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SHORT NOTE

Genetic stability of ectomycorrhizal fungi is not affected by cryopreservation at −130 °C or cold storage with repeated sub-cultivations over a period of 2 years

Charlotte Crahay[1] · **Françoise Munaut**[2] · **Jan V. Colpaert**[3] · **Stéphanie Huret**[4] · **Stéphane Declerck**[1]

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Abstract Cryopreservation is considered the most reliable method for storage of filamentous fungi including ectomycorrhizal (ECM) fungi. A number of studies, however, have reported genetic changes in fungus cultures following cryopreservation. In the present study, the genetic stability of six ECM fungus isolates was analyzed using amplified fragment length polymorphism (AFLP). The isolates were preserved for 2 years either by cryopreservation (at −130 °C) or by storage at 4 °C with regular sub-cultivation. A third preservation treatment consisting of isolates maintained on Petri dishes at 22–23 °C for 2 years (i.e., without any sub-cultivation) was included and used as a control. The differences observed in AFLP patterns between the three preservation methods remained within the range of the total error generated by the AFLP procedure (6.85%). Therefore, cryopreservation at −130 °C and cold storage with regular sub-cultivation did not affect the genetic stability of the ECM fungus isolates, and both methods can be used for the routine storage of ECM fungus isolates over a period of 2 years.

Keywords Cryopreservation · Ectomycorrhizal fungi · Genetic stability · AFLP

✉ Stéphane Declerck
stephan.declerck@uclouvain.be

[1] Earth and Life Institute, Applied Microbiology, Mycology, Université catholique de Louvain, Croix du Sud 2, bte L7.05.06, 1348 Louvain-la-Neuve, Belgium

[2] Earth and Life Institute, Applied Microbiology, Phytopathology, Université catholique de Louvain, Croix du Sud 2, bte L7.05.03, 1348 Louvain-la-Neuve, Belgium

[3] Center for Environmental Sciences, Environmental Biology Group, Universiteit Hasselt, Agoralaan, Gebouw D, 3590 Diepenbeek, Belgium

[4] Earth and Life Institute, Applied Microbiology, Mycology, Université catholique de Louvain, Mycothèque de l'Université catholique de Louvain (MUCL), Croix du Sud 2, box L7.05.06, 1348 Louvain-la-Neuve, Belgium

Introduction

Ectomycorrhizal (ECM) fungi are key organisms in forest ecosystems (Smith and Read 2008). Their application in timber production, afforestation, bioremediation, and their production as edible mushrooms are increasing continuously (Duponnois et al. 2011; Karwa et al. 2011). Therefore, their valorization requires the selection of strains of high biotechnological and economical value and their subsequent preservation over long periods under conditions that maintain their genetic, phenotypic, and physiological characteristics (Kuek 1994; Duponnois et al. 2011; Lalaymia et al. 2014).

ECM fungi usually are maintained in an actively growing state by continuous sub-cultivation (Brundrett et al., 1996; Kumar and Styanarayana 2002; Repáč 2011; Siddiqui and Kataoka 2011). The method comprises preserving ECM fungus stock cultures on nutrient agar Petri dishes/slants, usually at 2–5 °C and transfer of the isolates at regular intervals onto fresh synthetic medium at 20–25 °C before cold storage. Although sub-cultivation is effective for the short-term preservation of ECM fungi, it is costly, labor-intensive, space-consuming, and the frequent handling of the cultures increases the risk of contamination (Smith and Onions 1994). Furthermore, a decrease in the capability of the isolates to form ECM associations with their host plant after repeated sub-cultivation has been reported in several studies and is associated with a reduced effectiveness of the fungi to improve plant growth (Laiho 1970; Marx and Daniel 1976; Thomson et al. 1993).

