Mycologia, 92(4), 2000, pp. 810-817.
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Lyophilization of hypha-forming tropical wood-inhabiting Basidiomycotina

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Abstract: Mycelial isolates of tropical wood-inhabiting basidiomycetes are not normally considered candidates for preservation by lyophilization because these fungi do not form resistant propagules. This study investigated alternative methods of lyophilization of such mycelial isolates. Media components, freezing rates, dehydration and rehydration, and lyoprotectants were assessed for effects on viability. Results showed that these fungi can survive lyophilization. No noticeable changes in viability, growth rates, or morphological and biochemical characteristics occurred after lyophilization.

Key Words: basidiome, basidiomycete, bulbil, chlamydospore, conidium, cryopreservation, trehalose, water storage

INTRODUCTION

Mycelial isolates of tropical wood-inhabiting basidiomycotina usually grow well at optimal growth conditions on artificial media. However, these fungi present special problems with regard to long-term preservation, because in culture they do not form resistant propagules (basidiospores, conidia, bulbils or chlamydospores) but exist only as mycelia. Unlike wood-inhabiting basidiomycetous fungi from temperate climates, tropical isolates also present problems when stored cold (4 or 16 C) in sterile distilled water (Croan et al 1999). Maintaining the isolates by successive transfer on an artificial medium is labor intensive and increases genetic drift. Most fungi survive cryopreservation using liquid nitrogen (Daily and Higgins 1973, Hwang 1960, 1966, Hwang et al 1976, Kurtzman 1980, 1986, Maekawa et al 1990, Onions 1983, Smith 1988, Tan 1997). However, liquid nitrogen is expensive and requires constant resupply; the process entails a considerable expenditure of labor.

Lyophilization represents a potential alternative technique for long-term preservation. Most bacteria and spore- or conidium-forming fungi survive lyophilization. Lyophilized cultures in ampoules can be easily stored in a small space; require no maintenance, and can be shipped without special requirements. But fungi existing solely as vegetative hyphae usually do not survive lyophilization (Antheunisse 1973, Ellis and Roberson 1968, Haynes et al 1955, Rybnikar 1985, 1995, Schipper and Bekker-Holtman 1976, Smith 1984, Tan et al 1994). Chlamydospore- and bulbil-forming basidiomycetes can survive lyophilization (Croan et al 1999). However, most tropical wood-inhabiting basidiomycetes lack basidiospores, chlamydospores, conidia or bulbils. Thus, alternative lyophilization methods were sought which might be applicable for tropical wood-inhabiting basidiomycetes.

α–α–Trehalose, a naturally occurring, nonreducing disaccharide of two glucose units, is commonly found in bacteria (Kizawa et al 1995, Koh et al 1998, Yoshida et al 1995), yeasts (Zahringer et al 1998, Fernandez et al 1998), algae, insects, plants (Ring and Danks 1998), and animals or human organs (Schick et al 1991). Trehalose is known to help certain animals and plants to survive desiccation, high osmolarity, and damage by both freezing and heat. It is used to preserve biological materials (Kizawa et al 1995, Schick et al 1991) and as a stabilizer for unstable proteins, including dehydrated and frozen enzymes, diagnostic reagents, pharmaceuticals, and cosmetics (Yoshida et al 1995). When bacterial cells are freeze-dried with 100 mM trehalose as the lyoprotectant, the viability of *Bacillus thuringiensis* improves fourfold and that of *Escherichia coli* eightfold. The improved viability may be the result of the ability of trehalose to lower the temperature of the dry membrane phase transition and to maintain general protein structures in a dry state (Leslie et al 1995). These findings suggest that trehalose added to the growth medium and to the lyoprotectant might allow sufficient absorption by hyphae to protect cellular and organelle membranes.

The objective of this study was to develop a reliable, inexpensive, and simple method to preserve

Accepted for publication January 10, 2000.

