid nitrogen[a,b]

Class and species (strain no.)	Germination (%)[c]				Disease (%)			
	Fresh material	20 C Air	20 C Waterbath	40 C Waterbath	Fresh material	20C Air	20C Waterbath	40 C Waterbath
Myxomycetes								
Plasmodiophora brassicae	...	...	...	...	100	100	...	100
Oomycetes								
Phytophthora infestans ATCC 44725	46	40	40	40	100	100	100	100
Plasmopara viticola	...	51	48	51	100	80	95	96
Phytophthora cactorum (119)[d]	100	100	100	100	100	60	98	35
Pseudoperonospora cubensis	49	37	46	50	100	95	85	100
Ascomycetes								
Erysiphe cichoracearum	...	...	...	...	100	9	100	100
Erysiphe graminis f. sp. *hordei*	...	...	...	...	100	0	0	0
Monilinia fructigena (179)[d]	100	100	...	100	100	100	...	100
Venturia inaequalis (182)	64	54	45	62	50	100	90	90
Basidiomycetes								
Rhizoctonia solani (160)[d]	100	90	90	90	100	100	100	100
Hemileia vastatrix	84	6	8	87	100	0	0	95
Puccinia dispersa	43	0	0	48	100	3	0	100
Puccinia graminis f.sp. *tritici*	82	...	8	93	100	...	10	100
Uromyces phaseoli	47	...	57	54	100	...	100	86
Deuteromycetes								
Alternaria solani (3)	100	93	97	95	100	70	100	90
Botrytis cinerea (192)	98	30	98	52	100	52	100	82
Cercospora arachidicola (59)	90	68	76	90	100	65	80	100
Colletotrichum lagenarium (4)	76	74	61	68	100	86	52	43
Fusarium oxysporum f.sp. *lycopersici* (198)	100	100	...	100	100	100	...	100
Gloeosporium musarum (101)	92	79	83	87	71	97	99	90
Penicillium digitatum (155)	98	98	97	92	100	100	55	100
Pyricularia oryzae (283)	85	35	19	55	100	100	100	100
Septoria nodorum (135)	100	100	100	100	80	100	90	95
Verticillium albo-atrum (86)	100	85	83	60	100	100	100	100

[a] All fungi were slowly frozen, except *Erysiphe cichoracearum*, which was frozen rapidly.

[b] *Plasmodiophora brassicae, Rhizoctonia solani*, and all rust fungi were stored under dry conditions; *Pseudoperonospora cubensis* was suspended in DMSO; the rest of the fungi were suspended in skim milk-glycerol.

[c] The maximal standard error for germination was ±4.0.

[d] Estimated vigor of mycelial growth compared with isolates from fresh material.

digitatum have been stored for 8–9 yr, and *Plasmodiophora brassicae*, *Phytophthora infestans*, *Pythium ultimum*, *Podosphaera leucotricha*, *Rhizoctonia solani*, *Uromyces phaseoli*, *Hemileia vastatrix*, *Botrytis cinerea*, *Fusarium oxysporum* f. sp. *lycopersici*, *Gloeosporium musarum*, and *Verticillium albo-atrum* have been stored for 5–6 yr. We found no difference between storage in the vapor or liquid phase of nitrogen with any fungus.

We have not found appropriate freezing and storage conditions for successful preservation of conidia of *Erysiphe graminis* f. sp. *hordei* in liquid nitrogen, whereas *Podosphaera leucotricha* and *Erysiphe cichoracearum* retained viability with most procedures used.

Rust fungi. Our studies with rust fungi confirm that they do not survive cryogenic storage in liquid media but that survival is good under dry conditions (Table 2). Germination and disease development were significantly reduced after slow and intermediate rate of thawing of rust fungi *Hemileia vastatrix*, *Puccinia dispersa*, and *P. graminis* f. sp. *tritici* (and, to a lesser extent, *Uromyces phaseoli*). When urediospores were thawed rapidly at 40 C, almost 100% recovery was obtained compared with recovery from fresh material (Table 4).

DISCUSSION

This study confirms observations made by several authors (5,7,17,21) that damage to fungal spores can occur during freezing and thawing, and that cryoprotective solutions can have a decisive effect on survival of fungi. The data demonstrate that no single procedure is suitable for all fungi, but that each fungal class, family, or even species may require special handling for successful, long-term preservation. However, the results also indicate that for some fungi more than one or even all of the tested procedures are suitable.

Some fungi, such as *Rhizoctonia solani*, *Fusarium oxysporum* f. sp. *lycopersici*, and *Pyricularia oryzae* are equally well stored in water as in a protectant solution. However, cryogenic storage of other fungi, such as all representatives of the Oomycetes, *Colletotrichum lagenarium*, and, to a lesser extent *Cercospora arachidicola*, is dependent upon the proper cryoprotectant solution. In our tests, the protective solutions DMSO and the mixture SG offered good protection for most fungi against freezing and thawing damage. PG was tested on five fungi; two of the five fungi showed poor survival. From these preliminary results, it appears that PG is less suitable for fungi than SG and DMSO and that its advantages are restricted to cell cultures from plants (30).