So, maintaining ECM fungi in culture collections in an actively growing state not only requires a transfer on agar media every 3 to 6 months, but also should ideally include periodic inoculation of a host plant and subsequent re-isolation to retain/restore the ability to colonize plant roots (Marx 1981; Thomson et al. 1993). According to Marx (1981), passage through a host should be done at least every 4 years whereas Thomson et al. (1993) observed a decline in the ability of ECM fungi to colonize their host plant after less than a year. The latter authors concluded that re-isolation should be done more frequently than yearly. Storage in cold sterile water (Marx and Daniel 1976; Smith et al. 1994; Richter et al. 2008 and 2016) has proven to be an efficient alternative to continuous sub-cultivation. But it usually is considered that the period of cold storage in sterile water should not exceed 3 to 4 years (Kumar and Styanarayana 2002), although Richter et al. (2016) reported that 21 ECM fungus isolates survived 30 years with this method.

Cryopreservation at ultralow temperature is considered the most reliable method for long-term storage of most filamentous fungi (Smith 1998). It outperforms all other methods for preserving genetic stability and physiological properties (Hubálek 2003). The cryopreserved fungus cultures can be maintained in a physiologically stable state theoretically for unlimited time. In addition, cryopreservation requires little space for storage, and after initial cryopreservation, no regular handling procedures are required, considerably reducing the time needed for maintenance and the risk of contamination (Smith and Onions 1994).

Any successful cryopreservation should not only allow high survival rates of the fungus strains but also should ensure the maintenance of their genetic, phenotypic and physiological characteristics (Homolka 2014). With the development of molecular tools in the last decades, an increasing number of studies have been conducted to assess genetic stability after cryopreservation. Most confirmed the genetic stability of the fungi following cryopreservation (e.g., Singh et al. 2004; Voyron et al. 2009; Homolka et al. 2010; Lalaymia et al. 2013; Ryan et al. 2014) although Ryan et al. (2001) detected genetic polymorphism in two cryopreserved isolates of *Metarhizium anisopliae*. Similarly, Broughton et al. (2012) observed three polymorphic regions in two of five isolates of *Trichoderma* sp. after cryopreservation. The risk of genetic changes following cryopreservation suggests that tests evaluating the stability of specific characteristics of strains should be included before a cryopreservation protocol is applied on a large scale (Smith and Ryan 2012; Prakash et al. 2013).

Recently, Crahay et al. (2013a) developed a cryopreservation protocol named "cryovial protocol" based on the direct growth of ECM fungus mycelium in cryovials. This method allowed high survival rates of nearly 100 ECM fungus isolates belonging to 11 species (*Cortinarius* sp., *Hymenoscyphus* sp., *Hebeloma crustuliniforme*, *Laccaria bicolor*, *Lactarius rufus*, *Paxillus involutus*, *Pisolithus tinctorius*, *Rhizopogon luteolus*, *Suillus bovinus*, *S. luteus*, and *S. variegatus*). In order to ensure the reliability of the cryovial protocol, the evaluation of physiological and genotypic stability of ECM fungus cultures following cryopreservation needed to be tested. Physiological stability (the ability to colonize the plant root and to take up nutrients) of eight ECM fungus strains stored in the aforementioned study using the cryovial protocol was evaluated in the study of Crahay et al. (2013b). The authors observed that this protocol had minor effects on ability of the fungi to colonize roots of *Pinus sylvestris* plantlets and to subsequently uptake mineral nutrients.

The present study further aimed to evaluate the genetic stability of some of the ECM fungus isolates tested in the two aforementioned studies of the cryovial protocol. We evaluated and compared the genetic stability of six ECM fungus isolates maintained: (i) for 2 years at −130 °C using the cryovial protocol, (ii) at 4 °C including sub-cultivations, and (iii) at 22–23 °C without sub-cultivation. Genetic stability was assessed using the Amplified Fragment Length Polymorphism (AFLP) method.

Material and methods

Ectomycorrhizal fungi

Six ECM fungus isolates belonging to five genera and six species were analyzed (Table 1). From their initial isolation, the fungus isolates were maintained in culture collection by cold storage with regular sub-cultivation on modified Fries medium (MFM; Colpaert et al. 2000) and stored at 4 °C in the dark.