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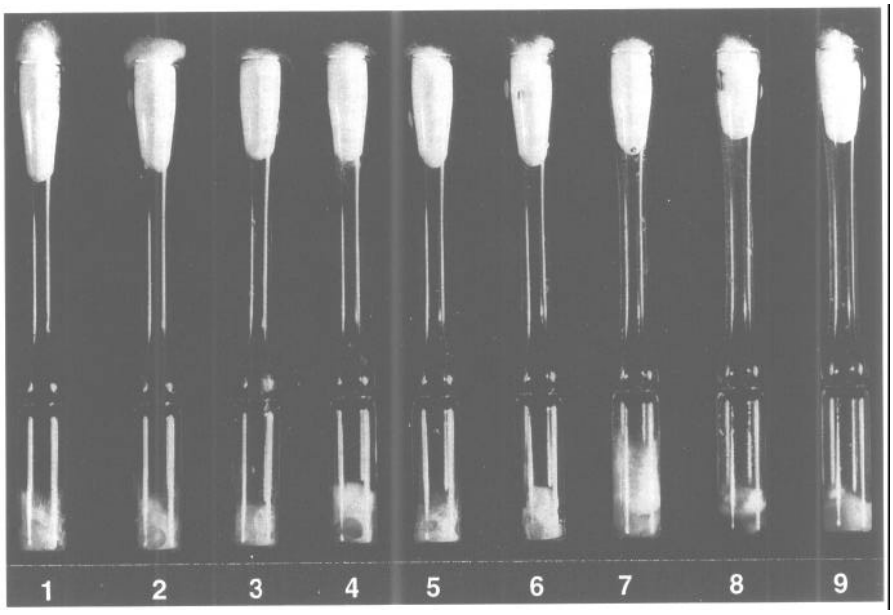

FIG. 1. Growth of mycelium/agar plugs (1-5) and mycelial fragments (6-9) in ampoules before lyophilization.

mycelial isolates of tropical wood-inhabiting basidiomycotina with no change in their original characteristics. The study investigated whether mycelia grown with trehalose as a carbon source and lyophilized using trehalose or inositol as a lyoprotectant could survive lyophilization.

MATERIALS AND METHODS

Fungi tested.—Tropical basidiomes were collected from woody substrates in moist to wet forests in Puerto Rico at the El Verde Field Station and at the Sabana Research Station in the Caribbean National Forest, Luquillo Mountains, and Guajataca. The specimens were collected during a 2-wk period in Jun 1996 and later identified by K. K. Nakasone. Thirty-three mycelial isolates, isolated from the context tissue or spore prints of these basidiomes, were selected for long-term preservation (see **TABLE I).**

Media.—The mycelium isolates were inoculated to malt extract agar (MEA) [1.5% (w/v) malt extract; Bacto, Difco, Detroit, Michigan], 2% (w/v) agar (Bacto, Difco), and growth medium (MPGT) [30 g malt extract, 2.5 g glucose, 5 g peptone, and 15 g (for broth) to 20 g (for agar medium) trehalose, per 1000 mL]. Other agar media were prepared by adding 0.5% (w/v) gallic acid (GAA) (Mallinckrodt Baker, Inc., St. Louis, Missouri) or tannic acid (TAA) (Mallinckrodt Baker, Inc.) to MEA, using the procedure of Davidson et al (1938). Tyrosine (0.2%, w/v; Tyr, Sigma) was added to 1.5% Bacto-agdr (Boidin 1958) with 0.5% malt extract (Nakasone 1990). All agar plates of 90-mm diam and MPGT agar plates of 30-mm diam were inoculated at the center with a plug of 6-mm diam taken from the margin of a young, actively growing colony. The plug was inoculated at the center of each plate and incubated at 24 C in the dark until measurable growth occurred.

Lyophilization.—Mycelium was lyophilized by two methods, (i) agar plugs with mycelium (mycelium/agar plug) and (ii) fragments from mycelial mats. For the mycelium/agar plug method, 3 to 5 agar plugs with mycelium of a stationary growth colony on MPGT agar were transferred into cotton-plugged 2-mL sterile constricted glass ampoules. The mycelium from the plugs in the ampoules was allowed to grow around the entire surface of the agar plugs for 1 to 3 wk at 24 C (FIG. 1, ampoules 1-5). Approximately 0.5 to 0.6 mL of a mixture of 10% (w/v) skimmed milk (Difco) with 10% (w/v) D(+) trehalose (Sigma, St. Louis, Missouri) or 10% (w/v) *myo*-inositol (Sigma) as the lyoprotectant was added to the ampoules.

