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Cryopreservation of the ectomycorrhizal mushroom *Cantharellus cibarius*

Eric DANELL[1] and Gunnar FLYGH[2]

Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, Box 7026, SE-750 07 Uppsala, Sweden.

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Cryopreservation of ectomycorrhizal fungi is often difficult, but needed for patent deposits and for enabling experiments on model strains with constant characteristics. In this study we report a protocol for successful cryopreservation of the ectomycorrhizal mushroom *Cantharellus cibarius*, together with a brief summary of some unsuccessful attempts. A slow freezing rate (0.3 °C min^{-1}) of agar cubes carrying the mycelium, and a gentle addition of the cryoprotectants sorbitol and DMSO were important features. This protocol was successfully repeated by ATCC who used it to deposit a patented strain of *C. cibarius*, and therefore the protocol should also be tested for other ectomycorrhizal basidiomycetes.

INTRODUCTION

Since the pioneer experiments by Hwang (1960), cryopreservation is considered the best preservation technique for filamentous fungi (Stalpers, de Hoog & Vlug 1987, Smith 1998). Protocols have been successfully applied on spores of arbuscular mycorrhizal fungi (Douds & Schenck 1990), but the maintenance of mycorrhizal basidiomycete mycelia is still rather difficult (Homolka *et al.* 2001). Since mycorrhizal mycelium can be used as inoculum for seedlings of economically important forest trees, and as inoculum for production of edible mushrooms, there is a need for maintaining selected strains for laboratory use during long periods of time. In our efforts to get a US patent of a selected *Cantharellus cibarius* strain, we were asked to develop a protocol for cryopreservation to be used for patent deposits by the American Type Culture Collection (ATCC). Our aim was therefore to develop such a protocol.

MATERIALS AND METHODS

We used the *Cantharellus cibarius* tissue culture strain SNGT2-A (ATCC 74488) originating from a fruitbody collected by the authors. The species identification of the mycelium was made by comparing PCR/RFLP of ITS rDNA from the isolated mycelium with the corresponding fruitbody (Danell 1994a, b, 1999).

We tried several cryopreservation protocols (Table 1), but only the successful Sorbitol/DMSO method will be described in detail below. It is based on a protocol developed for embryogenic suspension cultures of spruce by Find *et al.* (1993). For the successful protocol we used mycelial agar plugs, but we also tried other sources of mycelium without success, i.e. a hyphal suspension and agar mycelial plugs grown in liquid medium (Danell 1994a, b). We used three replicates for each trial.

Freezing

The mycelium was cultivated on MFM agar medium (25 ml in each 9 cm diameter Petri dish) for 25 d (Danell 1994a). In total 2 g of agar mycelia (fresh weight includes agar) were divided into small fractions of approx. 1 mm^3, and added to one flask of 20 ml liquid MFM. We used three replicates, i.e. 3 sets of agar cubes rather than testing growth from individual agar cubes. The reason was that the amount of mycelium in the agar cubes before freezing varied. To this flask, 105 µl 4 M sorbitol was added every 3 min for 30 min (in total 10 times). The flask was shaken each time and finally incubated on a rotary shaker for 24 h in darkness at room temp. The following day 117 µl of 4 M sorbitol were added every 3 min for 30 min and incubated as before. After 24 h the flask was placed in an ice bath (0 °C) and 117 µl dimethyl sulfoxide (DMSO) were added every 3 min for 30 min

Present addresses: [1]Museum of Evolution, Botany section, Uppsala University, Norbyv.16, SE-752 36 Uppsala, Sweden; and [2]Department of Veterinary Medical Chemistry, Swedish University of Agricultural Sciences, Box 575, SE-751 23 Uppsala, Sweden.

Table 1. Tested cryopreservation protocols. Cooling rate A means that five rates were tested: 0.5 °C min^{-1}, 1 ° min^{-1}, 10 ° min^{-1}, 30 ° min^{-1}, and 200 ° min^{-1}.

