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REVIEW

Maintenance and preservation of ectomycorrhizal and arbuscular mycorrhizal fungi

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Abstract Short- to long-term preservation of mycorrhizal fungi is essential for their in-depth study and, in the case of culture collections, for safeguarding their biodiversity. Many different maintenance/preservation methods have been developed in the last decades, from soil- and substrate-based maintenance to preservation methods that reduce (e.g., storage under water) or arrest (e.g., cryopreservation) growth and metabolism; all have advantages and disadvantages. In this review, the principal methods developed so far for ectomycorrhizal and arbuscular mycorrhizal fungi are reported and described given their distinct biology/ecology/evolutionary history. Factors that are the most important for their storage are presented and a protocol proposed which is applicable, although not generalizable, for the long-term preservation at ultra-low temperature of a large panel of these organisms. For ECM fungi, isolates should be grown on membranes or directly in cryovials until the late stationary growth phase. The recommended cryopreservation conditions are: a cryoprotectant of 10 % glycerol, applied 1–2 h prior to cryopreservation, a slow cooling rate (1 °C min^{-1}) until storage below −130 °C, and fast thawing by direct plunging in a water bath at 35–37 °C. For AMF, propagules (i.e., spores/colonized root pieces) isolated from cultures in the late or stationary phase of growth should be used and incorporated in a carrier (i.e., soil or alginate beads), preferably dried, before cryopreservation. For in vitro-cultured isolates, 0.5 M trehalose should be used as cryoprotectant, while isolates produced in vivo can be preserved in dried soil without cryoprotectant. A fast cryopreservation cooling rate should be used (direct immersion in liquid nitrogen or freezing at temperatures below −130 °C), as well as fast thawing by direct immersion in a water bath at 35 °C.

Keywords Cryopreservation · Lyophilization · Oil and water storage · Alginate beads · Culture collections · Genetic stability

Introduction

The importance of microbial culture collections as germplasm resources for basic and applied research, as well as for the white (industry), green (agro/food), grey (environment), blue (marine) and red (health) biotechnology sectors, is undisputable (Smith and Ryan 2012a, b; Houseknecht et al. 2012). However, their use often presupposes that the microorganisms are correctly identified and maintained under conditions that preserve their original properties over long periods (Smith 2012; Smith and Ryan 2012a, b). It is therefore common or desirable to preserve isolates under conditions that slow-down/arrest their metabolism, and to use at least two different methods to minimise the probability of genetic resources being lost. The preservation protocols must be robust, reproducible and, ideally, transferable to other repositories (Smith 2012; Smith and Ryan 2012a, b). All the steps must be controlled (e.g., culture preconditioning, storage conditions, reviving and stability assessment after storage) to guarantee the reproducibility of the method and the quality of the biological material. The development of quality management practices and biosecurity procedures are added-values and

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The names of mycorrhizal fungi mentioned in this review are those used at the time of their publication

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are gaining in importance under the auspice of international organizations such as the World Federation for Culture Collections (WFCC) (http://www.wfcc.info/collections/) and the Organization for Economic Co-operation and Development (OECD) (http://www.oecd.org/science/biotech/23547773.pdf).

While most fungal culture collections host mycorrhizal fungi (belonging principally to Basidiomycetes and Ascomycetes), a number of germplasm collections have been specifically developed for these root symbionts (e.g., Glomeromycota IN vitro COllection (GINCO: http://www.mbla.ucl.ac.be/ginco-bel), Centre for Mycorrhizal Culture Collection (CMCC: http://mycorrhizae.org.in/), the International Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM: http://invam.wvu.edu/) and the International Bank for the Glomeromycota (IBG: http://www.i-beg.eu/). The most widely used method for the bio-banking of these root symbionts is continuous sub-cultivation of the isolates either in vivo (e.g., in pots on plants) or in vitro (on excised roots or plants, or as vegetative hyphae, depending on the group of mycorrhizal fungi). However, these methods are time- and space-consuming, and prone to contaminations (Smith and Onions 1994). Moreover, the morphology, physiological activity and long-term genetic stability cannot be guaranteed (Marx and Daniel 1976; Thomson et al. 1993; Plenchette et al. 1996).

The present review reports on several methods developed so far for the maintenance and preservation of ectomycorrhizal (ECM) fungi and arbuscular mycorrhizal fungi (AMF), presents factors which are the most important for their storage and proposes protocols, applicable, although not generalizable, for the long-term preservation at ultra-low temperature (cryopreservation) of these organisms.

Fungal preservation at a glance

Various protocols, from sub-cultivation to methods that reduce (e.g., storage under water or oil) or arrest (e.g., cryopreservation) growth and metabolism have been developed for the maintenance/preservation of fungi (Smith et al. 2001). The most familiar is continuous growth by sub-cultivation (Smith and Onions 1994), typically used for short-term storage, where fungal cultures are most often grown on gelled medium and stored at room temperature, at temperatures close to 25 °C, or at lower temperatures (e.g., 4 °C) to increase the interval between sub-cultures (Smith and Onions 1994). Although this widely used method is simple and relatively cheap (specialized equipment is not required), it is time-consuming, labor-intensive and at risk for contaminations by mites or other unwanted organisms. In addition, viability may be lost and morphological as well as physiological characters may be altered during sub-cultivation (Thomson et al. 1993). Alternatives for the preservation of fungi are in sterile water or under oil, drying on silica gel, or storing in alginate beads or in soil at room or cold (e.g., 4–8 °C) temperatures (Smith and Onions 1994; Abd-Elsalam et al. 2010). Again, these methods are simple, do not require expensive equipment and are adapted for short-term preservation (2–5 years) but, similar to sub-cultivation, they present the risk of unwanted contaminations and could favor selection of mutants able to grow under difficult conditions (Smith and Onions 1994; Houseknecht et al. 2012).

Methods that arrest growth and metabolism are the most reliable for long-term preservation of fungi. Lyophilization (freeze-drying) and/or cryopreservation (i.e., storage at ultra-low temperature) are mandatory in international culture collections storing large numbers of fungi for extended periods (Smith and Onions 1994; Abd-Elsalam et al. 2010). Lyophilization consists of water removal by freezing, volatilization, and drying at low pressure and temperature under vacuum (Tan et al. 1995, 2007), and although it requires adequate equipment for freeze-drying, no specific infrastructure is needed to store the organisms following lyophilization (Tan et al. 1995, 2007). Cryopreservation consists in freezing the organisms and storage at temperatures most often below −130 °C in a freezer or at −196 °C in liquid nitrogen (LN), to completely arrest the metabolic activities for indefinite time periods (Mazur 2004). Viability of lyophilized and cryopreserved fungi depends mainly on the freezing rate, the use and nature of lyo- or cryoprotectants and the thawing rate (Tan et al. 1995; Mazur 2004); if not correctly applied, they may cause injuries to the fungi (Meryman 2007; He 2011). In addition to these parameters, various intrinsic factors (e.g., the age and physiological state of the culture) should be considered when preparing the organisms for lyophilization or cryopreservation (Smith and Onions 1994).

Most mycorrhizal fungi are preserved in collections by one or several of the methods cited above. If maintenance by metabolically active methods is common, they should be applied only if the fungal isolates cannot be preserved under conditions that arrest their metabolism or in addition to these methods. Here, we review the preservation methods currently used for ECM fungi and AMF, the economically most important groups of mycorrhizal fungi.

Methods of maintenance and preservation of ectomycorrhizal fungi

The maintenance/preservation of ECM fungi is rather delicate due to their intrinsic characteristics (Homolka et al. 2006). Under in vitro culture conditions, these organisms do not produce conidia and sexual structures are rarely formed (Sundari and Adholeya 1999, 2000a, b; Homolka et al. 2006). Hyphae, which is their dominant form in vitro, generally grow slowly in axenic culture (Molina et al. 2002; Homolka et al. 2006) and are usually more sensitive to environmental conditions than spores and conidia (Smith 1993; Tan et al. 1991). The most commonly used

method for the maintenance and propagation of ECM fungi is in vitro sub-cultivation on appropriate synthetic media (e.g., modified Fries Medium (MFM—Colpaert et al. (2000), Modified Melin Norkrans (MMN—Marx (1969)) and subsequent storage at ±25 °C (Corbery and LeTacon 1997; Siddiqui and Kataoka 2011). Reducing the storage temperature can extend the period between sub-cultures; for instance, Tibbett et al. (1999) stored a wide geographical range of *Hebeloma* isolates during 3 years at 2 °C. Although sub-cultivation is effective for the short-term preservation of ECM fungi, it is costly and time-consuming (Smith and Onions 1994; Kitamoto et al. 2002). Furthermore, it may reduce fungal ability to form mycorrhiza (Marx and Daniel 1976; Thomson et al. 1993; Di Battista et al. 1996) and may impact on some essential properties, like fungal capacity to improve plant growth (Marx and Daniel 1976; Thomson et al. 1993). Regular revitalization via passage on a suitable host plant has been proposed by Marx and Daniel (1976) and Thomson et al. (1993) at intervals of 4 years.

