Short Communication

# CONTROLLED RATE COOLING OF FUNGI USING A STIRLING CYCLE FREEZER

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## Abstract

The suitability of an 'N2-free' Stirling Cycle controlled rate freezer for the cryopreservation of fungi was assessed. In total, 77 fungi representing a broad taxonomic coverage were cooled using the $N_2$ free cooler following a cooling rate of -1$^{o}$C min$^{-1}$. In total 75 fungi survived cryopreservation, only a recalcitrant Basidiomycete and filamentous Chromist failed to survive. Of these, 15 strains were also cryopreserved using a traditional '$N_2$ gas chamber' controlled rate cooler and a comparison of culture morphology and genomic stability against non-cryopreserved starter cultures was undertaken. No changes were detected in genomic profile after preservation, suggesting that genomic function is not adversely compromised as a result of using '$N_2$ free' cooling. The results demonstrate the potential of '$N_2$-free' cooling for the routine cryopreservation of fungi in Biological Resource Centres. The technique has many advantages over traditional methodologies both from a scientific and logistical perspective.

**Keywords**: Cryopreservation. Genomic stability, PCR fingerprinting, Biological Resource Centre

## INTRODUCTION

Since 1949, when Polge *et al*. reported on successful freezing of avian spermatozoa (1), the technique of cryopreservation has been widely applied for the long-term conservation of cells. The utility of the application to fungi was noted in 1960 (2) and the resultant methodology has been widely accepted as the 'regime of choice' for fungal cryopreservation (3).

The aim of cryopreservation is to maintain the fungus in a viable state without change to its genomic integrity. There are reports of genomic instability following cryopreservation (e.g. 4), so cryopreservation methodology should be optimised to ensure that the prospect of damage at the molecular level is reduced. There are a number of techniques available to assess the success of cryopreservation in microorganisms at the molecular level and these include AFLP (5) and other PCR fingerprinting approaches (4, 6).

In Biological Resource Centres operating to OECD Best Practice Guidelines (3), cryopreservation is typically achieved by controlled rate cooling in the presence of a suitable cryoprotectant such as 10% glycerol, followed by storage in the vapour phase of liquid nitrogen. However, there are numerous protocols available for the cooling of fungi and these include use of traditional '$N_2$ gas chamber' controlled rate coolers and portable freezing containers such as Mr. Frosty (Nalgene, USA). There are advantages and disadvantages of using the different protocols, portable devices are not computer controlled and do not have in-built temperature recording while $N_2$ containers require the use of $N_2$ gas which is a potential risk of contamination and poses health and safety issues because of the hazardous nature of liquid nitrogen (7).

The use of a Stirling cycle freezer for cryopreservationis considered it to have significant advantages over traditional methodologies including, $N_2$ free operation, application of slow cooling rates, reduction of the risks of sample contamination and control of ice nucleation (7). The use of a Stirling cycle freezer for the cryocooling of fungi has not been reported.

The aim of this investigation was to evaluate the suitability of the Grant EF600 $N_2$ free 'Stirling cycle' controlled rate cooler for the cryopreservation of 77 fungi (representing a broad taxonomic diversity). A sub-set of 15 preservation-recalcitrant fungi was studied in more detail and results obtained with the EF600 were compared with those from a 'traditional' $N_2$ gas controlled rate cooler. A number of criteria were used to assess the success of cryopreservation regime including analysis of viability and culture morphology, plus the relative stability of genomic profile against non-preserved wild type cultures using a polymerase chain reaction (PCR) fingerprinting approach.

## MATERIALS AND METHODS

*Organism selection and growth*

A collection of 77 fungi (Table 1), representing the major fungal groups was selected. Of these, 15 recalcitrant strains (Table 2) were obtained from partner collections in the EU EMbaRC project (European Consortium of Microbial Resources Centres). The remaining 62 cultures were recent deposits obtained from the CABI genetic resources collection.

Before preservation cultures were maintained under optimal growth conditions, specific to the organism type. Cryopreservation was undertaken using a traditional '$N_2$ gas chamber' controlled rate cooler and an '$N_2$-free' cooler.