Preservation

Three preservation methods were considered. In the first method, the isolates were cryopreserved at −130 °C for 2 years using the cryovial protocol developed by Crahay et al. (2013a). Briefly, the mycelium of each ECM fungus isolate was grown in 2-mL sterile cryovials (Sarstedt, Germany) filled with 750 μL modified Fries medium (MFM; Colpaert et al. 2000) solidified with 10 g L^{-1} agar poured in a slope. The cryovials were incubated at 22–23 °C in the dark for 7 to 9 weeks and subsequently covered by 500 μL of glycerol (10% *v*/*v*) for 1–2 h before cryopreservation. Cryopreservation was applied by using a controlled cooling rate (8 °C min^{-1} from +20 °C to +4 °C; 1 °C min^{-1} from +4 °C to −50 °C; 10 °C min^{-1} from −50 °C to −100 °C), followed by direct transfer to a freezer at −130 °C (Sanyo, Japan) for 2 years. For revival, the cryovials were immersed directly in a water bath at 38 °C for 2 min. Each fungus plug in the cryovials was then carefully transferred to the center of a Petri dish (92 mm diameter) containing 30 mL of

Table 1 Differences between AFLP patterns of ECM fungi preserved for 2 years either by cryopreservation at −130 °C, by storage at 4 °C with regular sub-cultivations or at 22–23 °C without sub-cultivation

ECM species	Isolate code[a]	Collection date	Number of DNA fragments scored	DNA fingerprint differences (%)		
				NT vs. SC[b]	NT vs. CP	SC vs. CP
Hebeloma crustuliniforme	52208 (Hc1)	1998	434	1.15%	1.38%	0.69%
Laccaria bicolor	52210 (LbicLs1)	2005	317	5.99%	5.05%	2.84%
Pisolithus tinctorius	52222 (Pt14)	Unknown	384	2.08%	6.25%	6.77%
Rhizopogon luteolus	52199 (E1Rlu)	2000	345	6.67%	4.35%	5.22%
Suillus bovinus	52173 (P2Sbo)	2000	386	5.70%	5.70%	2.07%
Suillus luteus	52104 (MG1Slu)	2000	265	0.38%	1.13%	0.75%

[a] The MUCL number refers to the code number assigned to the isolate in the Mycothèque de l'Université catholique de Louvain (Louvain-la-Neuve, Belgium) and the number within parentheses refers to the original code of the isolate. The isolates were collected by Prof. J. Colpaert in the province of Limburg (Belgium) from *Pinus* sp. except *Pisolithus tinctorius* MUCL 52222 for which the collector, locality and host plant are unknown. Species identity of the isolates was confirmed by DNA sequencing of the internal transcribed spacer (ITS) region of rDNA

[b] NT = not transferred; SC = sub-cultured followed by storage at 4 °C; CP = cryopreserved

solid MFM and incubated at 22–23 °C for 4 weeks before DNA extraction and AFLP analysis.

In the second method, the ECM fungus isolates were maintained by continuous sub-cultivation. Briefly, the fungus cultures were transferred every 6 months onto fresh MFM medium (30 mL) in Petri dishes (92 mm in diameter) and incubated at 22–23 °C for approximately 2 weeks, until the mycelium was well established. Then, the cultures were stored at 4 °C until the next transfer.

In the third method, ECM fungus cultures were maintained on Petri dishes at 22–23 °C for 2 years, without any sub-cultivation. Briefly, these cultures were inoculated on 30 mL of solid MFM in Petri dishes (92 mm diameter). The plates were sealed with Parafilm and incubated at 22–23 °C. The Parafilm was changed regularly. After 2 years of storage, the viability of the cultures was confirmed (i.e., ability to growth on fresh MFM medium). These cultures were used as a control.

DNA extraction

For each ECM fungus isolate and preservation method, a small mycelium plug (~4 mm diameter) was sampled at the margin of the fungus cultures and transferred onto Petri dishes (92 mm diam.) containing 30 mL of MFM solidified with 10 g L^{-1} of agar (Scharlau, Spain), and covered with a sterile cellophane sheet (Hutchinson, France). The use of cellophane sheets facilitates the harvest of ECM fungus mycelium prior to DNA extraction. The cultures were grown for 4 weeks at 22–23 °C.