Storage in water, mineral oil, or alginate beads

Preservation of ECM fungi under water has also been reported, although less frequently. This consists in placing agar discs of the fungal culture on an agar slant in sterile tubes and covering it with sterile deionized water before storage at 4–25 °C (Smith and Onions 1994). Richter (2008) reported that the genus *Laccaria* (Electronic supplementary material (ESM) Table 1) is well-adapted to cold storage (i.e., 5 °C) in sterile water, while *Boletus*, *Lactarius*, *Paxillus*, *Scleroderma*, and *Thelephora* appear less adapted to this type of storage. Marx and Daniel (1976) showed that the survival of ECM fungi stored in sterile water at 5 °C was 100 % (ESM Table 1) after 1 year but was reduced for some isolates to 95 and 64 % after 2 and 3 years of storage, respectively. This method is therefore only useful for short-term preservation (2–5 years) and should be backed up with long-term storage methods (Smith and Onions 1994).

Since the preservation of fungi on agar slants after covering with sterile mineral oil at room or low temperatures (i.e., 4–8 °C) (Smith and Onions 1994) is appropriate for mycelium or non-sporulating fungi (Homolka and Lisá 2008; Perrin 1979), this method should as such be adapted to ECM fungi. However, little is known about ECM fungi preservation under mineral oil, although Perrin (1979) and Johnson and Martin (1992) reported that hundreds of Ascomycete and Basidiomycete ECM fungi remained viable up to 27 years of storage under mineral oil. While this method prevents dehydration of the fungi and slows down their methabolic activity and growth, the risk of contaminations and the selection of mutants adapted to grow under difficult conditions cannot be excluded (Smith and Onions 1994; Houseknecht et al. 2012).

Storage in alginate beads is often used to produce ECM fungal inoculum for plant inoculation. Hyphae are fragmented, mixed into a sodium alginate solution and dropped into a solution of calcium chloride (Paloschi de Oliveira et al. 2006), producing gelled beads (Maupérin et al. 1987). This procedure offers great flexibility because additives can be included in the beads that help to protect the fungi for long periods. The polymeric matrix of the alginate gel allows hyphae to grow inside the beads and extend outside. Several studies have demonstrated the success of this method of preservation for ECM fungi. Maupérin et al. (1987) maintained liquid-produced hyphae of *Hebeloma crustuliniforme* in alginate beads for at least 5 months at 4 °C (ESM Table 1), while Kuek et al. (1992) preserved eleven eucalypt ECM fungi belonging to *Paxillus*, *Laccaria* and *Hebeloma* (ESM Table 1) for 7 months entrapped within alginate beads stored at 25 °C. Using the same technique, Rodrigues et al. (1999) obtained 100 % viability with *Paxillus involutus*, and about 55 % with *Pisolithus tinctorius* (ESM Table 1), although viability significantly decreased after 60 days of storage. Paloschi de Oliveira et al. (2006) preserved hyphae of *Rhizopogon nigrescens* (ESM Table 1) immobilized in calcium alginate gel during 18 months at 8 °C.

Lyophilization and cryopresevation

It is often reported that ECM fungi are not adapted to lyophilization because of their growth characteristics (i.e., absence of conidia and few or no spores produced in vitro) (Tan et al. 1991; Smith and Onions 1994). However, a few studies have reported their successful lyophilization. Sundari and Adholeya (1999) tested different parameters (physiological growth conditions, culture age, the lyoprotectant and its concentration, the pre-freezing procedure, freeze-drying and rehydration programmes) to optimize the lyophilization process of *Laccaria fraterna*. Cultures from the late-growth phase or near to the stationary phase treated with 10 % DMSO as lyoprotectant survived freeze-drying. Morphological and physiological characteristics remained similar between freeze-dried and non-freeze-dried cultures. These authors successfully extended their protocol to 15 isolates belonging to *Laccaria*, *Amanita*, *Tricholoma*, *Thelephora*, *Pisolithus*, and *Scleroderma* (ESM Table 1). Using the same protocol, they investigated the stability of seven of these ECM fungal isolates by qualitative enzyme assays; all isolates survived lyophilization and showed stable enzymatic activity (Sundari and Adholeya 2000a, b). Obase et al. (2011), on the contrary, reported in their tudy that 34 ECM fungal isolates belonging to *Amanita*, *Cenococcum*, *Laccaria*, *Lactarius*, *Lepista*, *Paxillus*, *Pisolithus*, *Rhizopogon*, *Russula*, *Scleroderma*, *Suillus*, and *Tomentella* did not survive freeze-drying.

Although cryopreservation is the most reliable long-term preservation method used for fungi and a multitude of protocols have been developed for Basidiomycetes, Ascomycetes and Zygomycetes, only a few studies have focused strictly on

ECM fungi. This is probably because in vitro, these organisms only produce hyphae, which are more sensitive to environmental stresses than spores (Smith 1993). Lehto et al. (2008) and Dalong et al. (2011) showed that the lethal freezing temperature of different ECM fungal isolates (i.e., *Suillus luteus*, *Suillus variegatus*, *Laccaria laccata*, *Hebeloma* sp., *Cortinarius multiformis*, *Russula densifolia*, *Suillus granulatus*, and *Lactarius deliciosus*) was around −7 and −14 °C. Corbery and LeTacon (1997) tested the survival of different ECM fungal isolates (i.e., *H. crustuliniforme*, *Laccaria bicolor*, *P. involutus*, *P. tinctorius*, *Scleroderma flavidum*, *Rhizopogon luteolus*, *Thelophora terrestris*, and *Cenococcum geophillum*) after cryopreservation in 15 % glycerol (*v*/*v*) following different cooling rates (i.e., samples from colony margins were directly plunged in LN and placed in a freezer at −80 °C, or slowly cooled (~1 °C min^{-1}) before freezing in LN or at −80 °C). Survival depended largely on the fungal species or the isolate and on the cooling rate. For example, *L. bicolor*, *P. tinctorius* and *R. luteolus* survived slow freezing (~1 °C min^{-1}) (ESM Table 1), while the hyphae were injured at a faster cooling rate. *C. geophillum* was not affected by freezing, whether the cooling rate was slow, fast (i.e., in LN) or uncontrolled, while *T. terrestris* and *P. involutus* did not survive any freezing method. The authors suggested that the response to freezing of different species of ECM fungi was dependent on the physiology and water relationships of the hyphae. This is consistent with Smith's (1998) recommendation of a slow-cooling rate (1 °C min^{-1}) and application of glycerol as cryoprotectant for Basidiomycetes and Ascomycetes, and the absence of any obvious link between taxonomic group and response of fungi to freezing and thawing. Danell and Flygh (2002) also successfully cryopreserved *Cantharellus cibarius* (ESM Table 1) using slow (0.3 °C min^{-1}) controlled cooling and storage in LN followed by rapid thawing. Here, sorbitol (4 M) and DMSO (1 M) were used as cryoprotectants. Cryopreservation of the same isolate was ineffective, however, using trehalose (10 %) and glycerol (10 %) and cooling rates of 0.5, 1, and 10 °C min^{-1}.