Mycelial fragments were prepared as follows: a mycelium/agar plug from the margin of a young, actively growing colony on MEA was inoculated into 10 mL MPGT broth in a 125-mL Erlenmeyer flask and incubated until the mycelial mat covered the surface of the medium. The mycelial mat was then washed with sterile 0.005% Tween 20 in distilled water, torn into 4 to 6 pieces, and centrifuged. Approximately 0.2 to 0.3 g wet weight of the mycelial pellet was aseptically transferred into cotton-plugged 2-mL sterile constricted ampoules. The mycelial fragments in the ampoules were added to approx 0.3 to 0.4 mL of a mixture of skimmed milk with trehalose or inositol and allowed to grow around the surface for 1 to 3 wk at 24 C (FIG. 1, ampoules 6-9). An additional 0.2 mL of the same lyoprotectant was added to the ampoules with filamentous growth to make a final volume of 0.5 to 0.6 mL.

The ampoules were then transferred to the refrigerator (4 C) for 4 h to permit cold hardening (Smith 1984, Swoager 1971). Using liquid nitrogen as the coolant, the ampoules were slowly frozen (Hwang and Howells 1968, Kurtzman 1980, Smith 1984, Smith and Onions 1983, 1994, Tan et al 1995) in a microcomputer-programmed freezer (Cryomed Model 1010 programmable cooler, Stremikon, Mt.

TABLE I. Survival of mycelial isolates of tropical wood-inhabiting basidiomycotina after lyophilization

Fungus[a]	AN no.	SC no.	Mycelium/agar plugs		Mycelial fragments	
			Trehalose	Inositol	Trehalose	Inositol
Amethecium sp.*	14 653	15	5/5[b], FG	3/3, FG	5, FG	3, FG
Bourdotia sp.**	14 718	19	NS	NS	3, VSG	NS
Bourdotia sp.	14 726	21	3/5, SG	NG	3, SG	NS
*Ceriporiopsis mucida***	14 648	14	NG	NG	3, VSG, Fib	NS
Cerocorticium molle	14 802	24	5/5, FG	2/5, FG	3, SG	1-2, SG
Dendrothele sp.	14 799	23	5/5, FG	5/5, FG	4, SG	3, SG
Epithele sp.	14 686	17	3/5, SG	NS	3-4, SG	3, SG
Formitella supina	14 618	26	2/5, SG	NS	2-3, SG	NS
*Grammothele lineata**	14 659	8	5/5, MG	3/3, MG	5, MG	2-3, Mg
Hyphoderma ayresii	14 644	10	5/5, FG	5/5, FG	2-3, VSG	2-3, VSG
*Hyphoderma ayresii***	14 806	7	NS	NS	3-4, VSG	3, VSG
Hyphoderma ayresii	14 682	16	3/5, MG	NG, 1/5, VSG	3, VSG	NS
Hyphodontia lanata	14 792	31	NS	NS	3, VSG	NS
Hyphodontia sp.	14 658	34	5/5, Fils, FG	5/5, FG	1-2, SG	1, SG
Peniophora s.s.**	14 703	9	NS	NS	5, FG	2, FG
Peniophora s.s.*	14 734	22	5/5, Fils, FG	3/3, FG	5, Fils, FG	5, Fils, FG
Phanerochaete sp.	14 720	20	5/5, SG	5/5, SG	3-4, SG	NS
*Phellinus gilvus***	14 607	28	NS	NS	5, FG	NS/1, VSG
Phlebia "queletii"	14 728	32	5/5, Fils, FG	4/5, TG, FG	3-4, MG	2-3, MC
Poria sp.*	14 616	4	5/5, MG	3/3, MG	5, MG	3, MG
*Porogramme albocinctus***	14 612	3	NS	NS	5, Fils, FG	3, FG
Radulomyces sp.	14 791	18	5/5, FG	NS	3, FG	0-2 MG
Resinicium bicolor	14 642	12	5/5, Fils, FG	5/5, Fils, FG	5, FG	5, FG
Resinicium furfurellum	14 760	1	NS	NS	1-2, VSG	NS
Rigidosporus sp.**	14 608	1	NS	NS	1-2, VSG	NS
Schizopora radula	14 641	11	5/5, VSG	NS	3, VSG	NS
Schizopora flavipora	14 605	27	3/5, VSG	2/5, VSG	1, VSG	NS
Scytinostroma sp.	14 646	13	5/5, SG	2/5, SG	3, VSG	1, VSG
Scytinostroma sp.**	14 748	25	NS	NS	1-2, VSG	NS
Subulicystidium longisporum	14 673	33	NTa	NTa	NTa	NTa
Trechispora rigidia	14 758	30	5/5, VSG	NS	NTa	NTa
Tyromyces galactinus	14 609	2	5/5, MG	5/5, MG	NTa	NTa
Undetermined species*	14 620	5	5/5, Fils, FG	5/5, Fils, FG	5, Fils, FG	5, Fils, FG
Undetermined species	14 633	6	5/5, MG	3/5, MG	2-3, SG	NS