The thin-walled sporangia of Oomycetes, the resting spores of *Plasmodiophora brassicae*, and the conidia of *Colletotrichum lagenarium* were highly sensitive to the freezing step and very tolerant to all three thawing procedures. Maximum spore germination and disease development after storage were obtained by slow freezing and by slow, intermediate, or rapid thawing. Microscopic observations of sporangia of the Oomycetes showed extensive deformation, severe plasmolysis, and highly granulated plasma when frozen rapidly; sporangia frozen slowly appeared normal and were filled with homogeneous cytoplasm.

The survival of *Pyricularia oryzae* was reduced by about 50%

TABLE 5. Summary of useful cryoprotectant, freezing, and thawing procedures for storage of phytopathogenic fungi in liquid nitrogen and storage time[a]

Class and species (strain no.)	Procedure investigated: Protective solutions: Skim milk-glycerol (SG)	DMSO	None (dry)	Freezing conditions: Directly into liquid nitrogen	Stepwise to −40 C then into liquid nitrogen	Thawing conditions: Water 20 C	Water 40 C	Successful storage in liquid nitrogen (yr)
Myxomycetes								
Plasmodiophora brassicae			+	+−	+	+	+	6
Oomycetes								
Phytophthora infestans ATCC 44725	+	+		−	+	+	+	5
Plasmopara viticola	+	+		−	+	+	+	9
Phytophthora cactorum (119)	+	+			+	+	+	2
Bremia lactucae	+	+			+		+	3
Pythium ultimum (71)	+			−	+	+	+	5
Phytophthora citrophthora (262)	+				+		+	3
Pseudoperonospora cubensis		+			+	+	+	1
Ascomycetes								
Erysiphe cichoracearum	+−	+	−	+	+−	+	+	1
Erysiphe graminis f. sp. *hordei*	−	−	−	−	−	−	−	−
Podosphaera leucotricha	+		+	+	+	+−	+	5
Venturia inaequalis (182)	+			+	+	+	+	8
Monilinia fructigena (179)	+			+	+	+	+	3
Basidiomycetes								
Rhizoctonia solani (160)	+		+	+−	+	+	+	5
Uromyces phaseoli	−		+	+	+	+−	+	5
Hemileia vastatrix			+	+	+	−	+	5
Puccinia graminis f. sp. *tritici*	−	−	+	+	+	−	+	3
Puccinia dispersa	−		+	−	+	−	+	4
Deuteromycetes								
Alternaria solani (3)	+				+	+	+	4
Botrytis cinerea (192)	+				+	+	+−	5
Colletotrichum lagenarium (4)	+	+		−	+	+	+	1
Fusarium oxysporum f.sp. *lycopersici* (198)	+	+		+	+	+	+	5
Gloeosporium musarum (101)	+			+	+	+	+	5
Penicillium digitatum (155)	+			+	+	+	+	9
Pyricularia oryzae (283)	+	+		+	+	+	+	2
Cercospora arachidicola (59)	+	+−		+−	+	+	+	4
Helminthosporium gramineum (121)	+				+		+	3
Helminthosporium oryzae (201)	+				+		+	1
Septoria nodorum (135)	+			+	+	+	+	1
Verticillium albo-atrum (86)	+				+	+	+	6
Septoria apii (103)	+	+−		+	+	+	+	1

[a]Symbols: +, successful procedure; +−, partially successful procedure; and −, unsuccessful procedure.

during storage in liquid nitrogen regardless of the storage procedure chosen (Tables 2–4). However, survival even in water controls was sufficient to produce infections comparable to those obtained with fresh samples.

None of these various preservation procedures tested were suitable for *Erysiphe graminis*. Our results do not explain the failure, but illustrate that even individual species do have different requirements for liquid nitrogen storage.

Our results with the rust fungi corroborate the work done in other laboratories (5,17,18). Urediospores suspended in water or in one of our cryoprotectant solutions germinated poorly or not at all, regardless of the treatments, whereas spores preserved dry germinated well. Also, in our tests the thawing temperature had to be 40 C, whereas the rate of cooling had little or no effect on survival. These specific requirements of rust fungi for good survival during storage in liquid nitrogen suggest fundamental differences in the physiology and water relationships of these spores compared with spores of other fungi.

From this study, the suitability of long-term storage in liquid nitrogen for fungi from the different classes is apparent. The results show that with this method most fungi can be preserved without loss of viability and virulence, and that it can be used for a wider range of fungi than other methods of preservation. Therefore, it appears suitable for the maintenance of stock cultures and homogeneous infection material for routine testing.

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