Use of Uncontrolled Freezing for Liquid Nitrogen Storage of *Phytophthora* Species

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ABSTRACT

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Simple, inexpensive techniques were developed to allow storage of *Phytophthora* species in liquid nitrogen without the use of a programmable slow-freezing unit. Agar plugs containing mycelium were suspended in 1 ml of cryoprotectant (10% glycerol or 5% dimethyl sulfoxide) in screw-cap polypropylene vials and placed in freezers at −20 C and/or −80 C before storage in liquid nitrogen. High recovery rates (75–100%) were obtained for samples of *P. infestans* placed at −20 C for 24 min then −80 C for 60 min, and for samples placed at −80 C alone for 30, 45, 60, or 84 min before freezing in liquid nitrogen. Recovery rates of 68–100% were obtained after 7 days when subcultures of 10 different *Phytophthora* species were placed at −80 C for 60 min before storage in liquid nitrogen. Isolates of *P. infestans*, *P. boehmeriae*, and *P. megasperma* f. sp. *glycinea* frozen by this method showed no decrease in viability following at least 9 mo of storage in liquid nitrogen. The success of this technique may allow more widespread use of liquid nitrogen storage technology for *Phytophthora* spp.

Storage of *Phytophthora* species in liquid nitrogen is a procedure not widely used by plant pathologists and mycologists, even though it is generally regarded as the method of choice (1,13,14). Other less desirable and more laborious methods such as periodic transfer (3,8), storage under mineral oil (3,8,15), or storage in plant tissue (4,5,9) have been used more routinely by *Phytophthora* workers. One reason for this has been the prohibitively high cost of programmable slow-freezing units needed for liquid nitrogen storage of *Phytophthora* species. To survive freezing in liquid nitrogen many fungi, including *Phytophthora*, are reported to require a controlled temperature decrease of about 1 C per minute (1,2,6,7).

The technique of 'uncontrolled freezing' (1) involves the placing of samples in a mechanical freezer (−55 to −80 C) for a prescribed time before storage in liquid nitrogen. This method has been used successfully for storage of a variety of organisms (10,11,14), and is available to anyone having access to a mechanical freezer. Should such a method be effective for storage of *Phytophthora* species, it could gain wide usage by plant pathologists since −80 C freezers have become more widely available in many research institutions.

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The purpose of these studies was to determine whether uncontrolled freezing could be successfully applied to long-term storage of *Phytophthora* species in liquid nitrogen. Simple, inexpensive uncontrolled freezing techniques were developed that allowed high rates of recovery of 10 different *Phytophthora* species from storage in liquid nitrogen after time periods of up to 1 year.

MATERIALS AND METHODS

Isolates of *Phytophthora*. Four isolates of *P. infestans* (Mont.) de Bary were used in the majority of these studies: isolate 102 (ATCC 48716), isolate 106 (ATCC 48719), isolate 111 (ATCC 48720), and isolate 127 (ATCC 48723). All four isolates originated from New York state and were isolated from blighted potato plants between 1979 and 1982. Isolate 102 was chosen for use in these studies because it has slower growth and less sporulation in culture and was more difficult to maintain in storage than other isolates. Isolates of nine other *Phytophthora* species were obtained from various contributors.

Freezing experiments with pretreatments at −20 and −80 C. Isolates of *P. infestans* were grown for approximately 7 days on 20% V-8 juice agar in 9-cm-diameter petri dishes at 18 C in darkness. Four no. 2 cork borer plugs were cut from the colony margins and placed into 1 ml of sterile 10% (v/v) glycerol (in distilled water) in 2-ml screw-cap polypropylene vials. Three tubes containing four plugs per isolate per experiment were used in each experiment. The tubes were snapped into 29-cm aluminum canes (six tubes per cane) and covered with cardboard cryosleeves.

One treatment was plunged directly into liquid nitrogen (−196 C) while other canes containing samples representing the second, third, and fourth treatments were placed inside 51 × 51 × 419 mm stainless steel pipet cans (each treatment in a separate can) and treated as follows. The second treatment was placed at −20 C for 24 min and the canes then were removed from the steel can and plunged into liquid nitrogen. The third treatment was placed at −20 C for 24 min, then transferred to a −80 C freezer for 84 min. Samples were then removed from the can and plunged into liquid nitrogen. The fourth treatment was placed directly in a −80 C freezer for 84 min. Samples were then removed from the can and plunged into liquid nitrogen.

