Université catholique de Louvain

Faculté d'ingénierie biologique, agronomique et environnementale

Earth and Life Institute

Pole of Applied Microbiology (ELIM)

Laboratory of Mycology

Long-term preservation of Arbuscular mycorrhizal fungi

Thèse de doctorat présentée en vue de l'obtention du grade de Docteur en Sciences agronomiques et ingénierie biologique

Ismahen Lalaymia

Promoteurs:

Prof. Stéphane Declerck (UCL, Belgique)

Dr. Sylvie Cranenbrouck (UCL, Belgique)

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Louvain-La-Neuve, 2013

Acknowledgements

First and foremost, I would like to express my deep gratitude to my promoter, Professor Stéphane Declerck, for the opportunity he gave me to accomplish this PhD. Thank you for guidance, enthusiastic supervision, your confidence in me and the useful critiques of this research work.

I am grateful to Dr. Sylvie Cranenbrouck. Thank you Sylvie for your continuous encouragements and for the numerous stimulating discussions. Without your knowledge and help this study would not have been successful.

I am thankful to the European Community for financing of the VALORAM project and for providing the financial means and laboratory facilities to complete this project. Thanks are also addressed to the people involved in the VALORAM project.

To all of the colleagues who have been involved in this project and to the coauthors of the publications and manuscripts, I wish to express my sincere gratitude.

Special thanks are addressed to Céline Bivort and Stéphanie Huret for their advice for the molecular work.

Thanks to everyone in the CESAMM team, for your support, your friendships and the good time spend in and out of the laboratory. I would also like to extend my thanks to technicians and everyone in the Laboratory of mycology.

I am very grateful to all people that were constantly around me during these last four years. I also thank my friends (too many to list here,

but you know who you are!) for providing support and friendship that I needed.

Last, but not the least, I wish to express my love and gratitude to my beloved mom, dad, brothers, sister and my husband, who had always faith in me and my intellect even when I didn't have faith in myself. My hard-working parents have sacrificed their lives for my brothers, sister and me. Mom, dad, you provided me your encouragement, unconditional support; both financially and emotionally, and endless love and care through the duration of my life… I love you so much.

To my family and my husband

Thank you…

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List of abbreviations

ACP ACid Phosphatase

AFLP Amplified Fragment Length Polymorphism

ALP ALkaline Phosphatase

AMF Arbuscular Mycorrhizal Fungi/Fungus

ATCC American Type Culture Collection

ANOVA ANalysis Of VAriance

BAS Branched Absorbing Structure

C Carbon

Ca Calcium

CCFC Canadian Collection of Fungal Cultures

CFS Charcoal Filter paper Strips

CMCC Centre for Mycorrhizal Culture Collection

DC2 *Daucus carota* root clone no. 2

DMSO DiMethyl SulfOxide

DNA Deoxyribose Nucleic Acid

DSC Differential Scanning Calorimetry

ECM fungi ECtoMycorrhizal fungi

ERM Extra-Radical Mycelium

List of abbreviations

GINCO Glomeromycota *IN vitro* COllection

HPLC High Performance Liquid Chromatography

HSD Honest Significant Difference

HSPs Heat Shock Proteins

IBG International Bank of Glomeromycota

INVAM INternational culture collection of (Vesicular) Arbuscular Mycorrhizal fungi

IRM Intra-Radical Mycelium

LN Liquid Nitrogen

MSR medium Modified Strullu-Romand medium

MUCL Mycothèque de l'Université Catholique de Louvain

N Nitrogen

Na Sodium

OECD Organization for Economic Co-operation and Development

P Phosphorus

PCR Polymerase Chain Reaction

PIB% Percentage of Potentially Infective Beads

PVLG Poly-Vinyl alcohol Lactic acid Glycerin

PVS Plant Vitrification Solution

RAPD Randomly Amplified Polymorphic DNA

RC Root Compartment

List of abbreviations

RNA Ribose Nucleic Acid

RFU Relative Fluorescent Units

ROC Root Organ Culture

ROS Reactive Oxygen Species

Tg Glass transition Temperature

UPGMA Unweighted Pair Group Method of Arithmetic averages

WFCC World Federation for Culture Collections

Glossary

Branched absorbing structures: *Small groups of dichotomous hyphae formed by the extraradical mycelium of arbuscular mycorrhizal fungi involved in the uptake of nutrients from the environment* (Bago et al. 1998).