Homolka et al. (2003) tested the cryopreservation of three ECM fungi *Entoloma clandestinum*, *Scleroderma citrinum*, and *S. verrucosum* (ESM Table 1), among 250 Basidiomycetes isolates, following two methods. The first, named the original protocol (OP), used agar plugs sampled from an actively growing part of the colony, transferred into cryovials and submerged with 10 % glycerol (*v*/*v*). After pre-cooling to 7 °C, the cultures were cooled to −35 °C for 45–60 min and then immersed in LN. The second, named the cryovial protocol (CP), is slightly modified from the protocol of Hoffmann (1991). Sterile plastic straws, open at both ends, were used to sample agar colonized by the fungal hyphae grown on medium supplemented with 5 % glycerol (*v*/*v*) at 24 °C. The straws were then transferred into sterile cryovials, sealed and frozen to −70 °C at a programmed slow cooling rate of 1 °C min^{-1}, and then plunged into LN. After storage, the cryovials were thawed rapidly at 37 °C. None of the ECM fungi survived preservation with the OP method while with the CP method, on the contrary, *E. clandestinum* presented a 100 % survival rate and *S. citrinum* and *S. verrucosum* isolates 50 %. The extracellular laccase production of the fungi remained unchanged after cryopreservation (Homolka et al. 2003). As for other fungi tested in this study, the CP using a very slow cooling (1 °C min^{-1}) rate yielded the best results, which is consistent with the findings from several other studies (Corbery and LeTacon 1997; Smith 1998). Later, Homolka et al. (2006) compared the OP method to a perlite preservation (PP) protocol on 442 Basidiomycetes isolates including some ECM fungi (*Clavariadelphus pistillaris*, *E. clandestinum*, *L. laccata*, *L. proxima*, *S. citrinum* and *S. verrucosum* (ESM Table 1)). In the PP protocol, the fungal colony was grown directly on 200 mg of perlite in cryovials moistened with 1 ml of water and enriched with glycerol (final concentration 5 %). After 14 days at 24 °C, the cryovials were frozen at −70 °C following a slow-cooling rate of 1°C min^{-1} and direct immersion in LN. For both protocols thawing was rapid at 37 °C. Using the PP protocol, all the isolates survived 3-year storage versus 57 % with the OP. The activity of laccase before and after cryopreservation remained similar for almost all the Basidiomycetes and for all the ECM fungi tested.

In contrast, Kitamoto et al. (2002) successfully used rapid cooling, by direct transfer into a freezer at −85 °C, to preserve different ECM fungal isolates (e.g., *Boletus pulverulentus*, *Entoloma* sp., and *Hebeloma* spp.) for 10 years (ESM Table 1). Obase et al. (2011) tested 10 % skimmed milk (habitually used for the freeze-drying of microorganisms) as cryoprotectant in the cryopreservation of a large number of ECM fungal isolates (i.e., *Amanita ibotengutake*, two isolates of *Aminata* sp., *Cenococcum geophilum*, *Laccaria amethystina*, two isolates of *Lactarius* sp. *Lepista nuda*, *P. involutus*, *P. tinctorius*, four isolates of *Rhizopogon* sp., three isolates of *Russula* sp. *Scleroderma* sp., *S. granulatus*, *S. luteus*, *Suillus pictus*, *Suillus placidus*, and two isolates of *Tomentella* sp.). Agar disks of 30–45-day-old cultures were frozen in cryovials after preconditioning in skimmed milk for 1 h at 4 °C, stored for 3 h at −20 °C, and then transferred to −70 °C. Variation in revival was observed after rapid thawing at 37 °C, depending on the duration of storage. Most of the isolates did not survive or showed a reduction in growth after storage at −70 °C for more than 6 months as compared to controls; only 6 of the 23 fungal isolates did not differ from the controls (*C. geophilum*, *L. nuda*, two *Rhizopogon* sp. isolates and *S. granulatus* and *S. luteus*) (ESM Table 1).

Stielow et al. (2012) tested another protocol for the cryopreservation of 18 fungi among which several ECM fungi (*Hysterangium stoloniferum*, *Mutinus elegans*, *Gautieria morchelliformis*, *Melanogaster broomeianus*, *P. involutus*, *R. luteolus*, *Tapinella panuoides*, *Boletus edulis*, *Clitocybe gibba*,

Gymnomyces xanthosporus, and *Tuber borchii*). This protocol involved first growing fungal isolates on charcoal filter paper strips (CFS) placed on the surface of a culture medium for 3–5 weeks. The CFS were then collected, incubated in 10 % glycerol (*v*/*v*) for 1–2 min in a sterile Petri dish, and transferred into cryovials by layering the CFS on top of each other. The closed cryovials were placed 24 h in the gas phase of a LN tank (cooling rate of approx. 1–10 °C min^{-1} until −120 to −140 °C), before direct transfer into LN. This protocol was compared to a conventional straw preservation protocol which consists in pushing hyphae plugs, grown on medium flooded with a cryoprotectant (glycerol, 5 %*w*/*v*), into straws before transferring into cryovials and subsequent freezing in the gas phase of LN for 24 h before transfer into LN. In both cases, fungi were revived by rapid thawing to 25–30 °C. Viability was 100 % for 13 isolates with the CFS protocol (ESM Table 1) while, with the exception of *C. gibba* and *B. edulis*, none of the isolates survived freezing with the conventional straw technique.

Recently, Crahay et al. (2013a) developed a preservation protocol (Fig. 1) efficient for nearly one hundred ECM fungal isolates belonging to 8 species (*Cortinarius* sp., *H. crustuliniforme*, *L. bicolor*, *Lactarius rufus*, *P. involutus*, *Suillus bovinus*, *S. luteus*, and *S. variegatus*) (ESM Table 1). These authors compared the survival of the isolates using the straw protocol (SP) of Hoffman (1991) and the CP of Voyron et al. (2009). These protocols differed in the preparation of the isolates and their conditioning before cryopreservation. In both protocols, 10 % glycerol (*v*/*v*) was used and the same cooling rate was followed: 8 °C min^{-1} from +20 to +4 °C; 1 °C min^{-1} from +4 to −50 °C; 10 °C min^{-1} from −50 to −100 °C. Isolates were then directly transferred into a freezer at −130 °C and for revival, cryovials were directly thawed at +38 °C. With the SP (Hoffmann 1991), 2- or 4-week-old cultures (depending on the isolate) were submerged in the cryoprotectant for 1–2 h then hyphal plugs were pushed into straws and transferred in cryovials. With the CP (Voyron et al. 2009), hyphae of cultures grown directly in cryovials for 7–9 weeks were covered for 1–2 h with glycerol before cryopreservation. Survival rate of the cryopreserved ECM fungal isolates was greater with the CP as compared to the SP. Also, 89 % of the isolates survived with the CP suggesting its compatibility with a large set of ECM fungi and, contrary to the findings of Obase et al. (2011), two of three *P. involutus* isolates survived this preservation protocol. Following these observations, Crahay et al. (2013a) suggested that culture preparation is the key factor for ECM fungal survival to cryopreservation. In the CP, as in other successful studies (Homolka et al. 2003; Homolka et al. 2006; Stielow et al. 2012), the ECM fungi were grown directly in the cryopreservation containers (cryovials), thus avoiding manipulations and damage to hyphae before freezing. Hyphal density was also greater than with the SP and the whole colony rather than only the colony margin was cryopreserved. In addition, ECM fungal cultures were older with the CP (7–9 weeks) than with the SP (2–4 weeks) which is consistent with the general conclusion that filamentous fungi from the late exponential and stationary phases survive freezing better than actively growing cultures (Tanghe et al. 2003; Tan and Van Ingen 2004). When Crahay et al. (2013b) tested the symbiotic ability of eight ECM fungal isolates, cryopreserved for 6 months at −130 °C using the CP protocol, they found that this mode of preservation had no significant effect on the colonization rate of *P. sylvestris* roots, the concentration of ergosterol in the roots and substrate or the transport Pi and NH_4^+ from the substrate to the plant, as compared to the non-cryopreserved controls.