*Cryopreservation*

Conidial or spore suspensions (for zygo- and ascomycetous Fungi) or mycelial suspensions (for basdiomycetous Fungi and chromists) were collected and pre-treated with 10% (v/v) glycerol solution for 2 hours. 0.5 ml aliquots were then dispensed into 1.8 ml round bottom cryovials (Nunc Cat # 363401, UK). Samples were loaded into either a KRYO 10/16 (Planer, Sunbury UK) controlled rate cooler or an Asymptote EF600 Stirling cycle freezer (Cell Cryogenics Ltd, UK). Samples were cooled at -1˚C $min^{-1}$. For the Planer cooler the following regime was applied: -10$^{o}$C $min^{-1}$ to 5$^{o}$C; -1$^{o}$C $min^{-1}$ to -8$^{o}$C; -6.6$^{o}$C $min^{-1}$ to -22$^{o}$C; -3$^{o}$C $min^{-1}$ to -30$^{o}$C; -5.5$^{o}$C $min^{-1}$ to -50$^{o}$C; -1$^{o}$C $min^{-1}$ to -35$^{o}$C; -10$^{o}$C $min^{-1}$ to -50$^{o}$C; then hold for 3 min at -50$^{o}$C. For the EF600 a strict linear cooling rate of -1 ˚C $min^{-1}$ (from 5$^{o}$C to -30$^{o}$C) was applied. After cooling, samples were immediately transferred to LN vapour phase ultra-cold storage (<-150$^{o}$C) for at least 24 hours. When required, samples were resuscitated by rapid warming in a water bath held at 36$^{o}$C.

*Viability, Growth Rate and Culture Characteristics*

Baseline culture characteristics were established before cryopreservation. Post cryopreservation, samples were allowed to recover in culture for a period of 7 days before transfer to a suitable medium for analysis Viability post preservation was assessed visually (e.g. see Fig. 2), with atypical growth noted as "attenuated". Culture characteristics were assessed using the following criteria. Pigmentation: colour, intensity, diffusing (into media) or non-diffusing (held within mycelium), exudate (presence/absence of globules of liquid on the surface); Mycelial and hyphal form: cottony (flocculose), aerial (present or absent), hyphal aggregation (stranding, etc.), sectors (including number, typical or atypical morphology), colony edge (smooth, rigid etc.), point growth (area of more rapidly growing mycelium at the colony edge); Sporulation: Intensity; degree, colour, type of sporulation (such as conidia and chlamydospores).

*PCR Fingerprinting*

Two PCR fingerprinting methods were used: inter-simple sequence repeat anchored [ISSR]-PCR and variable number tandem repeat [VNTR]-PCR. Fingerprinting was undertaken on pre and post cryopreservation cultures. Mycelium was collected from cultures grown on distilled water malt agar plates (MADW; 20 g $l^{-1}$ toffee malt extract, 20 g $l^{-1}$ agar Oxoid, UK) at 22 ˚C for 1-4 weeks. Biomass (approx. 2.4 g) was removed with a sterile spatula and DNA was extracted using a Plant DNeasy® extraction kit (Qiagen Ltd., Crawley, UK) following manufacturer's instructions, with the exception of omission of the suggested addition of Proteinase K to the lysis buffer as this was deemed unnecessary.

*Primers and polymerase chain reaction conditions*

Methodology for ISSR PCR was adapted from the method described by Grünig (8). Amplification reactions with TGT primer (5′-VHVTGTTGTTGTTGTTGT-3″) were undertaken in volumes of 20 µl containing 0.5 µl primer, 0.8 µl of each dNTP (100 mM stock, Promega Ltd., Southampton, UK), PCR buffer 10× *Tth* (Qiagen Ltd., Crawley, UK), 0.05 U µl-1 *Tth* DNA-polymerase (Qiagen Ltd, Crawley, UK) and 1 µl of stock solution of template DNA. Polymerase chain reaction conditions were as follows: an initial denaturation for 3 min at 94˚C, followed by 35 cycles of denaturation step at 94˚C for 1 min, annealing step at 46˚C for 1 min and extension step at 72˚C for 2 min. A final extension step (72˚C for 10 min) was included. The samples were held at 4˚C until further processing occurred. PCR reactions were performed in duplicate per extract. Conditions for VNTR PCR were adapted from a method proposed by Bridge (9). Amplification reactions with MR primer (5′-GAGGGTGGCGGTTCT-3′) were undertaken in volumes of 25 µl containing 0.5 µl primer, 2.0 µl of each dNTP (100 mM stock, Promega Ltd., Southampton, UK), PCR buffer 10× *Tth* (Qiagen Ltd., Crawley, UK), 0.05 U $\mu l^{-1}$ *Tth* DNA-polymerase (Qiagen Ltd, Crawley, UK) and 1 µl of stock solution of template DNA. Polymerase chain reaction conditions were as follows: an initial denaturation for 6 min at 95˚C, was followed by 39 cycles of denaturation step at 95˚C for 1 min, annealing step at 45˚C for 1 min and extension step at 72˚C for 1 min. A final extension step (72˚C for 5 min) was included. The samples were held at 4˚C until further processing occurred. PCR reactions were performed in duplicate per extract.