Further experiments were performed varying the time length of −80 C pretreatments necessary for survival of *P. infestans* in liquid nitrogen. Four plugs of isolates 102, 106, 111, and 127 were placed in 1 ml of 10% glycerol in 2-ml screw-cap polypropylene vials. One set of tubes (three replications per treatment) was then plunged directly into liquid nitrogen, while other sets were placed in stainless steel pipet cans and pretreated in a −80 C freezer for 15, 30, 45, 60, and 84 min before plunging into liquid nitrogen.

Twenty-two isolates comprising nine different *Phytophthora* species were divided into three groups of manageable size for freezing experiments. For each group, three experiments were performed in which four no. 2 cork borer plugs in 1 ml of 10% glycerol or 5% (v/v) dimethyl sulfoxide (DMSO) in 2-ml screw-cap polypropylene vials were frozen in liquid nitrogen following a 60-min pretreatment at −80 C (three tubes per isolate per experiment for a total of 12 plugs per isolate per experiment).

Samples in all experiments were thawed 7 days after freezing by removing the tubes from liquid nitrogen and placing them in a 37 C water bath for 2–4 min. The four plugs from each tube were then plated on one 9-cm-diameter petri dish of 20% V-8 juice agar and incubated at 18 C in darkness.

Plugs were rated for the presence or absence of mycelial growth 7 days after plating. Percent recovery for each isolate was determined from the number of plugs with viable mycelium divided by the total number of plugs frozen per isolate per experiment. Data were analyzed by analysis of variance, and means were compared using the Waller-Duncan Bayesian *k*-ratio *t* test (12).

Freezing rates inside the −80 C freezer

were monitored with a digital thermometer containing a type k thermocouple probe. The probe was placed in a stainless steel pipet can inside a 2-ml screw-cap polypropylene vial containing 1 ml of cryoprotectant (10% glycerol or 5% DMSO) and four agar plugs.

RESULTS

In the first series of experiments involving both −20 and −80 C pretreatments of samples before freezing in liquid nitrogen, high recovery rates were obtained only when samples were given a −80 C pretreatment or a pretreatment at −20 and −80 C (Table 1). Low recovery resulted for samples plunged directly into liquid nitrogen and those given a pretreatment only at −20 C before freezing in liquid nitrogen (Table 1).

Since little difference in percent recovery was observed between samples given a pretreatment at −20 and −80 C and those receiving a pretreatment at −80 C alone, further experiments centered upon defining important parameters for use of the −80 C pretreatment. Samples receiving a −80 C pretreatment of 30, 45, 60, or 84 min survived freezing in liquid nitrogen while those receiving no pretreatment or a 15-min pretreatment showed very low recovery (Table 2).

Use of the digital thermometer to monitor the freezing rate of the samples inside the stainless steel pipet cannister at −80 C revealed that 20–25 min were required for the samples to reach −40 C, the temperature at which controlled freezing is generally halted when using programmable slow-freezing units. The average freezing rate in these experiments from room temperature down to −40 C was about 3 C per minute, but rates of up to 5 C per minute were noted during the earliest and last 5–6 min of the freezing run.

Six experiments were performed to evaluate the reproducibility of the uncontrolled freezing method for recovering *P. infestans* from liquid nitrogen storage. In these experiments, the same four isolates used in the previous experiments were frozen in liquid nitrogen following a 60-min pretreatment at −80 C (Table 3). All four isolates were consistently recovered from liquid nitrogen storage with mean percentage recoveries ranging from 67 to 99%. Isolates showed differences in their ability to survive liquid nitrogen storage, but no significant differences in recovery were observed between samples frozen in 10% glycerol and those frozen in 5% DMSO (Table 3). Isolate 102, which had proven difficult to maintain in storage by other methods, consistently survived freezing in liquid nitrogen but showed the lowest percent recovery of the four isolates tested (Table 3).

Results of freezing experiments with nine different *Phytophthora* species indicated that a 60-min pretreatment at −80 C before freezing in liquid nitrogen allowed survival of all species tested, with mean recoveries ranging from 79 to 100% (Table 4).