Anastomosis: *The fusion of two fungal hyphae, allowing the cytoplasm and possibly the nuclei of two individuals to mix* (Sanders and Croll, 2010).

Appressorium: *A flattened hyphal organ that facilitates the penetration of cells or tissues of other organisms* (Parniske, 2008).

Arbuscular mycorrhizas: *Widespread type of endomycorrhizal interactions involving fungi of the phylum Glomeromycota, the hyphae of which reach the root inner cortex and develop highly branched exchange structures called arbuscules* (Bonfante and Genre, 2010).

Arbuscule: *Highly branched structure produced by arbuscular mycorrhizal fungi inside the cell lumen of their host. Arbuscules are considered to be the key element of the symbiotic nutrients exchanges between the plant and the fungus* (Bonfante and Genre, 2010).

Auxiliary cells: *Clustered swellings on external hyphae. These are often ornamented by spines or knobs and are characteristic of Scutellospora and Gigaspora* (http://mycorrhizas.info/vam.html#stages).

Cooling rate: *The known or estimated change in temperature over a set time* (Shaw and Jones, 2003).

Glossary

Cryoinjury: *Injury caused by cryopreservation leading to death or cells lysis* (Shaw and Jones, 2003).

Cryopreservation: *Ultra-low temperature storage (usually in liquid nitrogen at ca. -135 to -196°C) of living cells, tissues and organs capable for resuming normal function after revival* (Day et al. 2008).

Crystallization: *Transition of water molecules from a liquid state into ice* (Shaw and Jones, 2003).

Dehydration: *Reducing the water content of a cell to reduce the likelihood of damaging intracellular ice formation when the cell is cooled* (Shaw and Jones, 2003).

Differential scanning calorimeter: *Apparatus used to measure small changes in temperature (as calories). Sensitive enough to measure the release of heat during ice crystallization and the uptake of heat during melting* (Shaw and Jones, 2003).

Ectomycorrhiza: *Symbiosis between higher plants and fungi belonging to Asco- and Basidiomycetes, in which fungal hyphae surround the root tips and develop between epidermal cells but never enter the cell lumen* (Bonfante and Genre, 2010).

Endomycorrhiza: *Group of mycorrhizal symbioses involving fungal penetration inside living cells of the root epidermis and cortex* (Bonfante and Genre, 2010).

Extraradical mycelium: *Hyphal network that develops in the rhizosphere, in which it absorbs inorganic nutrients that are transferred to the host plant through intraradical hyphae* (Bonfante and Genre, 2010).

Glossary

Freezing: *The formation of ice crystals* (Shaw and Jones, 2003).

Freezing point: *The temperature at which water, ice and vapour can coexist at atmospheric pressure. Ice can nucleate or be seeded at this temperature, but forms more easily at temperatures < 0°C* (Shaw and Jones, 2003).

Glass transition temperature: *The temperature at which vitrifying solutions change to from the solid, stable glass-like state* (Shaw and Jones, 2003).

Heterogeneous nucleation: *The formation of ice nuclei triggered by surfaces or impurities* (Shaw and Jones, 2003).

Homogeneous nucleation: *The spontaneous formation of ice nuclei not triggered by surfaces or impurities* (Shaw and Jones, 2003).

Intraradical hyphae: *Network of hyphae from mycorrhizal fungi that colonizes the host root tissues* (Bonfante and Genre, 2010).