Methods of maintenance and preservation of arbuscular mycorrhizal fungi

While ECM fungi can grow axenically, AMF should be associated obligatorily with roots of a compatible host plant to complete their life cycle. Their in vitro culture on excised root systems is limited to a number of species and therefore most short to long-term maintenance/preservation methods are based on pot cultures. The lifestyle of these fungi is of major importance for their maintenance/preservation since, whatever the mode of cultivation (in vitro or in vivo), their propagules (spores, vesicles, colonized root pieces) need to survive the preservation process and be able to re-associate to a plant root. Compared to ECM fungi, the mycelium of AMF is often less abundant and they produce spores either inside and/or outside roots. Some genera also produce intraradical vesicles, structures involved in storage and reproduction (Smith and Read 2008). Spores, and to a lesser extent vesicles, are adequate for the preservation of AMF, while the mycelium alone is probably inappropriate. Several preservation methods, using propagules produced either in vivo or in vitro, have been developed and tested on AMF isolates.

In vivo maintenance of AMF

AMF are most often maintained on host plants in greenhouse or growth chamber conditions. Pot culture to produce spores of a single member of AMF was first described by Mosse (1959) and such in vivo cultures are currently used for research purposes, mass-propagation (see review by IJdo et al. 2011), or in culture collections (e.g., IBG, INVAM) for germplasm conservation and distribution. Different methods can be used to initiate in vivo cultures (see review by IJdo et al. 2011) starting from soil containing AMF, propagules (i.e., spores, colonized roots) isolated from field soil or from pot culture. These are mixed or layered in pots with sterilized substrate and using a suitable plant as host, newly produced propagules are obtained after several weeks or months according to the species. The propagules are then used as starter inoculum to initiate pure pot cultures (one

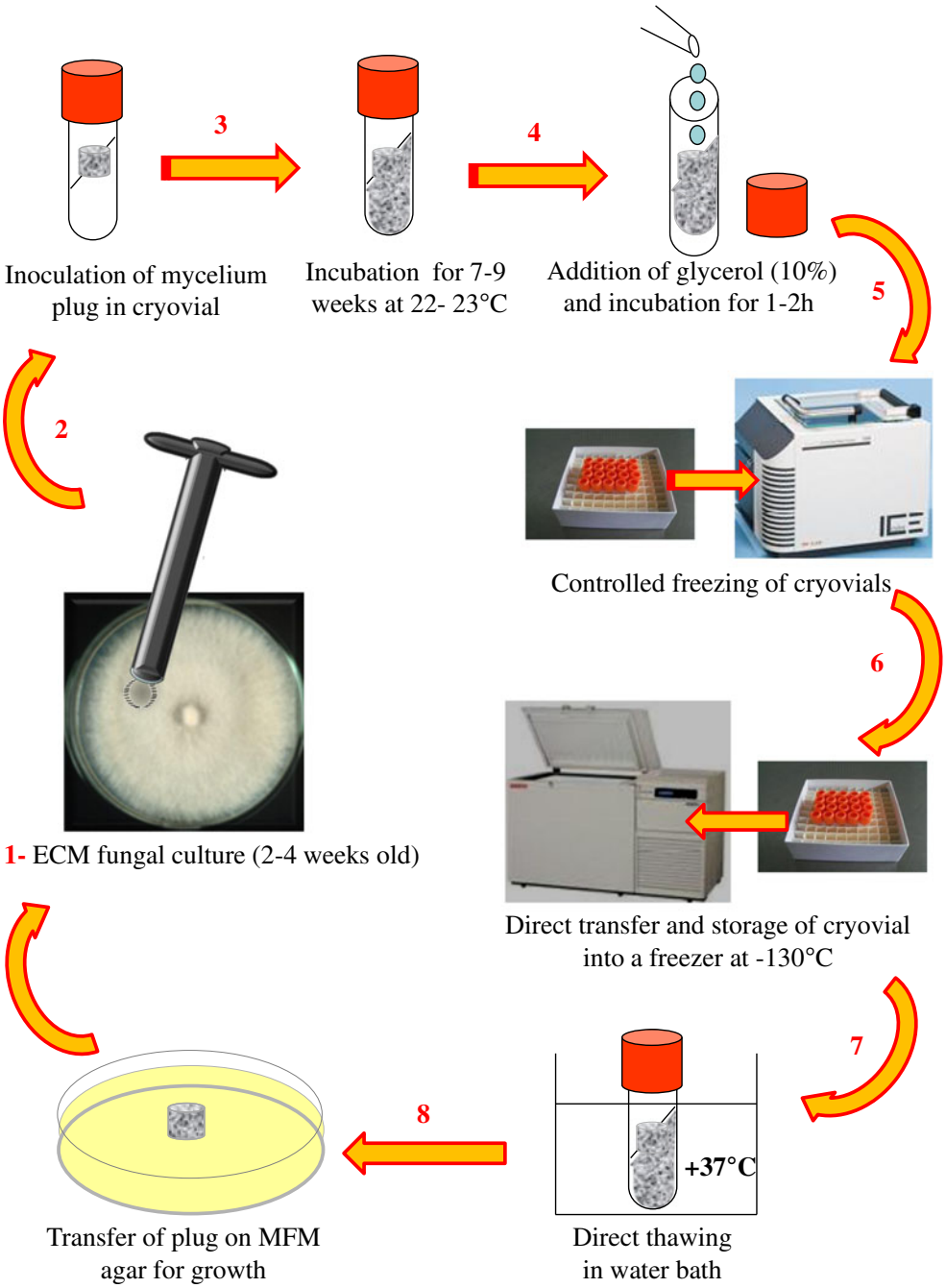

Fig. 1 Protocol for the cryopreservation of ECM fungi (from the method developed by Crahay et al. 2013a). (*1*) Isolates are grown on agar medium in Petri plates and incubated in the dark at 22–23 °C for 2 to 4 weeks. (*2*) A mycelium plug of ~4 mm diameter is sampled from the margin of the growing colony and inoculated into a 2-ml sterile polypropylene cryovial containing 750 μl of sterilized (121 °C for 15 min) MFM agar medium poured in a slope. (*3*) Cryovials are incubated at 22–23 °C in the dark for 7 to 9 weeks and (*4*) 500 μl sterilized (121 °C for 15 min) glycerol cryoprotectant solution (10 %*v*/*v*) is added into the cryovial for 1 to 2 h before cryopreservation. (*5*) Cultures are cryopreserved by controlled decreases in temperature (8 °C min^{-1} from +20 to +4 °C; 1 °C min^{-1} from +4 to −50 °C; 10 °C min^{-1} from −50 to −100 °C). (*6*) The cultures are directly transferred into a freezer at −130 °C. (*7*) For revival, the isolates are directly thawed in a water bath at +38 °C for 2 min. (*8*) Viability is checked by transferring plugs of cultures on 30 ml MFM in a Petri dish and incubated at 22–23 °C

AMF species) in adequate, preferably sterilized, substrate. For each mode of production, inoculum should be, as far as possible, free of contaminants and correctly identified. Leek (*Allium* spp.), Plantago (*Plantago lanceolata* L.), and Bahia grass (*Paspalum notatum Flugge*) are excellent host plants commonly used for the maintenance of a wide range of AMF isolates. International culture collections such as INVAM and IBG maintain their isolates on actively growing plants in pots. Sudangrass (*Sorghum sudanense* (Staph.) Piper) and sorghum (*Sorghum vulgare* L.) are routinely used for AMF maintenance at INVAM and isolates are sub-cultured after 6 to 18 months (Morton et al. 1993), while the host plants used at IBG are mainly leek (*Allium* spp.), clover (*Trifolium pratense* L.), parsley (*Petroselinum crispum* L.) and *Tephrosia* sp., a tropical woody legume, and isolates are sub-cultured every 1 to 2 years to minimize the risk of viability loss and contaminations (Redecker, personal communication, 2013; http://www.i-beg.eu/).

Maintaining AMF in pot cultures offers the advantage to be applicable to a large set of AMF species. This method remains therefore the easiest way to keep isolates, even though pot-cultures may occupy important surfaces and need constant

attention (e.g., watering, plant cleaning, AMF viability assessment). Importantly, the risk of contaminations by unwanted organisms (e.g., bacteria and fungi), loss of isolates due to inappropriate handling (e.g., risk associated with the presence of grazing organisms and plant diseases) is not negligible and thus necessitates the development of alternative methods to maintain AMF isolates viable, pure and stable over long periods.