During August 1985, five agar plugs of 94 different *P. infestans* isolates from the United States, the United Kingdom, Canada, and Mexico and one isolate of *P. boehmeriae* Sawada in 10% glycerol were frozen in liquid nitrogen after a 60-min pretreatment in a −80 C freezer. Five replicate vials of each isolate were frozen. On 9 May and 22 August 1986 (after approximately 9 mo and 1 yr, respectively), samples were tested for viability by thawing in a 37 C water bath and plating on 20% V-8 juice agar. Nineteen of the 94 *P. infestans* isolates and the single *P. boehmeriae* isolate frozen during August 1985 were plated on 9 May 1986. All 19 isolates of *P. infestans* and *P. boehmeriae* were successfully recovered. *Phytophthora boehmeriae* grew from all agar plugs plated, and *P. infestans* grew from all but one of the agar plugs plated.

On 22 August 1986, 66 of the 94 isolates of *P. infestans* frozen during August 1985 were tested for viability,

Table 1. Percent recovery from liquid nitrogen of four isolates of *Phytophthora infestans* following pretreatment at −20 and −80 C

	Recovery (%)[y]				
Treatment[x]	Isolate 102	Isolate 106	Isolate 111	Isolate 127	Mean[z]
1 (−196)	0	0	3	0	1 a
2 (−20, −196)	0	3	0	0	1 a
3 (−20, −80, −196)	100	100	97	100	99 b
4 (−80, −196)	100	75	100	89	91 b

[x]Treatment 1 = direct plunge into liquid nitrogen (−196 C); treatment 2 = −20 C for 24 min, then plunge into liquid nitrogen (−20, −196); treatment 3 = −20 C for 24 min, then −80 C for 60 min, then plunge into liquid nitrogen (−20, −80, −196); treatment 4 = −80 C for 84 min, then plunge into liquid nitrogen (−80, −196).

[y](Number of mycelial plugs from which growth occurred divided by the total number of plugs frozen per experiment) times 100. Data are means of three experiments.

[z]Main effect means for each treatment averaged over all isolates. Means followed by the same letter do not differ significantly according to the Waller-Duncan Bayesian *k*-ratio *t* test, *k* = 100 (12).

Table 2. Percent recovery from liquid nitrogen of four isolates of *Phytophthora infestans* following pretreatment in a −80 C freezer for different time intervals

	Recovery (%)[y]				
Pretreatment time at −80 C (min)	Isolate 102	Isolate 106	Isolate 111	Isolate 127	Mean[z]
0	0	3	0	0	1
15	0	36	3	0	10
30	100	100	100	100	100
45	97	100	100	100	99
60	97	100	100	100	99
84	97	100	100	100	99

[y](Number of mycelial plugs from which growth occurred divided by the total number of plugs frozen per experiment) times 100. Data are means of three experiments.

[z]Main effect means of pretreatment time averaged over all isolates. The *F* value for the effect of pretreatment time was highly significant ($P = 0.001$), while the *F* value for isolate effects was nonsignificant. The coefficient of variation was 17%.

Table 3. Percent recovery from liquid nitrogen of four isolates of *Phytophthora infestans* following pretreatment in a −80 C freezer for 60 min

	Recovery (%)[y]			
Cryoprotectant	Isolate 102	Isolate 106	Isolate 111	Isolate 127
10% Glycerol	68	93	76	99
5% Dimethyl sulfoxide	67	83	72	99
Mean[z]	67 a	88 ab	74 a	99 b

[y](Number of mycelial plugs from which growth occurred divided by the total number of plugs frozen per experiment) times 100. Data are means of six experiments.

[z]Main effect means for each isolate averaged over cryoprotectants. Means followed by the same letter do not differ significantly according to the Waller-Duncan Bayesian *k*-ratio *t* test, *k* = 100 (12).