Lyopholization: *Is the removal of water by freezing and volatilization and drying at low pressure and temperature under vacuum* (Tan et al. 2007).

Mycorrhizas: *Plant-fungal symbioses that are typically mutualistic, obligate and based on an exchange of photosynthates for soil minerals* (Bidartondo, 2005).

Nucleation: *The first event in ice crystal formation. At the molecular level, individual water molecules join. The change from a liquid to a crystalline state is associated with a loss of energy. Nucleation is a stochastic process* (Shaw and Jones, 2003).

Glossary

Propagule: *Any structure that is capable of giving rise to a new mycelium by asexual or sexual reproduction* (modified from Zaid et al. 2001).

Recrystallization: *A complex process, usually associated with warming, in which individual ice crystals within a solution change size. Small crystals commonly shrink while large crystals become larger* (Shaw and Jones, 2003).

Supercooling: *The phenomenon at which aqueous solutions remain in liquid states when cooled below the freezing point* (Zachariassen and Kristiansen, 2000).

Thawing: *Melting of ice crystals* (Shaw and Jones, 2003).

Vesicles: *Intercalary (-o-) or terminal (-o) hyphal swellings formed on internal hyphae within the root cortex. These may form within or between cells. Vesicles accumulate lipids and may develop thick wall layers in older roots. They are storage organs which may also function as propagules* (http://mycorrhizas.info/vam.html#stages).

Vitrification: *Solidification of liquid solution to amorphous glass, by extreme elevation of viscosity during ultrarapid cooling by the addition of vitrificant solution of high cryoprotectant concentrations* (Sakai and Engelman, 2007).

Vitrifcation solution: *Solutions which, on cooling, can form a solid, non-crystalline, amorphous glass phase* (slightly modified from Shaw and Jones, 2003).

Summary

Arbuscular mycorrhizal fungi (AMF) are obligate root symbionts, forming associations with most existing terrestrial plants. The plants obtain inorganic nutrients (e.g. N, P) via their fungal partners in exchange of which they provide the fungi with carbon compounds. AMF improve plant growth, health and productivity and as such, represent key organisms in agro-ecosystems.

Currently, AMF diversity is maintained via continuous culture; *in vivo* on trap plants under greenhouse facilities, and *in vitro* in association with excised, transformed or non-transformed roots. However, these methods are time and work-consuming and the risks of contaminations and loss of genetic stability are not excluded.

The objective of this thesis was to develop a long-term preservation method adapted to different AMF species and genera that guarantee their viability and stability over unlimited time of storage.

In a first step, we tested different long-term preservation protocol (e.g. lyophilization, vitrification, cryopreservation) on *Rhizophagus sp.* MUCL 43204 produced *in vitro.* Cryopreservation appeared the most reliable method and was used in a second step on a large number of AMF isolates cultured *in vitro.* After 6 months storage at -130 °C, the viability of AMF isolates and their ability to colonize plant roots and reproduce the life cycle were tested. In a third step, we evaluated the morphology, physiological activity and genetic stability of 6 months-cryopreserved AMF

isolates belonging to the genus *Rhizophagus.* Finally we applied the cryopreservation protocol developed on a large array of AMF isolates cultured either *in vitro* or *in vivo.*

Our results demonstrated that the cryopreservation by encapsulation-drying and storage at -130°C of AMF propagules was effective, at least for 6 months, for different *Rhizophagus* isolates cultured *in vitro.* The method comprised five steps (1) the encapsulation in alginate beads of AMF propagules (i.e. spores and mycorrhizal root pieces) isolated from 5 months old cultures, (2) the incubation overnight in trehalose (0.5M), (3) the drying at 27°C for 2 days (i.e. at 8.1 ± 4.6% of beads water content), (4) the cryopreservation at -130 C in a freezer following a 2 steps 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°C to -130°C and (5) the direct thawing in a water bath set at +35°C. The morphology, physiological activity and genetic stability evaluated after 6 months storage at -130°C were similar to the non-cryopreserved controls. This method was also successfully applied to isolates belonging to *Glomus*, *Claroideoglomus*, *Septoglomus*, *Paraglomus* and *Gigaspora* cultured either *in vitro* or *in vivo.*