In vitro maintenance of AMF

The cultivation of AMF in vitro on root organs is a method developed in the late fifties which is nowadays used in several laboratories for fundamental as well as applied research (Fortin et al. 2002; Declerck et al. 2005). This method consists in the association of AMF propagules (spores, colonized root fragments, isolated vesicles) with an excised root, transformed or not, on a synthetic growth medium and under sterile growth conditions (see for details Fortin et al. 2002; Declerck et al. 2005). Starting from the mid-nineties, the in vitro cultivation of AMF was extended to photosynthetically active plants (Elmeskaoui et al. 1995; Hernandez-Sebastia et al. 1999; Voets et al. 2005; Dupré de Boulois et al. 2006). Although this system was developed for fundamental research (e.g., transport studies—De Jaeger et al. 2011, gene expression analysis—Gallou et al. 2011), it has potential for the in vitro maintenance of AMF isolates.

In vitro cultivation of AMF is currently the most promising method to produce pure contaminant-free inoculum (see review of IJdo et al. 2011). Tens of isolates are maintained throughout the world, among which more than 40 in GINCO (see for details http://www.mbla.ucl.ac.be/ginco-bel). However, in vitro cultures are nowadays only successful for a limited number of species, and maintenance is done essentially via sub-cultivation, which is time and energy-consuming. The risk of contaminations during sub-cultivation is not excluded as well as the loss of infectivity after several successive sub-cultures (Plenchette et al. 1996). Thus, the development of alternative methods to maintain AMF isolates viable, pure and stable over long periods are necessary. Different short and long-term AMF preservation methods have been developed in the past and most have used in vivo produced AMF propagules (spores and vesicles).

Preservation by drying or cold storage

Using in vivo-produced spores from 6-month-old pot cultures, Tommerup and Kidby (1979) preserved four AMF species (i.e., *Glomus caledonium*, *Acaulospora laevis*, *Glomus monosporum*, and *Gigaspora* sp. by L-drying under vacuum (ESM Table 1); spores preserved in soil survived L-drying better than those extracted from soil before L-drying. Furthermore, slow dehydration of spores, by 5 months at 22 °C and then drying 21 days over silica gel, before L-drying increased their viability (ESM Table 1). Later, Tommerup (1988) reported preservation of in vivo-cultured *Acaulospora*, *Glomus Scutellospora* species for 8 years at 4 °C after L-drying under vacuum (ESM Table 1). This is consistent with the findings of Ferguson and Woodhead (1982) that AMF could be successfully stored in soil for at least 4 years at 5 °C following air-drying. Young (1982) reported that, even kept at room temperature, spores of *Glomus*, *Acaulospora*, *Gigaspora*, and *Sclerocystis* species in dried soil were able to germinate and colonize roots after 2 to 8 years of storage. At INVAM, most of the AMF isolates produced in pot cultures are stored in dried soil/substrate in a walk-in cold room at 4 °C for different time periods depending on the genera. Spores of *Gigaspora* species cannot be stored longer than 8 months because of their rapid degradation, those of *Scutellospora* species degrade less rapidly, and spores of *Acaulospora* and *Entrophospora* species are the most resistant to storage (see http://invam.wvu.edu/methods/inocula-storage/refrigeration-storage). For *Glomus* species, finally, dark spores (whether large or small) tend to lose viability faster than small pale or colourless spores. Kuszala et al. (2001) successfully preserved six in vivo-produced Glomalean species belonging to the genera *Glomus* and *Acaulospora* up to 20 months in osmosed water at 4 °C, as well as at room temperature, after drying propagules extracted from 6- to 8-month-old pot cultures at room temperature for 3–4 days (ESM Table 1). Kuszala and Gianinazzi-Pearson (2011) preserved spores, sporocarps and/or mycelium of isolates belonging to *Glomus*, *Acaulospora*, *Gigasopra*, *Scutellospora* extracted from 6- to 11-months-old pot cultures (IBG), in osmosed water at 4 °C, at ambient temperature, at +27 °C or at +37 °C (ESM Table 1).

Cold storage at 4 °C has also been applied to in vivo-produced AMF propagules encapsulated in alginate beads, prior to field application (Strullu and Plenchette 1991). However, propagule viability and infectivity decreased with long-term storage, for example intraradical vesicles/spores of *G. intraradices* encapsulated in alginate beads and stored at 4 °C had a reduced infectivity after 62 months of storage (Plenchette and Strullu 2003). Mugnier and Mosse (1987) found that storage of in vivo cultured sporocarps of *G. mosseae* under saturated salt solutions at 4 °C maintained viability for at least 4 years.

Lyophilization and cryopreservation

Few studies have focused on lyophilization as a method for storage of AMF. According to early work by Dalpe (1987), single-stage lyophilization can be effective for AMF isolates having spores with a thin wall such as *Glomus* species (e.g., *G. clarum*, *G. macrocarpum*, and *G. pustulatum*). Later, Kuszala et al. (2001) reported successful storage by lyophilization for in vivo cultures of 10 AMF isolates belonging to *Glomus* and *Acaulospora* for up to 28 months (ESM Table 1). Kuszala and Gianinazzi-Pearson (2011) successfully lyophilized dried

spores, sporocarps, mycelium, and/or mycorrhizal roots fragment (i.e., at room temperature up to 1 week) of isolates belonging to *Glomus* and *Acaulospora* genera extracted from 6- to 11-months-old pot culture (IBG) (ESM Table 1).

Tommerup and Bett (1985) were the first to successfully preserve AMF at ultra-low temperature. Pot cultures containing AMF were slowly cooled to −40 °C before freezing to −196 °C. Douds and Schenck (1990) also observed that slow drying of soil-based pot cultures containing AMF spores, followed by direct freezing at −60 to −70 °C, was effective for several AMF isolates belonging to *Glomus*, *Gigaspora*, *Entrophospora*, *Acaulospora*, and *Scutellospora*. However, storage was restricted to 3 months (ESM Table 1) and most of the *Scutellospora* or *Gigaspora* isolates and a *G. clarum* isolate subsequently had a very low rate of spore germination. These authors suggested that the water content of soil prior to freezing was a determinant parameter for the success of the method. Kuszala et al. (2001) tested the preservation by direct freezing at −18 or −80 °C of a large number of in vivo cultured AMF isolates belonging to 16 species and 4 genera (*Glomus*, *Acaulospora*, *Gigaspora* and *Scutellospora*) (ESM Table 1). Propagules were wet-sieved from 6- to 18-month-old pot cultures (IBG) and dried at room temperature for 3–4 days before cold treatment. All the isolates were infective and produced spores in pot cultures after 26 months at −18 °C, and some after several months at −80 °C (ESM Table 1). Direct storage at −18 and −80 °C was also successful for several months (Kuszala and Gianinazzi-Pearson 2011) for dried spores, sporocarp, mycelium and/or frangments of mycorrhizal roots (i.e., at room temperature up to 1 week) of different isolates of *Glomus* and *Acaulospora* from 6- to 11-months-old pot culture (IBG) (ESM Table 1).

Kuszala et al. (2001) further succeeded cryopreservation of AMF propagules by immersion in LN of sievings of *Glomus* and *Acaulospora* isolates (IBG) extracted from 6- to 18-month-old pot cultures and dried at room temperature for 3–4 days before storage (ESM Table 1). Kuszala and Gianinazzi-Pearson (2011) cryopreserved for up to 39 months in LN, dried spores (i.e., at room temperature up to 1 week) of *Glomus* and *Acaulospora* isolates extracted from from 6- to 11-months-old pot culture (IBG) (ESM Table 1).

Lalaymia et al. (2013b) confirmed and extended this work on the cryopreservation of AMF belonging to *Claroideoglomus*, *Septoglomus* and *Paraglomus* (ESM Table 1) which were isolated from at least 5 month-old soil-based pot cultures and cryopreserved one month at −130 °C following encapsulation-drying as developed by Lalaymia et al. (2012) (see the detailed protocol below for in vitro produced isolates and Fig. 2). After 1-month storage at −130 °C, isolates were able to colonize plant roots in pots and produce new spores (Lalaymia et al. 2013b).