Table 4. Percent recovery from liquid nitrogen of nine species (22 isolates) of *Phytophthora* following a 60 min pretreatment in a −80 C freezer

Isolate no.	Species of *Phytophthora*	Recovery (%)[y] Cryoprotectant 10% Glycerol	5% DMSO	Mean[z]
300	*P. drechsleri*	100	86	93 abc
301	*P. drechsleri*	100	94	97 abc
302	*P. capsici*	100	100	100 a
303	*P. capsici*	94	89	92 abc
304	*P. capsici*	97	100	99 ab
305	*P. capsici*	100	100	100 a
306	*P. capsici*	100	92	96 abc
307	*P. capsici*	100	97	99 ab
308	*P. pseudotsugae*	97	92	94 abc
309	*P. megasperma*	100	100	100 a
310	*P. cryptogea*	100	86	93 abc
311	*P. cactorum*	100	83	92 abc
312	*P. megasperma* f. sp. *glycinea*	100	92	96 abc
313	*P. m.* f. sp. *glycinea*	100	80	90 abc
314	*P. m.* f. sp. *glycinea*	97	94	96 abc
315	*P. m.* f. sp. *glycinea*	92	75	83 abc
316	*P. m.* f. sp. *glycinea*	100	100	100 a
317	*P. m.* f. sp. *glycinea*	100	58	79 bc
325	*P. bohmeriae*	100	94	97 abc
326	*P. palmivora*	100	94	97 abc
327	*P. palmivora*	78	78	78 c
328	*P. palmivora*	94	97	96 abc
Mean[z]		98 a	90 b	

[y](Number of mycelial plugs from which growth occurred divided by the total number of plugs frozen per experiment) times 100. Data are means of three experiments.
[z]Main effect means for isolates and cryoprotectants. Means followed by the same letter do not differ significantly according to the Waller-Duncan Bayesian *k*-ratio *t* test, $k = 100$ (12).

including duplicates of the 19 isolates tested for viability on 9 May 1986. Sixty-five of the 66 isolates of *P. infestans* plated were successfully recovered, including all 19 of those already tested for viability on 9 May. A single U.S. isolate (isolate 109) was not recovered because of bacterial contamination. Losses of pathogenicity have not been observed for *P. infestans* isolates frozen by the uncontrolled freezing method.

In another experiment to test longevity of *Phytophthora* isolates frozen by the uncontrolled freezing method, six isolates of *P. megasperma* f. sp. *glycinea* Kuan & Erwin, frozen both in 10% glycerol and 5% DMSO following a 60-min pretreatment at −80 C, were successfully recovered when plated after 9 mo in liquid nitrogen, with recoveries ranging from 67 to 100%.

DISCUSSION

Several different *Phytophthora* species can be successfully frozen and stored in liquid nitrogen if they are first placed at −80 C or at both −20 and −80 C before freezing in liquid nitrogen. Pretreatment of samples at −80 C alone gave results equivalent to pretreatment at both −20 and −80 C before freezing in liquid nitrogen.

A −80 C pretreatment of at least 20–25 min is required if *Phytophthora* samples are to survive freezing in liquid nitrogen. This length of time is required for the sample temperature to decrease to −40 C, the temperature below which freezing injury will not occur when samples are plunged into liquid nitrogen. However, pretreating samples at −80 C for longer time intervals up to 84 min did not reduce sample recovery. In these studies, a standard −80 C pretreatment of 60 min was chosen for routine use, and it allowed successful recovery from liquid nitrogen storage of all 10 *Phytophthora* species tested.

Our studies also indicated that many *Phytophthora* species can withstand freezing rates substantially greater than the 1 C per minute previously thought necessary for survival. The freezing rate during the −80 C pretreatments averaged approximately 3 C per minute, but rates of up to 5 C per minute were noted during portions of the freezing run. However, our samples were frozen as mycelium on agar plugs, rather than as suspensions of spores and/or mycelium used by other workers (13). When frozen as mycelium in contact with agar, the fungus may be more resistant to freezing injury than when frozen as a spore suspension.

Samples of three different *Phytophthora* species frozen by the uncontrolled freezing method showed high levels of recovery from liquid nitrogen after time periods ranging from 9 months to 1 year. Since high recovery was obtained after these time periods, it seems likely that *Phytophthora* species should also retain viability in liquid nitrogen storage for the longer time periods observed by other workers (2,13).

It is hoped that the results of these studies will allow liquid nitrogen storage technology to be used more widely by *Phytophthora* workers. Liquid nitrogen is relatively inexpensive, and large-capacity liquid nitrogen storage tanks are now available with static holding times for liquid nitrogen of up to 9 months. In addition, −80 C freezers of the type used in these studies are now common in many university departments and research organizations. Thus, most phytopathologists and mycologists should have ready access to the equipment necessary for application of this technique to long-term storage of *Phytophthora* species.

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