Ours findings highlights the possibility to use the encapsulation-drying method for the cryopreservation and storage at -130°C of AMF isolates produced either *in vitro* or *in vivo.* These results further open the door to isolates that remain recalcitrant to different forms of long-term preservation at ultra-low temperature.

Introduction

Introduction

Soil microorganisms are key-components of most agricultural system. They exert multiple functions, from detrimental (e.g. root and leaf pathogens) to beneficial (e.g. plant growth promoters and pathogen antagonists), impacting yield as well as sustainability of agro-ecosystems.

Among these microorganisms, arbuscular mycorrhizal fungi (AMF) are of particular importance. These obligate symbionts colonize the roots of nearly 80% of plant families (Newman and Reddell, 1987; Treseder and Cross, 2006; Smith and Read, 2008) among which most agricultural crops. They transport nutrients (in particular phosphorus) from the soil to the plant, in exchange of carbohydrates provided by the plant (Smith and Read, 2008). They improve plant resistance to biotic (e.g. roots pathogens, grazers …) and abiotic (e.g. soil nutrients deficiencies, heavy metal contaminations…) stresses and increase their productivity (Smith and Read, 2008).

At present, around 250 AMF species have been identified (Schüßler and Walker, 2010). It is obvious that with the fast development of molecular tools for identification (Stockinger et al. 2010), this number will markedly increase in the close future. It is therefore essential to develop/support culture collections that preserve this biodiversity under the highest standard of quality and that guarantee the distribution of well-identified, pure and genetically-stable microorganisms for basic research and application in sectors such as agriculture and environment.

Currently AMF are kept in collections using continuous cultivation on trap plants for the vast majority of species and *in vitro*, mostly associated

with genetically transformed roots, for a restricted number of species. These modes of preservation require considerable work (Douds et al. 1990), present a risk of contamination for the *in vivo* cultures, and do not exclude somaclonal variation as shown for *in vitro* cultures (Plenchette et al. 1996). Therefore, the development of methods allowing the long-term preservation of these microorganisms under stable conditions appears essential.

Few studies have been conducted in the past on the long-term preservation of AMF (e.g. drying and freezing of soils containing spores, lyophilisation…) (Douds and Schenck, 1990; Dalpé, 1987). However, none of these techniques were able to guarantee the physiological and genetic stability over long-term periods.

In 2000, Declerck and Van Coppenolle (2000) cryopreserved for the first time an AMF isolate cultured *in vitro*. *Rhizophagus sp*. MUCL 41835 (synonym *Glomus sp*. MUCL 41835 (Schüßler and Walker, 2010)) spores encapsulated in alginate beads with trehalose as cryoprotectant and cryopreserved for a few hours at -100°C retained their potential for germination and colonization of roots. However, this technique was not tested on other strains and over long periods of storage. In addition, nor the genetic stability neither the physiological stability were addressed in this study.

Cryopreservation is recognized as the most reliable technique for the long-term preservation of fungi (Smith, 1998; Homolka et al. 2007). Below -130°C, cell division and metabolic activities are arrested (Mazur, 1984; Smith and Onions, 1994; Benson et al. 2006). Under these conditions, microorganisms can be maintained without change for periods theoretically