[a] Single asterisk (*) denotes trehalose-grown mycelium/agar plugs of isolates that survived after lyophilization; ** Mycelial fragments of isolates grown in MPGT broth that survived after lyophilization. No asterisk designates survival as both agar plugs with mycelium and mycelial fragments.

[b] Fractions indicate number of mycelium/agar plugs; numerator is number of plugs that survived after lyophilization; denominator is total number of plugs. Whole numbers indicate quality of growth: 1-2 = very poor, 3 = moderate, 4 = good, 5 = excellent. NTa denotes not tested (no growth obtained in liquid growth medium); Fils, filamentous growth; TG, transparent growth. Growth rates are denoted by NS, no survival; VSG, very slow growth, 3-5 wk; SG, slow growth, within 2-3 wk; MG, moderate growth, 1-2 wk; FG, fast growth, within 3 d to 1 wk.

Clemens, Michigan) to about -45 C at -1 C/min, then frozen to -90 C at -10 C/min. The frozen samples were lyophilized in an ethanol-dry ice bath. The ampoules were then sealed under vacuum and stored in the refrigerator.

The sealed ampoules were opened after a few days to a few weeks and then again after 1 yr of storage. Sterile distilled water was added to the lyophilized cultures and left for approx 10 min to permit water absorption. These reconstituted lyophilized specimens, whether mycelium/agar plugs or mycelial fragments, were then inoculated on MEA plates to determine their viability. The growth rate of viable cultures was determined by measuring the diameter of the fungal colonies on MEA every 2 to 3 d during 2 wk incubation at 24 C. The results were compared to those obtained within a period of a few days to a few weeks and compared to those obtained again after 1 yr of storage.

Gallic acid, tannic acid, and tyrosine agar medium reactions.—Specific strain characteristics were compared before and after preservation to ascertain the stability of the iso-

lates. The viability of the isolates before and after preservation was measured by the ability of vegetative hyphae to regrow on MEA plates. Growth characteristics and agar medium reactions on gallic acid, tannic acid, and tyrosine were also compared before and after preservation.

A mycelium/agar plug (6-mm diam) of a young, actively growing margin of the colony on MEA plates was inoculated at the center of the gallic acid, tannic acid, and tyrosine agar plates. The growth rate and color reactions were recorded at 1- and 2-wk intervals after incubation in the dark at 24 C. The rate of radial growth was also compared to that of growth on 1.5% MEA plates. The reaction of the diffusion zone to the fungal colony of the tropical mycelial isolates was rated according to the method described by Davidson et al (1938) with minor revisions (see TABLE II). Two additional reactions were also recorded: (i) diffusion zone light yellow, formed under the inoculum plug, and (ii) diffusion zone black, formed under the inoculum plug on the tyrosine agar medium when no growth takes place.

RESULTS

Mycelium of the following basidiomycetes grown on MPGT agar (growth medium) and harvested as plugs during the stationary phase survived lyophilization when the plugs were frozen by slow cooling in skimmed milk alone or a mixture of skimmed milk with inositiol or trehalose: *Amethecium* sp., *Grammothele lineata*, *Peniophora* s. s. (AN 14734), *Poria* sp., and an undetermined species (AN 14620) (TABLE I). The isolates exhibited filamentous growth and/or grew faster compared to nonlyophilized isolates. Mycelium on agar plugs grown on MEA under the same conditions did not survive lyophilization. Nor did the five trehalose-grown mycelial isolates subjected to rapid freezing by direct immersion into the ethanol-dry ice bath.