*Electrophoresis conditions*

Aliquots (11 µl) of amplification products were mixed with 5 µl loading buffer (sucrose – 40 g; bromophenol blue – 0.05 g; $dH_2O$ to 100 ml) and separated in 1.5% (w/v) SeaKem LE agarose (Lonza Ltd., Basel, Switzerland) gels containing SafeView Nucleic Acid Stain (NBS Biologicals Ltd., Huntington, UK) at 5 µl per 100 ml 0.5x TBE buffer. Electrophoresis was

undertaken in a midi gel tank (Hybaid, UK) in 0.5x TBE buffer (TBE stock solution 5x: 54 g Tris base, 27.5 g Boric acid, 20 ml 0.5 M EDTA (pH 8.0)) following the protocol for electrophoresis buffers (Sambrook and Russel, 2001). Aliquots (10 µl) of 100-bp or 1kb ladder (25 ng/ µl) Invitrogen Ltd., Paisley, UK) were added as a DNA size marker standard in the first and last lanes of each gel. Gels were visualised using a U:Genius gel documentation system (Syngene, UK). Images were stored as TIF bitmaps for subsequent use.

## RESULTS

A total of 75 Fungi (97%) were cryopreserved successfully using the Stirling cycle freezer (Table 2) which included representatives from each main fungal Phylum and the filamentous Chromists.

Of the 15 recalcitrant fungi subjected to preservation by both the Stirling cycle and $N_2$ Chamber coolers, 12 were recovered after cooling with both controlled rate coolers; the basidiomycetous fungus *Paxillus involutus* MUCL 52217 was not recovered after cooling with either the '$N_2$ free' or '$N_2$ gas' control rate coolers; The highly preservation recalcitrant Chromist *Saprolegnia diclina* IMI 308259 was recovered only after cooling with the '$N_2$ free' controlled rate cooler. However, the Chromist, *Phytophthora citrophthora* IMI 396200 was recovered only after cooling with a 'traditional' $N_2$ gas controlled rate cooler. In common with other 'cryo' regimes, a delayed onset of growth was evident with most of the cultures after preservation. Altered culture morphology was evident in *Phytophthora citrophthora* (Fig 1) preserved using the traditional controlled rate cooler although anatomical features were typical. Changes were not evident after cooling using the Stirling cycle freezer. No changes were detected in genomic profiles after preservation, suggesting that genomic function is not adversely compromised as a result of using '$N_2$-free' cooling (Fig 2).

**Table 2 List of isolates and their viability post preservation**

| Isolate | Collection number | Taxonomy | KRYO N2 cooler | EF600 Stirling Cooler | PCR fingerprint (compared to pre-preservation) | Culture Characteristics |
|---|---|---|---|---|---|---|
| *Aspergillus ibericus* | MUM 3.49 | Ascomycete | V | V | TYPICAL | TYPICAL |
| *Botrytis elliptica* | CBS 108966 | Ascomycete | V | V | TYPICAL | TYPICAL |
| *Conidiobolus rhysosporus* | CBS 141.57 | Zygomycete | V | V | TYPICAL | TYPICAL |
| *Coniophora olivacea* | CECT 20145 | Basidiomycete | V | V | TYPICAL | TYPICAL |
| *Diplocarpon rosae* | IMI 381057 | Ascomycete | V | V | TYPICAL | TYPICAL |
| *Hebeloma crustiliniforme* | MUCL 52208 | Basidiomycete | V | V | TYPICAL | TYPICAL |
| *Laccaria bicolor* | MUCL 52210 | Basidiomycete | V | V | TYPICAL | TYPICAL |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Mortierella alpina* | CECT 2977 | Zygomycete | V | V | TYPICAL | TYPICAL |
| *Paxillus involutus* | MUCL 52217 | Basidiomycete | N | N | N/A | N/A |
| *Penicillium expansum* | MUM 0.07 | Ascomycete | V | V | TYPICAL | TYPICAL |
| *Phytophthora citrophthora* | IMI 396200 | Chromist | V | N | TYPICAL | ATYPICAL (N2 cooler) not significantly attenuated. |
| *Ramularia variabilis* | CBS 434.67 | Ascomycete | V | V | TYPICAL | TYPICAL |
| *Saprolegnia diclina* | IMI 308259 | Chromist | N | V* | TYPICAL | TYPICAL |
| *Suillus luteus* | CECT 20236 | Basidiomycete | V | V | TYPICAL | TYPICAL |
| *Trichophyton rubrum* | MUM 10.132 | Ascomycete | V | V | TYPICAL | TYPICAL |