unlimited. On a practical level, the material is kept in a small volume, protected from contamination and with reduced maintenance (Engelmann, 2004; Homolka et al. 2001). This preservation method was successfully applied to different tissues of plant and animal (Moges et al. 2004) and with many microorganisms (Smith and Onions, 1994). It outperformed all other methods for preserving the genetic and physiological properties (Homolka et al. 2001). The development of methods of preservation at ultra-low temperature generally requires fastidious research and several criteria must be taken into consideration (Engelmann, 2004). The aim of all the methods of cryopreservation is to minimize the impact of dehydration and intracellular and extracellular freezing to preserve the various cellular elements and cells' three-dimensional architecture. A multitude of factors affect the effectiveness of cryopreservation in microorganisms (species, isolates, cell size and form, cell water content, growth medium composition, lipid content and composition of the cells), but the most important are cooling and thawing rates, cryoprotectant and temperature of storage (Smith and Onions, 1994; Mazur, 2004; Hubálek, 2003; Benson, 2008).

In the present study we investigated several important cryopreservation factors to develop a long-term preservation method for AMF that insure the stability of these microorganisms. The technique was tested on the survival of several AMF species as well as on their morphology, physiology and genomic stability.

Outline of the thesis

Outline of the thesis

Within this thesis, three major objectives are followed:

1. The development of a method allowing the long-term preservation at ultra-low temperature of AMF.

2. The evaluation of the morphological, physiological and genetic stability of AMF following cryopreservation.

3. The extension and/or adaptation of the cryopreservation method developed, to AMF isolates belonging to different species and genera cultured either *in vitro* or *in vivo*.

The successive steps to achieve these objectives are presented below and schematized in Fig.1.

Outline of the thesis

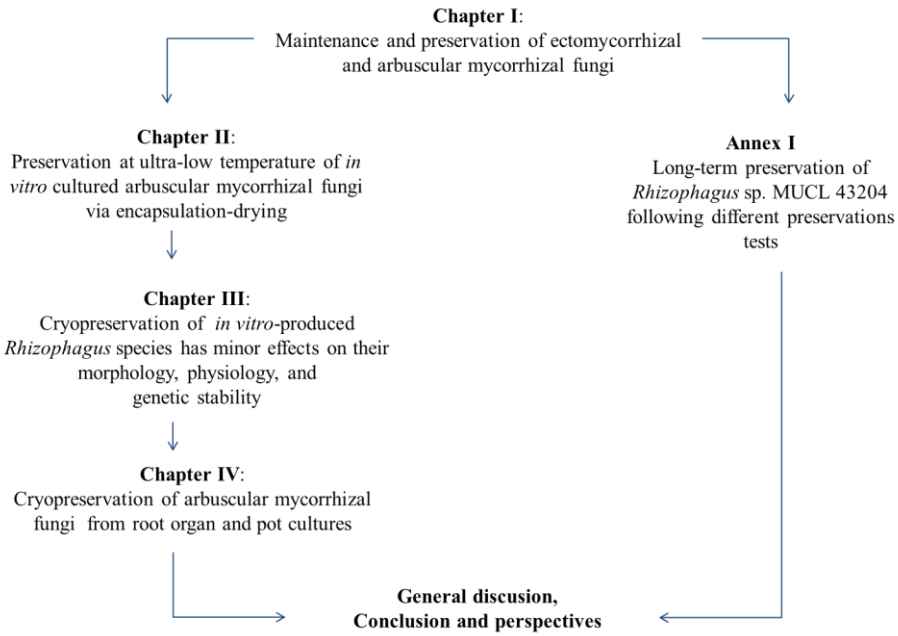

Figure 1 Outline of the thesis

In the **introduction**, we present the subject and the general objective of the thesis. In the **Context of the Study**, the state of the art relative to the study is reviewed. This chapter contains a brief overview on AMF and their ecological importance. We emphasize the difficulties of their maintenance and describe the principal maintenance/preservation methods used so far with a particular attention to the cryopreservation method and the extrinsic and intrinsic factors that affect cell survival and recovery during this process.