Freezing medium	Cooling rate	Mycelial source	Incubation time
Modified Fries Medium (MFM; Danell 1994a)	A	30 agar plugs	24 h
Distilled water	A	30 agar plugs	24 h
10% (v/v) glycerol	A	30 agar plugs	24 h
10% (w/v) trehalose	A	30 agar plugs	24 h
Sorbitol/DMSO	0.3 °C min^{-1}	2 g agar plugs, hyphal suspension, 2 g agar pregrown in liquid MFM	6 d and 72 h

(i.e. 10 times). The flask was shaken after each addition. After the DMSO treatment, the flask was left still for 30 min. Cryotubes (NUNC, Denmark) kept on ice were each filled with 1–1.5 ml of the agar slurry, using a 5 ml plastic pipette tip with a cut edge. The caps were tightly closed and the tubes were transferred to the cryopreservator (Kryo 10 series II, Planer Products, Sunbury on Thames). The freezing program started at −1 ° for 10 min. Then the temperature was lowered by 0.3 ° min^{-1} until −16 °. This temperature was kept for 15 min and then lowered by 0.3 ° min^{-1} until −35.5 °. Finally the temperature was dropped by 25 ° min^{-1} until −50 °. At −50 °, the tubes were transferred to a liquid nitrogen container (−196 °).

Thawing

After 6 d in liquid nitrogen, the cryotubes were placed in a plastic jar with 45 ° sterile water for 3–4 min, and then transferred to 4 ° sterile water. When thawed the tubes were dipped in 70% ethanol for sterilisation. The tubes were dried in a laminar flow and all agar cubes from each tube were poured onto three sterile filter papers (30–40 mm diam) on MFM agar medium in a Petri dish. After 1 h, the filter papers were transferred to new MFM Petri dishes to minimize the amount of DMSO transferred with the filter papers. After 24 h, the filter papers were transferred to 5 cm Petri dishes with liquid MFM (no agar). The filter papers were tilted to allow the agar cubes to slid down into the medium. The agar mycelium was incubated at 20 ° in darkness.

This freezing protocol was repeated by ATCC with the following changes: agar cubes were transferred with 0.5 ml liquid suspension to cryotubes. After 72 h in liquid nitrogen the material was thawed in a 55 ° water bath for 90 s, and viability studied. Four agar cubes in tubes kept on dry ice were sent from ATCC back to our laboratory for thawing, viability test and species identification using PCR/RFLP of rDNA ITS.

RESULTS AND DISCUSSION

Our successful protocol resulted in the recovery of mycelia from all three sets of agar. In the repeated experiment by ATCC, the *Cantharellus cibarius* recovered from all 4 agar plugs. The patent for the strain SNGT2-A was granted by the ATCC (Danell 2001). The recovered mycelia were incorporated into the strain collection used for mycorrhiza synthesis (Danell 1994a, b), since no morphological or physiological changes were observed when compared with unfrozen mycelia of the same strain. All other protocols (Table 1) failed with one exception: after a 10% (w/v) trehalose treatment, a cooling rate of 1 ° min^{-1} and a storage of 24 h, a mycelium from one single agar plug of 30 (3.3%) was revived. It is not known why a cooling rate of 0.5 ° min^{-1} and the common DMSO substitute glycerol did not work. The very slow freezing rate and the slow addition of cryoprotectants might be a key. The combined positive effects of sorbitiol and DMSO are described by Find *et al.* (1993). Sorbitol has not been found in NMR studies in vegetative mycelium of the *C. cibarius* strain SNGT2-A, but trehalose, mannitol, erythritol and arabitol are synthesised by the fungus (Rangel-Castro, Danell & Pfeffer 2002). Using cryogenic light microscopy for our exact protocol and organism may reveal when extreme care is necessary (Smith & Thomas 1998). Our gentle protocol should also be tried for other ectomycorrhizal fungi, but cryogenic microscopy might be needed to tune the freezing program.

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