V = Viable; A = Attenuated; NV= Non-viable
CBS- Centraalbureau voor Schimmelcultures, Netherlands; MUCL- Université Catholique de Louvain, Belgium; CECT- Colección Española de Cultivos Tipo, Spain; IMI-CABI, UK
* only 2 of 5 replicates viable

**Table 1 List of fungi viable following cooling using the EF600 Stirling Cryocycler**

| Classification | Species |
|---|---|
| *Basidiomycota* | *Chondrostereum purpureum, Coniophora olivaceae, Hebeloma crustiliniforme, Laccaria bicolor, Pyrofomes demidoffii, Sporobolomyces ruberrimus, Suillus luteus* |
| Ascomycota | *Acremonium strictum, Aureobasidium pullulans* var. *pullulans, Aspergillus fumigatus*, *Aspergillus ibericus, Aspergillus repens, Beauveria amorpha, Bloxamia leucophthalma, Botrytis cinerea, Botrytis elliptica, Brachysporium bloxamii, Chaetomium globosum, Chloridium botryoideum* var. *minutum, Cladobotryum dendroides, Cochliobolus heterostrophus, Colletotrichum sublineolum, Cordana pauciseptada, Cryptocoryneum condensatum, Cryptosporiopsis actinidiae, Cylindrocarpon permirum, Dictyosporium toruloides, Diplocarpon rosae*, *Eladia saccula, Fusarium avenaceum, Fusarium incarnatum, Fusarium oxysporum, Fusarium proliferatum, Fusarium sacchari, Fusarium striatum, Geotrichum candidum, Glomerella septospora, Helicoma perelegans, Heteroconium citharexyli, Hyphopichia burtonii, Hypocrea parapilulifera, Hypomyces rosellus, Lecanicillium lecanii, Metarhizium* sp.*, Nectria coronata, Paecilomyces variotii, Penicillium expansum, Penicillium ochrochloron, Phomopsis* sp.*, Podospora austroamericana, Ramularia variabilis, Setosphaeria turcica, Stachybotrys cylindrospora, Stenella musae, Tetracoccosporium aerium, Thermomyces ibadanensis, Trichoderma atroviride, Trichophyton rubrum, Verticillium tricorpus, Volutella ciliata* |
| Zygomycota | *Conidiobolus rhysosporus, Dimargaris* sp.*, Mortierella alpina*, *Mucor racemosus* f. *sphaerosporus, Rhizomucor pusillus, Umbelopsis isabellina* |
| Chromista | *Phytophthora nicotianae, Phytophthora niederhouserii, Pythium irregulare, Pythium ultimum*, *Saprolegnia declina* |

**Figure 1.** Culture Morphology in Phytophthora citrophthora, exhibiting changes after cryopreservation using a traditional 'N2 gas chamber' controlled rate cooler. Before cryopreservation (left image) and After cryopreservation (right image**)**