Survival rates increased when the mycelium on agar plugs grown on growth medium containing trehalose were allowed to continue to grow until filamentous mycelial growth was observed all around the agar plugs in ampoules (FIG. 1, ampoules 1-5). The following six mycelial isolates survived lyophilization in skimmed milk with either trehalose or inositol as a lyoprotectant: *Dendrothele* sp., *Hyphoderma ayresii* (AN 14644), *Hyphodontia* sp., *Phanerochaete* sp., *Resinicium bicolor*, and *Tyromyces galactinus* (TABLE I). Good growth was obtained after lyophilization. The following 12 isolates survived lyophilization in the presence of trehalose but had poor viability or did not survive in the presence of inositol: *Bourdotia* sp. (AN 14726), *Cerocorticium molle*, *Epithele* sp., *Fomitella supina*, *Hyphoderma ayresii* (AN 14682), *Phlebia "queletii," Radulomyces* sp., *Schizopora radula*, *Sch. flavipora*, *Scytinostroma* sp. (AN 14646), *Trechispora rigida*, and an undetermined species (AN 14633).

Before lyophilization, mycelial fragments grown on MPGT broth of the remaining isolates were allowed to continue to grow in ampoules in skimmed milk with either inositol or trehalose (FIG. 1, ampoules 6-11). These mycelial fragments survived lyophilization. However, the mycelium/agar plugs of the same isolates grown on MPGT agar did not survive with lyoprotectant containing trehalose or inositol. The mycelial fragments grown on MPGT broth survived with the trehalose-containing lyoprotectant but they survived poorly or did not survive at all with inositol-containing lyoprotectant. The fungi tested were *Bourdotia* sp. (AN 14718), *Ceriporiopsis mucida*, *Hyphoderma ayresii* (AN 14806), *Hyphodontia lanata*, *Peniophora* s. s. (AN 14703), *Phellinus gilvus*, *Porogramme albocintus*, *Rigidoporus* sp., and *Scytinostroma* sp. (AN 14748). By contrast, the mycelial fragments grown in MPGT broth survived but myceliun/agar plug grown on MPGT agar of the same isolates did not survive lyophilization in skimmed milk with trehalose (TABLE I). *Scytinostroma* sp. (AN 14748.00) survived very poorly. It grew very slowly on the growth medium and even more slowly on MEA plates. *Subulicystidium longisporium* did not grow at all on the growth medium, but a trace of growth occurred after 4 to 5 wk incubation on MEA. This isolate was not tested for lyophilization.

Although several strains survived lyophilization as mycelial fragments but not as plugs, all strains surviving as plugs also survived lyophilization when mycelial fragments were used. The mycelium-agar plugs thrived only in the presence of trehalose, but survived poorly or did not survive at all in the presence of inositol-containing lyoprotectant. *Trechispora rigida* and *Tyromyces galactinus* did not grow in the liquid growth medium. They grew poorly on the growth agar medium and thus their mycelium/agar plugs were lyophilized. The isolates that survived lyophilization after a few days to a few weeks also proved viable and stable after 1 yr of storage.

Growth of about half the mycelial isolates apparently was stimulated after lyophilization compared to the growth of nonlyophilized cultures. These isolates exhibited filamentous growth **(TABLE** I, Fils) and/or grew faster **(TABLE** I, FG) on MEA plates immediately after lyophilization compared to nonlyophilized isolates. The other surviving isolates grew slowly after lyophilization, but normal growth characteristics were usually obtained after the second transfer to MEA plates. Lyophilization affected the rate of growth, but none of the isolates demonstrated changes in morphological and anatomical characteristics.

The original isolates cultured from the context tissue or spore prints of tropical basidiocarps were compared to the isolates that survived the preservation

TABLE II. Gallic acid, tannic acid, and tyrosine media reactions of isolates[a]