After 4 weeks, the fungus cultures were scraped from the surface of the cellophane and ground in liquid nitrogen to a very fine powder using a mortar and a pestle. DNA extraction was performed using a CTAB-protocol (modified from Amalfi 2016). The powdered mycelia was macerated in 800 μL of 2X CTAB lysis buffer (i.e., 2% (*w*/*v*) CTAB (hexadecyltrimethylammonium bromide), 100 mM Tris-HCl pH 8, 20 mM EDTA, 1.4 M NaCl, 2% (*w*/*v*) PVP-40], 2.4 μL of 0.3% (*v*/*v*) of β-mercapto-ethanol and 10 μL of proteinase K (20 mg/mL) for 45 min at 60 °C with periodic gentle mixing. The samples were centrifuged 5 min at 13000 rpm. The supernatant from each tube was loaded onto a filtration spin column (QIAshredder, Qiagen, Germany) placed on a 1.5-mL Eppendorf tube previously filled with 4 μL of RNase A (100 mg/mL). Each tube was centrifuged 2 min at 13000 rpm and incubated for 1 h at room temperature. After filtration and RNase treatments, 600 μL of the suspension was transferred into a new Eppendorf (1.5 mL) and centrifuged with an equal volume of chloroform: isoamyl alcohol (24:1) at 13000 g for 5 min. A 500 μL volume of the supernatant was transferred into a new Eppendorf tube and the previous step was repeated.

DNA was precipitated from 400 μL of the supernatant by addition of 0.8 volume of isopropanol (4 °C), followed by mixing, incubation for 10 min at −20 °C, and centrifugation at 4 °C and 16,000 g for 5 min. The resulting pellet was washed with 600 μL of 70% ethanol (−20 °C), centrifuged at 4 °C and 16,000 g for 5 min and left to dry 1 h. DNA was suspended in 50 μL TE (10 mM Tris-HCl, 1 mM EDTA, pH 8) and incubated 20 min at 60 °C. The integrity and quality of the genomic DNA was verified by electrophoresis in 1% agarose gel.

AFLP reaction

The AFLP procedure was conducted as described by Voyron et al. (2009) with minor modifications. Seven μL of extracted genomic DNA was digested with 2 μL of the mixture of EcoRI and MseI restriction enzymes and 1 μL of their 5× buffer. Pre-selective amplification was performed in a final volume of 20 μL instead of 25 μL with EcoRI + 0 and MseI +0 used as pre-selective primers. Selective amplification was carried out with the three following primer combinations: (i) EcoRI + C-MseI, (ii) EcoRI + G-MseI + C, and (iii) EcoRI + T-MseI + C

with EcoRI primers labeled with D4 WellRED dye (Sigma-proligo, Beckman Coulter license, USA). Fragment separation and detection were performed using the CEQ™ 2000xl DNA Analysis System (Beckman Coulter, USA, USA) under the conditions indicated by Voyron et al. (2009).

Data analysis

Fragments were analyzed with the automated scoring software CEQ 8000 with a maximum bin width of 1.00 nucleotide and a minimum fluorescence threshold of 500 relative fluorescent units (RFU) and were scored as binary data (1 for presence and 0 for absence). The AFLP electropherogram of each isolate preserved under the three methods were examined in order to correct manually erroneously scored peaks from the automated analysis. Only fragments with a size between 80 and 350 base pairs (bp) were considered for the analysis. Fragments present in negative controls were excluded from the analysis. The difference rates were then calculated as the number of fragments that differed between two AFLP patterns expressed as a percentage of the total number of fragments considered (Bonin et al. 2004).

Reproducibility of the AFLP

To assess the reproducibility of the AFLP procedure, a culture of *S. bovinus* MUCL 52173 was extracted twice and subjected to two independent AFLP reactions with the primer combinations described above. One half of the culture was harvested and extracted under the conditions described above, and the remaining half of the culture was harvested and extracted the next day. *Suillus bovinus* MUCL 52273 was selected to assess reproducibility because the DNA extraction and the AFLP protocols described above were developed on this isolate in a preliminary test and had proved to be suitable. The fragments analyzed as described above provided an estimate of the total error generated by the AFLP. The difference rates between the preservation methods were compared to the error rate of the AFLP procedure in order to determine if the difference rates between the preservation methods for each isolate were likely to have resulted from the error generated during the AFLP procedure (i.e., were the differences less than the total error) or from differences between the preservation methods (differences greater than the total error).