For in vitro cultures of AMF, Addy et al. (1998) were the first to demonstrate the survival of extraradical hyphae/spores of *G. intraradices* at temperatures below 0 °C (i.e., at −12 °C) when slowly cooled before freezing (ESM Table 1). Declerck and Van Coppenolle (2000) developed a method for the cryopreservation at −100 °C of in vitro-produced spores of the AMF *Rhizophagus* sp. MUCL 41835. This was based on the encapsulation of spores in alginate beads followed by their incubation in trehalose (0.5 M) before cryopreservation at −100 °C in a two-step temperature decrease (slow (1 °C min^{-1}) from +20 to −35 °C and rapid (18 °C min^{-1}) from −35 to −100 °C). Spores remained viable after 3 h of cryopreservation at −100 °C and were able to reproduce the fungal life cycle in vitro. However, storage was not tested for longer periods, and spore germination decreased or was not observed when storage temperature was decreased to −140 or −180 °C. Based on some factors selected by Declerck and van Coppenolle (2000) (encapsulation in alginate beads, trehalose as cryoprotectant, rapid thawing after cryopreservation), Lalaymia et al. (2012, 2013b) improved and extended the method to 19 isolates produced in vitro belonging to the genera *Rhizophagus*, *Glomus*, *Claroideoglomus*, and *Gigaspora*. By drying the beads containing propagules before cryopreservation and applying a faster cooling rate, as compared to Declerck and van Coppenolle (2000), the AMF isolates were preserved at −130 °C for up to 6 months (ESM Table 1). This method comprises five steps (Fig. 2): (1) encapsulation of propagules (spores, mycorrhizal root pieces from 5-month-old in vitro cultures) in alginate beads, (2) incubation overnight in 0.5 M trehalose, (3) drying of the beads during 48 h at 27 °C to 8.1±4.6 % of bead water content, (4) cryopreservation at −130 °C following a two-step decrease in temperature: a fast decrease (~12 °C min^{-1}) from room temperature (+20 °C) to −110 °C followed by a slow decrease in temperature (~1 °C min^{-1}) from −110 to −130 °C, and (5) direct thawing in a water bath (+35 °C). According to Lalaymia et al. (2012, 2013b), this cooling rate permitted better survival after cryopreservation than preservation in LN or following the cooling procedure used by Declerck and van Coppenolle (2000). After 1- to 6-months storage at −130 °C, isolates maintained their capacity to form mycorrhiza in vitro and produce new spores. The percentage of root colonization, number of spores produced and architecture of the extraradical hyphae (e.g., hyphal length, presence/number of branched absorbing structures, number of anastomosis) did not differ between the cryopreserved and non-cryopreserved isolates (Lalaymia et al. 2013a). Similarly, no differences were noted in alkaline and acid phosphatase activities in the extraradical hyphae. Finally, the genetic stability (assessed on 3 *Rhizophagus* isolates), estimated by AFLP, did not differ between the cryopreserved and non-cryopreserved isolates (Lalaymia et al. 2013a).

Discussion and conclusions

Significant progress has been made over the last few years in the ex-planta maintenance/preservation of mycorrhizal fungi.

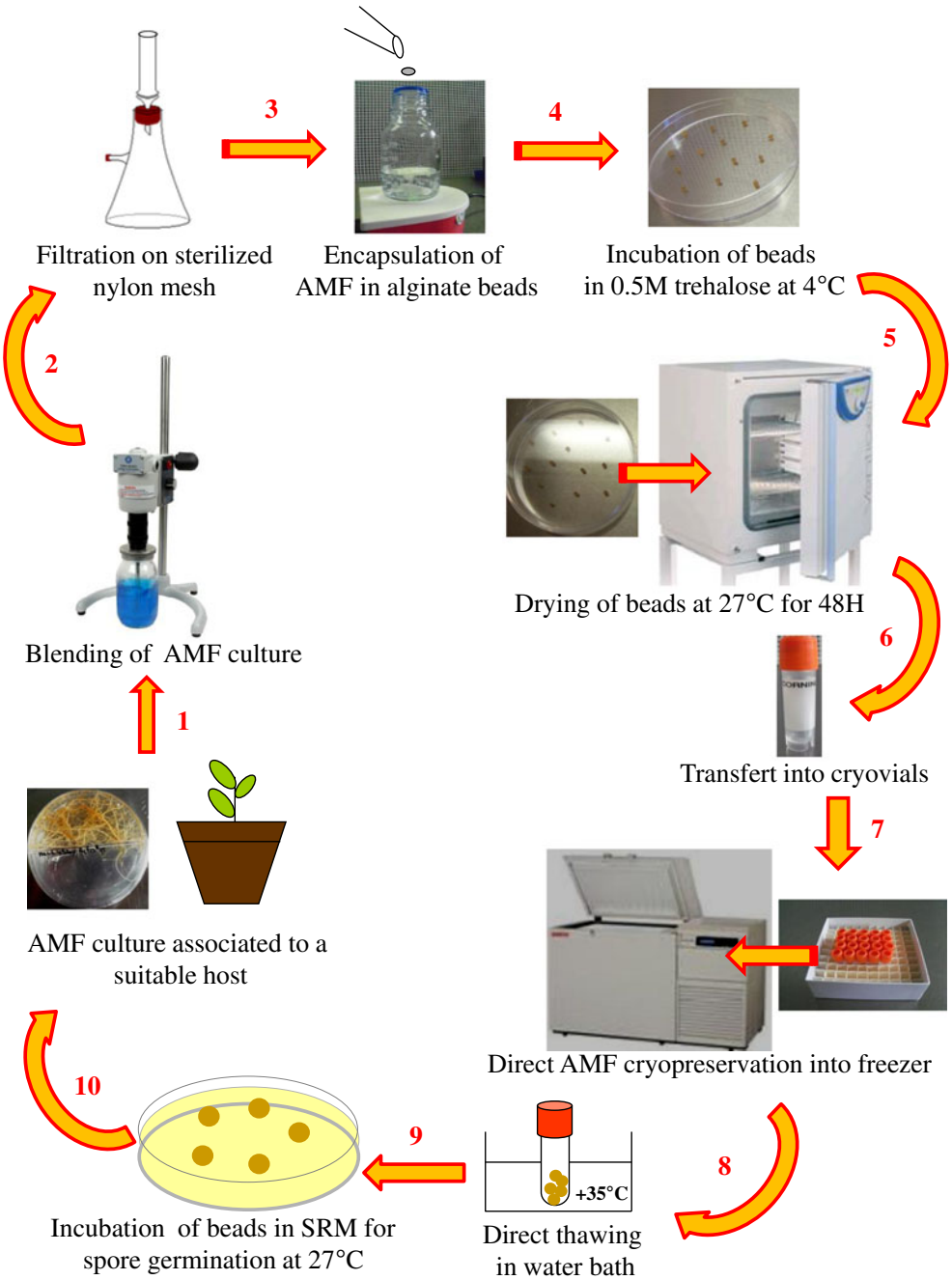

Fig. 2 Protocol for the cryopreservation of AMF (from the method developed by Lalaymia et al. 2012). (*1*) Gelling medium, containing spores and roots of a 5-month old in vitro culture, is extracted from the Petri dishes, poured into 100 ml of sterilized (121 °C for 15 min) deionized water and subsequently blended twice for 30 s at 20,000 rpm in a sterilized (121 C for 15 min) mixer. (*2*) The mixture is filtered on a sterilized (121 °C for 15 min) nylon filter (40 mm). (*3*) The supernatant (spores and mycorrhizal/non-mycorrhizal root pieces) is encapsulated in 2 % (*w*/*v*) solution of sodium alginate (50±5 propagules per bead). (*4*) The encapsulated propagules are incubated overnight in 0.5 M trehalose and (*5*) dried at 27 °C for 48 h (bead water content of approximately 8.1±4.6 %). (*6*) Beads are transferred into 2-ml cryovials. (*7*) The cryovials are cryopreserved in a freezer at −130 °C following a two-step decrease in temperature: a fast decrease (~12 °C min^{-1}) from room temperature (+20 °C) to −110 °C followed by a slow decrease in temperature (~1 °C min^{-1}) from −110 to −130 °C. (*8*) For revival, the encapsulated AMF propagules are directly plunged in a water bath at +35 °C. (*9*) The beads are dropped into sterilized (121 °C for 15 min) MSR medium, cooled in a water bath to 40 °C, and incubated at 27 °C for germination. (*10*) After 4-weeks incubation, beads containing germinated propagules are associated with an excised root under in vitro culture conditions to reinitiate the fungal life cycle. Lalaymia et al. (2013b) adapted as follows this cryopreservation protocol to in vivo produced propagules: Pot cultures, at least 5 months old, are sampled. Spores are collected by wet sieving and decanting, while roots are collected with forceps and blended in a mixer in 100 ml deionized water for 30 s at 20,000 rpm, and filtered as above. The spores and the supernatant of the blended roots are mixed together and encapsulated in alginate beads, dried, cryopreserved, and thawed as described above. After thawing, the encapsulated propagules are placed directly in contact with roots of plants in pots containing a sterilized (2×15min at 121 °C, with 12-h interval) substrate. The plants are grown for at least 8 weeks in a growth chamber before assessing the fungal viability following cryopreservation