Fungus	Incub. (wk)	MEA GR (mm)	GAA GR (mm)	GAA rxn	TAA GR (mm)	TAA rxn	Tyr GR (mm)	Tyr rxn
Amethecium sp.	1	75	0	−	0	−	63	−
	2	90+	0	−	0	−	90+	−
Bourdotia sp.	1	72	0	−	0	−	66	−
	2	90+	0	−	0	−	90+	−
Boudotia sp.	1	64	0	−	0	−	46	−
	2	90+	0	+ +	0	+ +	90+	+
Ceriporiopsis mucida	1	12	0	+ +	10	+	10	−
	2	29	0	+ + +	23	+ + +	18	+
Cerocorticium molle	1	32	0	+	0	+	18	−
	2	72	0	+ +	35	+ + +	35	±
Dendrothele sp.	1	42	0	+	25	+ +	21	−
	2	72	0	+ +	43	+ +	45	±
Epithele sp.	1	88	0	+	33	+ +	88	−
	2	90+	0	+ + +	62	+ +	90+	+
Fomitella supina	1	34	0	+	0	+	25	−
	2	60	0	+	12	+ + +	45	2
Grammothele lineata	1	50	0	+	23	+ + +	71	−
	2	90+	0	+ + + +	62	+ + + +	90+	+ +
Hyphoderma ayresii	1	32	0	−	0	−	20	−
	2	64	0	−	0	+	90+	+ +
Hyphoderma ayresii	1	58	0	−	0	−	20	−
	2	90+	0	±	0	−	90+	+
Hyphoderma ayresii	1	78	0	−	0	−	70	+
	2	90+	0	+	0	+	90+	+
Hyphodontia lanata	1	25	0	+	0	−	0	−
	2	44	0	+ +	0	±	0	+ *
Hyphondontia sp.	1	34	0	−	0	−	0	−
	2	67	0	−	0	−	0	−
Peniophora s. s.	1	70	34	+ + +	43	+ +	75	+
	2	90+	63	+ + + +	76	+ + +	90+	+ + +
Peniophora s. s.	1	86	14	+ + +	56	+ +	45	−
	2	90+	20	+ + + +	70	+ +	86	−
Phanerochaete sp.	1	46	21	−	0	−	42	−
	2	78	31	−	0	−	90+	−
Phellinus gilvus	1	43	12	+	25	+	0	+
	2	70	14	+ + + +	45	+ +	0	+ +
Phlebia "queletii"	1	69	0	+	0	−	70	+
	2	90+	0	+ +	0	+	90+	+ +
Phlebia "queletii"	1	69	0	+	0	−	70	+
	2	90+	0	+ +	0	+	90+	+ +
Poria sp.	1	68	0	+	6	+	61	−
	2	90+	0	+ +	10	+ +	90+	+
Porogramme albocinctus	1	70	0	+	26	+ + +	71	−
	2	90+	0	+ + + +	41	+ + + +	90+	+ +
Radulomyces sp.	1	34	0	+	0	+	9	−
	2	62	0	+ +	0	+	12	±
Resinicium bicolor	1	77	0	+	0	−	50	−
	2	90+	0	+ +	0	±	90+	−
Rigidoporus sp.	1	20	0	±	0	−	22	−
	2	70	0	+	0	−	55	+
Schizopora radula	1	45	0	+	28	+	29	±
	2	76	0	+ + +	49	+ +	47	+
Schiropora flavipora	1	54	0	+	0	−	0	−
	2	90+	0	+ +	0	−	0	−

TABLE II. Continued

Fungus	Incub. (wk)	MEA GR (mm)	GAA GR (mm)	GAA rxn	TAA GR (mm)	TAA rxn	Tyr GR (mm)	Tyr rxn
Scytinostroma sp.	1	45	0	+	27	+ +	38	–
	2	74	0	+ + +	51	+ + +	60	+
Scytinostroma sp.	1	60	0	+	34	+	40	–
	2	90+	9	+ + +	60	+ +	60	+
Subulicystidium longisporium	1	0	0	–	0	–	0	–
	2	8	0	–	0	–	0	–
Trechispora rigida	1	tr	0	+	0	±	8	–
	2	12	0	+	0	±	14	+
Tyromyres galactinus	1	18	0	+	0	+	10	–
	2	31	0	+ +	0	+ + +	90+	+ +
Unknown sp.	1	64	0	+	0	+	61	–
	2	90+	0	+ + +	0	+ +	90+	+ +
Unknown sp.	1	25	0	+	13	+ + +	15	–
	2	55	0	+ +	34	+ + +	36	+ +

[a]GAA, TAA, and TyrA reactions: Radial growth rate and color reactions were recorded after 1 and 2 wk incubation. First row of data indicates measurement after 1 wk incubation; second row, measurement after 2 wk. 90+ designates maximum growth; GR, growth radius; rxn, reaction; 90+, maximum growth, — no change, ± diffusion zone light yellow, formed under inoculum plug, + diffusion zone light to dark brown, formed under inoculum plug at center of colony or under inoculum plug when no growth takes place, + + diffusion zone light to dark brown, formed under most of colony or under inoculum plug in the event that no growth takes place, +++ diffusion zone light to dark brown, extending short distance beyond margin of colony or inoculum plug when no growth takes place, + + + + diffusion zone dark brown, extending considerably beyond margin of colony or inoculum plug when no growth takes place, * diffusion zone black instead of light or dark brown.

process. The agar medium reactions of the original isolates to gallic acid, tannic acid, and tyrosine were basically the same as that of mycelial isolates that survived after lyophilization (TABLE II).