Results and discussion

The total error estimated for the AFLP fingerprintings derived from *S. bovinus* MUCL 52173 extracted twice and subjected to two independent AFLP reactions was 6.85%. All cryopreserved ECM fungus isolates showed differences in their AFLP patterns when compared to the control isolates, but those differences were less than the total error generated by the extraction and the AFLP procedure (i.e., 6.85%) (Table 1). Therefore, cryopreservation appeared to be a reliable method for preserving the genetic stability of these ECM fungus isolates as reported by other studies with ECM fungi and other fungi (Voyron et al. 2009; Homolka et al. 2010; Lalaymia et al. 2013; Ryan et al. 2014).

In the AFLP patterns of the six isolates maintained for 2 years at 4 °C by continuous sub-cultivation and the control isolates (i.e., not transferred during the 2-year period), no differences were observed greater than the error (6.85%). Similarly, no differences in the AFLP patterns for the six isolates maintained for 2 years by cryopreservation at −130 °C and by continuous sub-cultivation were observed which were greater than the error (6.85%). So, we concluded that both cold storage with regular sub-cultivation and cryopreservation at −130 °C can be used for the routine storage of ECM fungus isolates over the time investigated (i.e., 2 years). Compared to cryopreservation, however, regular sub-cultivation is time-consuming and contamination is possible (Smith and Onions, 1994) especially when the ECM fungus isolates require periodic inoculation on a suitable host plant followed by subsequent re-isolation to retain/restore the ability to colonize plant roots (Thomson et al. 1993). Therefore, cryopreservation is a much more manageable technique than sub-cultivation for the long-term storage of ECM fungus isolates. In addition, a higher reliability of cryopreservation compared to sub-cultivation might be apparent after periods of storage longer than 2 years. Indeed, prolonged periods of storage would have required additional subcultures for the cultures maintained by the sub-cultivation method and thus might have increased the risk of genetic drift (Butt et al. 2006).

Despite the observed lack of genetic differences between cryopreserved and sub-cultured cold-stored isolates, we cannot totally exclude mutagenic effects of the cryovial protocol on the genome of the ECM fungus isolates investigated. Indeed, in our study, a relatively low fraction of the genome was screened by AFLP markers. For reference, Harding (2004) estimated that typically for plants with large genome sizes (~1 Gb), only ~0.001% of the genome was screened. In the present study, approximately 0.1% of the genome of *L. bicolor* was screened using AFLP (sum of the size of the 317 fragments generated by AFLP for *L. bicolor* = ~60 kb; genome size of *L. bicolor* = ~65 Mb, Martin et al. 2008).

In a previous study, Crahay et al. (2013b) tested the ability of eight ECM fungus isolates after cryopreservation for 6 months at −130 °C or after storage at 4 °C to colonize *Pinus sylvestris* roots and to transport inorganic phosphate and ammonium from the substrate to the plant. The six ECM fungus isolates considered in the present study originated from the same batch of cultures that was used in the study of Crahay et al. (2013b). Some samples were thawed and revived after 6 months of cryopreservation for the evaluation

of phenotypic stability and others were thawed after 2 years for the genetic stability assessment. For most isolates, cryopreservation did not impair the ability of the fungi to colonize the root system of *P. sylvestris* seedlings and to take up P_i and NH_4^+, supporting the functional stability of cryopreserved ECM isolates. The isolate *S. bovinus* MUCL 52173 cryopreserved for 6 months, however, was considerably affected by the cryopreservation process as it exhibited a reduced ability to form mycorrhizas and to take up P_i after cryopreservation compared to non-cryopreserved cultures. Nevertheless, no differences in the AFLP patterns greater than the total error were detected after 2 years of cryopreservation. Although the observed phenotypic differences might have had a genetic origin, that was not detected by the AFLP with the primers and restriction enzymes used. Alternatively, these phenotypic changes might be a reversible response to stress and have an epigenetic origin as reported by some studies on plants, algae and animals (e.g., Harding 2004; Kaity et al. 2008; Peredo et al. 2008; Johnston et al. 2009; Peredo et al. 2009).