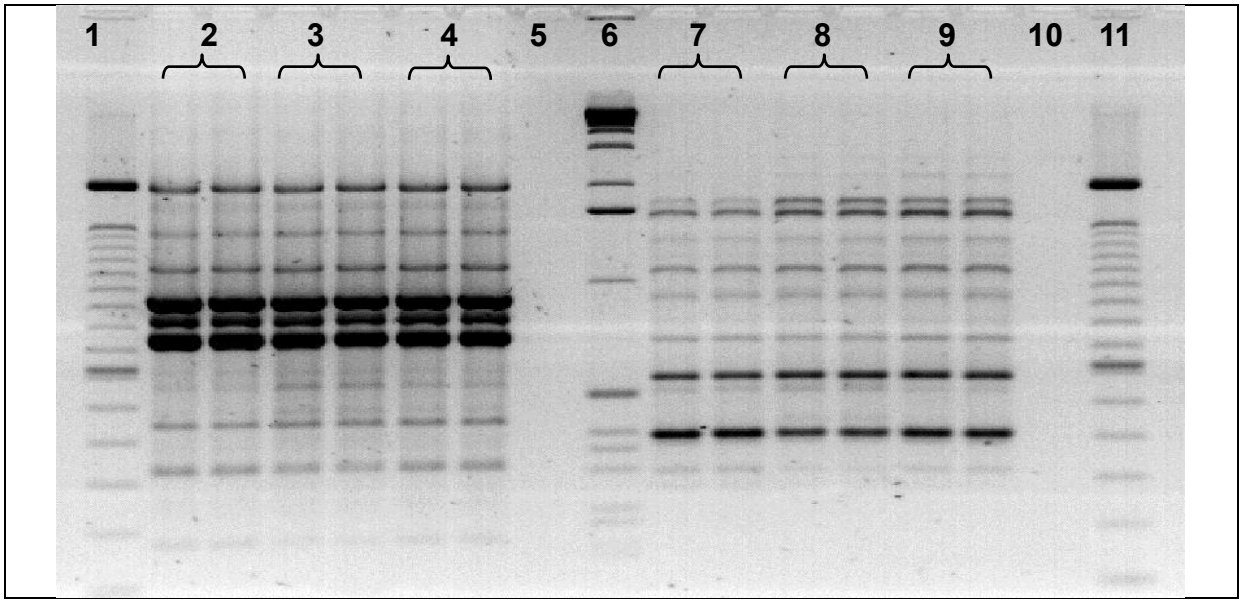


**Figure 2**. ISSR PCR fingerprint profile (using TGT primer) of Botrytis elliptica (lanes 2-4). No polymorphisms were detected, suggesting that the organism retained genomic integrity post preservation. VNTR PCR fingerprint profile (using primer MR) of Botrytis elliptica (lanes 7-9). No polymorphisms were detected, suggesting that the organism retained genomic integrity post preservation.

Lanes: 1. 100 bp ladder; 2. Non-preserved; 3. EF600 '$N_2$ free' Stirling cycle cooler; 4. traditional $N_2$ controlled rate cooler; 5. Negative control; 6. 1Kb Ladder; 7. Non-preserved; 8. EF600 '$N_2$ free' Stirling cycle cooler; 9. traditional $N_2$ controlled rate cooler; 10. Negative control; 11. 100 bp Ladder.

## DISCUSSION

Recovery of fungi after cooling using the Stirling cycle cooler was excellent, as demonstrated by the 97% of fungi from all major groups that were recovered, including recognised preservation recalcitrant strains. Of the two strains that did not survive, both were known to be preservation recalcitrant and further optimisation of methodology such as

controlled ice nucleation, use of alternative cooling rates / cryoprotectants etc. could result in successful preservation. The successful preservation and recovery of the highly recalcitrant *Saprolegnia declina* is worth a specific note as this fungus could not be preserved using the nitrogen cooler but did survive after cooling with the Stirling cooler. Only two of five replicates were viable, but the method applied provides a basis for further protocol optimisation. The control of freezing is much more finite with the Stirling cooler, lessening the variation in ice nucleation between replicates. This provides a platform for further protocol optimisation.

Culture morphology was unchanged pre- and post-preservation in all viable samples (from both cooling treatments), except in *Phytophthora citrophthora* preserved using the nitrogen cooler. However, although differences were evident in the culture plates, microscopical observation revealed that anatomical characters were typical of the genus. Further, analysis of the molecular fingerprinting results revealed 100% similarity between pre- and post-preservation samples. Changes in culture morphology following cryopreservation are not uncommon (Ryan 2001), and because anatomical and molecular characters appeared consistent, the phenotypic changes evident in this strain may be recoverable with repeated culture.

Overall, the lack of polymorphisms in the PCR fingerprinting profiles suggests that genomic stability has not been compromised after cooling with the Stirling cooler. However, a limitation of the molecular methods used is that a mutation could occur outside of the regions of the genome that were targeted by the PCR primers and, hence, remain undiscovered. A multi-primer approach was used in this investigation to increase coverage of the genome, but the use of additional primer sets would further enhance coverage The PCR methods utilised provide a rapid means to assess stability, but is best-suited to 'one-off' investigations carried out at a single time as reproducibility may be problematic (6).

Although alternative low temperature, mechanical freezing and nitrogen devices are available, the simple operation, flexibility of use, rapid turnaround times (between runs) and low running costs of the Stirling cooler are impressive. In a Biological Resource Centre, the fact that the unit can be operated in a clean, sterile environment reduces the risk of sample contamination during preparation and preservation. One additional advantage of the Stirling unit is that samples were in direct contact with the cooling plate, ensuring consistent cooling of all samples and temperature uniformity between them ($<1.0^{o}$C) and control (to 0.1 $^{o}$C). This may improve reproducibility over samples cooled in a chamber, but further investigation would be required. Further, the technology allows the application of 'true' linear cooling rates, this is advantageous over cooling in nitrogen coolers where the nucleation process results in deviation from the pre-programmed controlled rate, even when the projected deviation is compensated for.

In conclusion, Stirling cycle, nitrogen free cooling is a viable alternative to existing nitrogen cooling approaches for the routine cryopreservation of fungi in Biological Resource Centres and is suitable for the development of protocols for cryopreservation recalcitrant fungi (such as Basidiomycetes and Chromists). This study is the first report of the successful application of Stirling cycle cooling to microorganisms.

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