Some of the methods developed with pot- and in vitro-cultured isolates are adequate for short-term storage using standard infrastructures and equipment (e.g., sub-cultivation, preservation in alginate beads or in dried soil), while others can be applied to long-term storage (i.e., cryopreservation or lyophilization) but require specific equipment and infrastructures. Short and long-term methods for maintenance/preservation of ECM fungi and AMF are summarized below and factors essential for their storage over long periods are emphasized. Finally, a protocol applicable, although not generalizable, to a large panel of these organisms is proposed.

Ectomycorrhizal fungi

For ECM fungi, sub-cultivation at room temperature or preferably at cold temperatures (i.e., ~4 °C), or under conditions that slow down fungal growth (e.g., under water and oil), is adequate, easy-to-apply in the laboratory and enables maintenance of some ECM fungi for periods up to 20 years (Richter 2008). However, this approach presents disadvantages of being time-consuming, prone to contamination and risks of genetic instability over time. Thus, methods such as lyophilization and cryopreservation that arrest fungal metabolism are more appropriate for the long-term preservation of ECM fungi, and preferable (infrastructure and equipment permitting) if the objective is to keep the organisms under stable conditions for long periods, as in the case of culture collections.

From the various lyophilization/cryopreservation protocols detailed above, six parameters appear paramount for the long-term preservation of ECM fungi:

1. *The preconditioning of the culture prior to preservation*. Growing the fungus on a carrier (e.g., perlite—Homolka et al. (2006), charcoal filter paper strips—Stielow et al. (2012)) or directly in cryovials (Crahay et al. 2013a) on a growth medium (e.g., MFM) submerged with a suitable cryoprotectant (most often glycerol) prior to preservation, and eventually at cold temperature (close to 4 °C), greatly enhances the survival of ECM fungi. Growing the fungi on a carrier or in a cryovial avoids excessive manipulations of the organism that may damage the colony/hyphae before preservation (see below, the next parameter). Flooding the culture with a cryoprotectant (generally 1–2 h before cryopreservation) or growing the culture on a medium supplemented with a cryoprotectant (Homolka et al. 2006) introduces protective compounds into the fungal cytoplasm and further increases survival of the organism during cryopreservation (Smith and Onions 1994; Tan et al. 1991). Finally, pre-exposure of the fungal culture to cold temperatures (generally 4 °C) and/or heat shock can enhance resistance to freezing by inducing the accumulation of trehalose/polyols and the production of heat shock proteins (HSPs) that protect cells from preservation stresses and damage (De Virgilio et al. 1990; Neves et al. 1991; Neves and Francois 1992; Tan and van Ingen 2004; Tereshina 2005).
2. *The integrity of the hyphae prior to preservation.* It is essential that the fungal culture is not physically damaged prior to cryopreservation in order to minimize leakage of cytoplasmic contents (Houseknecht et al. 2012) and to avoid the propagation of ice formation inside the hyphae (Smith and Onions 1994; Smith 1998). Growing ECM fungi on a carrier or in cryovials, as described above, avoids unnecessary manipulations of the organisms and as such prevents injury to the mycelium.
3. *The age of the culture*. Fungal cultures in late or stationary growth phases are most often better adapted to survive freezing or freeze-drying than young cultures (Smith and Onions 1994; Smith 1998; Sundari and Adholeya 1999; Tan and van Ingen 2004; Houseknecht et al. 2012). It is suggested that in the late-growth phase, the fungal cells are stressed and thus produce and accumulate compounds, such as trehalose (Van Laere 1989), polyols, and polysaccharides (Fuller et al. 2004) which are involved in the protection of membranes and proteins from denaturation (Fuller et al. 2004; Tan and van Ingen 2004).
4. *The cryoprotectant.* Although this is not a generality, glycerol seems the most reliable (and therefore the most used) cryoprotectant for ECM fungi, as for the majority of filamentous fungi (Smith 1998). Glycerol penetrates and protects hyphal contents during cooling, and prevents the cells from excessive dehydration.
5. *The cooling rate.* Slow cooling of cultures (~1 °C min^{-1}) seems most appropriate for the majority of ECM fungi. Mycelium, which is the predominant form of ECM fungal colonies on synthetic medium, is rich in water. Therefore it is essential to dehydrate, at least partially, the hyphae to avoid injury due to water crystallization. During slow cooling, the formation of ice is gradually initiated outside the hyphae, resulting in the gradual increase of the cryoprotectant outside the hyphae and thus, by osmotic effect, the dehydration of hyphae by water leakage (Mazur 2004).
6. *The thawing of ECM fungi.* Fast thawing by direct immersion in a water bath (at a temperature between 35 and 37 °C) is often reported to prevent the risk of ice recrystallization occurring during slow thawing.

In conclusion, it can be recommended for most ECM fungi to grow the organisms on membranes or directly in cryovials until the late stationary phase for pre-conditioning and to avoid injuries to the hyphae due to excessive manipulations. Glycerol (often at 10 % concentration) applied 1–2 h before cryopreservation should be used as cryoprotectant. The decrease in temperature should be slow (1 °C min^{-1}) until storage (ideally at temperatures below −130 °C), while thawing should be fast by direct plunging in a water bath at 35–37 °C.

Arbuscular mycorrhizal fungi

The maintenance/preservation via sub-cultivation is the most widely used method for AMF, whether in vitro on synthetic growth medium in association with transformed roots or in vivo in association with plants in the greenhouse. Similarly to ECM fungi, sub-cultivation is time-consuming, presents a risk of contaminations and does not guarantee genetic stability over long periods. For the long-term storage it is thus recommended to freeze-dry or cryopreserve the AMF at ultra-low temperatures (i.e., ideally below −130 °C).

From the lyophilization/cryopreservation protocols reviewed above, six factors appear to be particularly important for the long-term preservation of AMF:

1. *The drying of propagules.* Drying of soil containing AMF propagules or drying of propagules isolated from either in vivo or in vitro cultures and included or not in a carrier, before long-term preservation, is a key factor for the lyophilization /cryopreservation of these organisms (Douds and Schenck 1990; Lalaymia et al. 2012, 2013b). For isolates produced in pots, drying of soil containing AMF propagules is generally achieved at room temperature, during 2–4 days before preservation, depending on the soil moisture. For isolates produced in vitro, the drying of the carrier (alginate beads containing AMF propagules) is crucial and should be preceded by incubation in a cryoprotectant (ideally trehalose) at 27 °C for 2 days. (Lalaymia et al. 2012, 2013b). Drying may reduce ice crystallization during freezing and induce (if applied as a pre-conditioning step several days before preservation) fungal stress and, thus, the endocellular production of natural protectants such as trehalose (see explanation above—Tan et al. 1991; Smith and Onions 1994; Tan and Van Ingen 2004; Ocon et al. 2007), which could help to reduce ice crystal size or to convert the fungal cytoplasm into a glassy state during rapid freezing, thus improving the chances of survival (Tan and van Ingen 2004).
2. *The culture age.* Whatever their mode of production (in vitro or in vivo), AMF propagules isolated from cultures in the stationary phase of growth are the most suitable for long-term preservation. Propagules in this phase of growth are more resistant to cryopreservation, as shown recently by Lalaymia et al. (2012) with in vitro produced isolates. Fungal colonies at the end of the exponential and stationary phase are more resistant to freezing and thawing than cultures in the early growing stages (Smith and Onions 1994; Smith 1998; Tan and van Ingen 2004). At the late-growth phase (frequently associated with stress conditions), fungal cells accumulate compounds like trehalose (Van Laere 1989), polysaccharides and glycoproteins to protect the intra and extracellular mycelium integrity.
3. *The carrier.* AMF propagules cultured in vivo are usually conserved in the soil substrate in which they are produced. Tommerup and Kidby (1979) reported that spores preserved in soil survive L-drying better than spores isolated from soil. Encapsulation in alginate beads has been reported as mandatory for the cryopreservation of in vitro-cultured AMF (Declerck and Van Coppenolle 2000; Lalaymia et al. 2012). The preservation of the propagules within a carrier may further facilitate their handling, by protecting them against the potentially toxic or osmotic effects of the cryoprotectant during treatment and from mechanical and oxidative stress during cryopreservation (Suzuki et al. 2005; Sakai and Engelmann 2007).
4. *The cryoprotectant.* Most long-term preservation methods applied so far to AMF have been achieved without cryoprotectant and with propagules produced in vivo (pot-cultured organisms). Interestingly, Declerck and Van Coppenolle (2000) and Lalaymia et al. (2012) reported that the use of cryoprotectants (trehalose) with in vitro-produced cultures and pot-cultured isolates is a key factor for the success of cryopreservation. Trehalose has the capacity to interact with cell membrane phospholipids to maintain their fluidity during freezing and desiccation (Crowe et al. 2001).
5. *The cooling rate.* Contrary to ECM fungi, fast cooling seems to be preferable for AMF. Cryopreservation of different species produced in vivo by directly placing propagules in LN (Kuszala et al. (2001); Kuszala and Gianinazzi-Pearson (2011)) or direct cooling of in vitro produced AMF in a freezer (Lalaymia et al. 2012) have both been found to be effective for the preservation of isolates. This could be related to the fact that AMF propagules (spores and/or vesicles) are less rich in water as compared to hyphae. Slow cooling of propagules, encapsulated or in soil containing, may result in excessive drying leading to propagule shrinkage and subsequent death.
6. *The thawing rate and temperature.* Whatever the cooling rate (slow or fast), fast thawing is generally the preferred to prevent the risk of ice recrystallization after cryopreservation (Mazur 1984, 2004). Predried, cryopreserved AMF propagules from either in vitro or in vivo cultures have been successfully revived after fast thawing in a water bath at +35 °C (Declerck and Van Coppenolle 2000; Lalaymia et al. 2012, 2013b). Reviving of cryopreserved in vivo cultured propagules also seems to be successful by slow thawing at room temperature (Kuszala et al. 2001). This could be explained by the fact that no or very few ice crystals are formed during fast cooling of dried propagules, thus, even if the thawing is slow, the recrystallization process do not occur or occurs only weakly.

In conclusion, it can be recommended for AMF (whether cultured in vivo or in vitro) that propagules from cultures in the

late or stationary phase of growth be used to avoid lyophilization/cryopreservation stresses. Also, lyophilization/cryopreservation of AMF propagules should be conducted in a carrier (e.g., soil or alginate beads) to protect them from preservation stresses and facilitate their manipulation, and the carrier should be dried before lyophilization/cryopreservation to prevent water crystallization outside and inside the propagules. Trehalose (0.5 M) can be used as cryoprotectant for in vitro-cultured AMF propagules, while in vivo-cultured propagules can be preserved in dried soil without cryoprotectant. Cooling to low temperatures should be fast, either by direct immersion in LN (at −196 °C) or in the freezer to temperatures below −130 °C, and subsequent fast thawing is recommended by direct immersion in a water bath at 35 °C to prevent ice recrystallization. Monitoring fungal integrity after preservation.

Stability following preservation

It is essential to assess the biological stability of ECM fungi and AMF following preservation, since long-term preservation at ultra-low temperature or following freeze-drying may result in morphological, physiological, and genetic modifications in microorganisms (Smith and Ryan 2012a, b). The effects of freezing on the integrity of fungi can be checked instantly using cryogenic light microscopy (Smith and Thomas 1998); however, growth rate, culture morphology, metabolic activity, and genetic stability are the parameters most frequently used for fungi (Smith and Ryan 2012a, b). For ECM fungi and AMF, morphological characteristics of spores or conidia (e.g., form and color) and hyphae can be checked by microscopy, and culture growth rate, number and abundance of spores or conidia can be quantified. Architecture/ramification of hyphae and culture pigmentation can also be assessed (Voyron et al. 2009; Lalaymia et al. 2012, 2013a), as well as fungal ability to establish symbiosis with a host plant (Lalaymia et al. 2013a, b; Crahay et al. 2013b). Enzymatic activities detected before and after preservation are another indication of fungal integrity (Lalaymia et al. 2013a) and these may be extended using APIZYM, a system based on the simultaneous detection of different enzymatic activities applied to other fungi (Ryan et al. 2001; Smith et al. 2001; Smith and Ryan 2012a, b). Likewise, detection of secondary metabolite production using HPLC used to compare viability of other organisms before and after cryopreservation (Ryan et al. 2001) may be applicable to ECM fungi and AMF.

Even if fungi are viable after preservation and exhibit the same morphology and physiological characteristics as before preservation, their genetic stability could be affected (Ryan et al. 2001). The possibility of damage to DNA and induction of mutations upon cryopreservation of fungal structures has been addressed in several studies (Ryan et al. 2001; Voyron et al. 2009). The use of PCR primers specific to particular regions of the genome (e.g., ITS and IGS) was for a long time the most widely used technique to assess genetic stability after long-term preservation. This technique may produce reproducible data but, because of its specific nature, a large part of the genome is not analyzed. Alternatively, randomly amplified polymorphic DNA and amplified fragment length polymorphism analysis, which reproducibly detect a large number of polymorphic loci, can be used and have provided evidence for stability after cryopreservation in fungi belonging to different phylum, including mycorrhizal fungi (Voyron et al. 2009; Camelini et al. 2012; Lalaymia et al. 2013a).

Future directions

For many ECM fungi and AMF, the methods for short-term maintenance (e.g., sub-cultivation in soil or in vitro, storage under water or oil) are adequate to be applied routinely in research laboratories. Conversely, many mycorrhizal fungi remain recalcitrant to long-term preservation, notwithstanding the extended list of mycorrhizal fungi reported in ESM Table 1. The research for straightforward conservation methods is most often empirical but remains essential to increase the number of mycorrhizal fungi kept under stable conditions for long-term periods. From a methodological point of view, several easy-to-apply pre-conditioning methods can be assayed. For instance, it is advisable to grow fungi on nutrient-poor medium before freezing or freeze-drying in order to stimulate their entry into a stationary growth phase and the subsequent production of natural protectants such as trehalose and glycerol (Smith and Onions 1994). It is also conceivable to test a light treatment to stimulate the fungal growth before freezing and freeze-drying. In this context, Tan and Van Ingen (2004) reported that exposure to light enhances sporulation of some fungi with the production of large quantities of disaccharides and polyols in spores and conidia, which could enhance the resistance of the fungi to low temperature storage. In addition, light cryomicroscopy and differential scanning calorimetry could be useful monitoring methods when trying to improve or adjust the lyophilization/cryopreservation procedures. They can help to visualize and measure physical changes in water (freezing, melting, glass transition) during cooling and warming and thus evaluate effects on the mycorrhizal fungi.

Regarding so far uncultivable ECM fungi and AMF and for those with insufficient propagule production in vivo or in vitro, lyophilization/cryopreservation in association with a suitable host plant could be an alternative. The lyophilization/cryopreservation of dried fragments of mycorrhizal roots or their cryopreservation following encapsulation in alginate beads could offer good and practical methods for mycorrhiza preservation. The cryopreservation of root sections surrounded by an ECM fungal mantle or sections of AMF mycorrhizal

roots may be an elegant approach to reinitiate the fungal life cycle, especially for AMF. Cryopreservation of plant roots has been achieved for several plants species (Dereuddre et al. 1991; Sakai 2004) and opens the door to the cryopreservation of mycorrhizal roots. It is also conceivable that the cryopreservation methods cited above may be directly applied to field samples of uncultivable or recalcitrant fungi. For example, direct extraction and encapsulation in beads of field-collected AMF spores may safeguard their diversity until adequate methods are developed for their cultivation in vitro or in vivo.

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