The error rate of 6.85% due to AFLP is slightly higher than error rates below 5% usually reported for plants and animals (Bonin et al. 2004). For fungi, error rates of AFLP rarely have been evaluated, but some studies reported rates ranging from 3.9% (Voyron et al. 2009) to 20% (Keirle et al. 2014). Every step of the AFLP procedure can generate errors (Bonin et al. 2004). The calculation of the error rate is thus highly step-dependent, which results in difficulty in comparing errors among studies (Crawford et al. 2012). In our experiment, the error rate of 6.85% was calculated for the whole AFLP procedure, i.e., from the extraction to the scoring process. Errors during the AFLP reaction may have multiple causes such as low-quality DNA, amplification artifacts, biochemical anomalies, electrophoresis discrepancies, laboratory temperature variations, material, and protocol (Bonin et al. 2004). Those affect AFLP patterns (especially the intensities and positions of the peaks) that are subsequently erroneously analyzed (Harding 2004; Mikulášková et al. 2012).

Because of the relatively high error rate as well as the low genome coverage of the AFLP, we recommend further assessing genetic stability at the molecular level in combination with other techniques such as morphologic, phenotypic, and/or biochemical approaches as already proposed by Harding (2004).

Our control cultures were maintained for 2 years on MFM plates at 22–23 °C without any transfer. These cultures were still viable after 2 years of storage. Possible reasons they survived for 2 years are that (i) ECM fungi are very slow growing fungi (Kumar and Styanarayana 2002), (ii) about 30 mL of MFM agar was poured in the Petri dish (while a volume of 20 mL is usually used for ECM fungi; Brundrett et al. 1996), and (iii) the plates were sealed with Parafilm that was regularly changed which reduced desiccation of the growth medium. As the cultures aged, however, secondary metabolites and autolytic enzymes might have accumulated in the growth medium as reported by Paterson and Lima (2013). Some of those have been reported to have mutagenic effects. For instance, it was shown that isovelleral and necatorin, two mutagenic toxins, were produced by ECM species belonging to *Lactarius* and *Russulaceae* (Sterner et al. 1982; Liu 2005). Therefore, genetic variations that may have arisen in those cultures after 2 years of storage on nutrient agar cannot be excluded from affecting the AFLP patterns of the control isolates. Nevertheless, many studies have reported that it is not the duration in axenic culture per se that promotes changes of the fungus, but rather the rate of sub-cultivation (e.g., Hajek et al. 1990; Butt et al. 2006).

In conclusion, the cryovial protocol is a suitable cryopreservation method, not only to reach high viability rates of a large set of ECM fungus isolates as demonstrated in Crahay et al. (2013a) but also to maintain important phenotypic characteristics (e.g., root colonization and nutrient transport) (Crahay et al. 2013b) as well as genetic integrity of the ECM fungus isolates for the part of genome that we investigated. Our results provide fungus culture collections an efficient and reliable method for the maintenance of ECM fungi—usually considered recalcitrant to cryopreservation—as an alternative to the widely used continuous sub-cultivation method. Though superiority of cryopreservation over cold storage with regular sub-cultures was not demonstrated after 2 years of preservation, cryopreservation seemed the most reliable method of storage because it decreased the necessity for laborious sub-cultivations with the attendant risk of contamination and need for passage through a host at regular intervals. Because of the relatively high error rate as well as low genome coverage of the AFLP, we recommend using such markers in combination with phenotype analysis as an additional confirmation of stability after cryopreservation. The evaluation of the physiological and genetic stability of cryopreserved ECM fungi needs to be extended to additional replicates and isolates, and to longer periods of storage than 2 years, but our results support the high promise of cryopreservation.

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