DISCUSSION

Tropical mycelial isolates generally grow well under optimal growth conditions of temperature, aeration, and humidity with artificial media. They generally grow poorly or not at all at lower temperatures (i.e., temperatures below 24 C, depending on fungus) and they have a much higher optimum temperature (28-36 C) than do isolates from temperate areas. These tropical isolates also grow poorly or not all after storage in distilled water in 4 C. They grow somewhat better when taken from 15 C distilled water storage, although even at that temperature not all survive. The cold temperature is detrimental to tropical mycelial isolates, in contrast to isolates from wood-inhabiting basidiomycetous fungi from temperate climates (Croan et al 1999). Tropical mycelial isolates grow as vegetative hyphae without thick-walled bulbils, chlamydospores, or conidia, which are conducive to survival under unfavorable conditions for extended periods.

Mycelium/agar plugs of five mycelial isolates grown on a growth medium containing trehalose survived lyophilization when skimmed milk was used alone or in combination with inositiol or trehalose. Intracellular trehalose accumulates in the stationary growth phase and acts as carbohydrate storage (Lillie and Pringle 1980). A recent study demonstrated that trehalose is an important protectant for yeast (Singer and Lindquist 1998). It enables yeast to survive dehydration, freezing, or ethanol stress; it also prevents native proteins from denaturing, suppresses the aggregation of denatured proteins (Singer and Lindquist 1998), and acts as an osmotic stress protectant (Hounsa et al 1998). Intracellular trehalose in mycelium/agar plugs sufficed to protect five isolates with or without trehalose or inositol. The isolates also exhibited filamentous growth and/or grew faster compared to nonlyophilized isolates. Mycelium/agar plugs grown on malt-extract agar did not survive, possibly because there was no intracellular trehalose accumulation to protect the cells in the mycelial isolates. When the trehalose-grown mycelium/agar plugs were directly frozen by immersion in a dry ice-ethanol bath, they did not survive lyophilization, possibly because intracellular solutes leaked during the transition from active life to a rapidly frozen state. This suggests that a slower cooling rate followed by

the lyophilization process may be necessary to assure the viability of these tropical basidiomycetes.

When trehalose-grown mycelium/agar plugs or mycelial fragments in ampoules were allowed to continue to grow, survival rates increased considerably, possibly because additional trehalose was absorbed intracellularly, thus protecting the cells in the mycelial isolates.

The isolates that survived lyophilization only in the presence of trehalose but had poor viability or did not survive in the presence of inositol might not have had enough intracellular trehalose accumulation to protect the cells. Trehalose-grown isolates apparently absorbed additional trehalose intracellularly during the process of lyophilization in the presence of trehalose-containing lyoprotectant. The presence of trehalose protected these isolates because the isolates survived poorly or did not survive in the presence of inositol. Viability in the presence of trehalose as the lyoprotectant by *Bacillus thuringiensis* and *Escherichia coli* was reported to improve considerably (Leslie et al 1995). Trehalose might have accumulated rapidly and thus protected the bacterial cells during freezing and rehydration.

All trehalose-grown mycelium/agar plugs or mycelial fragments of isolates that exhibited filamentous growth on/in growth medium survived lyophilization in presence of trehalose as the lyoprotectant. These trehalose-grown isolates appeared to be protected against cell damage in the presence of trehalose but not all by inositol. The fact that some isolates exhibited filamentous growth and/or grew faster on MEA plates immediately after lyophilization may have been due to the survival of the more vital parts of mycelia through lyophilization. The other surviving isolates grew slowly after lyophilization, but normal growth rates were usually obtained after the second transfer to MEA plates. Irrespective of whether growth rates were affected by lyophilization, none of the isolates demonstrated changes in morphological and anatomical characteristics or biochemical properties (agar medium reactions of tannic acid, gallic acid, and tyrosine), suggesting that no major genetic changes occurred.

In summary, this technique demonstrates an effective method for preserving tropical wood-inhabiting basidiomycetes and possibly many other basidiomycetes as well.

ACKNOWLEDGMENT

I wish to thank Dr. K.K. Nakasone for the identification of tropical wood-inhabiting basidiomycotina.

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