Outline of the thesis

In **Chapter I,** we describe the most recent and efficient methods developed so far for the long-term preservation of ectomycorrhizal fungi (ECM) and AMF. We present the parameters that are critical for fungi maintenance/preservation, discuss the advantages and disadvantages of the methods and highlight their most obvious field of application.

Chapter 1 is submitted as a review to the journal Mycorrhiza (Lalaymia L, Cranenbrouck S, Declerck S.) Maintenance and preservation of ectomycorrhizal and arbuscular mycorrhizal fungi.

In **Chapter II,** we develop a method for the long-term preservation at ultra-low temperature of AMF. Several experiments are conducted with fungal isolated cultured *in vitro* on root organ cultures. The effects of culture age, spores encapsulation in alginate beads, the rate of water content in alginate beads and the cooling rates are investigated. Viability of 12 different AMF isolates belonging to 5 species is assessed after different periods of cryopreservation. Their potential to associate roots *in vitro* and to initiate a new fungal life cycle is evaluated.

Results of Chapter II are published in Fungal Biology (Lalaymia L, Cranenbrouck S, Draye X, Declerck S. 2012) Preservation at ultra-low temperature of *in vitro* cultured arbuscular mycorrhizal fungi via encapsulation-drying. Fungal Biology 116: 1032-1041.

Outline of the thesis

To validate the AMF long-term preservation technique, we evaluate in **Chapter III** their morphology, physiology and genetic stability after 6 months of cryopreservation. Studies are focused firstly on the viability and the morpho-features, secondly, on the metabolic enzymatic activities, and finally, on the genetic variations evaluated by means of the Amplified Fragment Length Polymorphisms technique.

Results of Chapter III are published in Mycorrhiza (Lalaymia L, Declerck S, Cranenbrouck S. 2013) Cryopreservation of *in vitro*-produced *Rhizophagus* species has minor effects on their morphology, physiology, and genetic stability. Mycorrhiza DOI 10.1007/s00572-013-0506-y.

The number of AMF isolates cultured *in vitro* is still restricted and much lower as compared to the number of isolates cultured in pot cultures. In **Chapter IV**, we extend/validate the long-term cryopreservation method developed in Chapter II, to other AMF cultured either *in vivo* on trap plants or *in vitro* on root organs. After one month cryopreservation we assess their potential to colonize roots under *in vivo* or *in vitro* culture conditions.

Results of Chapter IV are published in Mycorrhiza (Lalaymia L, Declerck S, Naveau F and Cranenbrouck S. 2013). Cryopreservation of arbuscular mycorrhizal fungi from root-organ and pot cultures. Mycorrhiza DOI: 10.1007/s00572-013-0525-8.

Outline of the thesis

During this thesis different other preservation methods are tested. In **Annex I**, we summarize the principal results obtained and discuss the reasons for failure.

The pertinence of this thesis is summarized in the **General Discussion**. In the **Conclusion**, the major outcomes of this research are presented.

Finally, in the **Perspectives**, we present some future directions to better comprehend the bottlenecks of AMF preservation to improve the application of cryopreservation on a growing set of AMF.

Context of the study

Context of the study

1- Arbuscular Mycorrhizal Fungi

Arbuscular mycorrhizal fungi (AMF) are obligate root symbionts belonging to the Phylum Glomeromycetes (Smith and Read, 2008). These microorganisms are the most ancient mycorrhizal fungi (Schüβler et al. 2001). Their origin dates back 400 million years ago (Remy et al. 1994). Their co-evolution with plants is the direct reason for their prevalence in most if not all environments and their non-specific relation with roots of more than 80% of land plants (Smith and Read, 2008; Bonfante et al. 2009). The composition of AMF communities influences the composition of plant communities as well as functioning of the ecosystem (van der Hejden and Sanders, 2002; van der Heijden and Scheublin, 2007; Wagg et al. 2011; Verbruggen et al. 2012). AMF receive carbohydrates from the host plant necessary for their life cycle, in exchange of which they supply the plants with nutrients (e.g. phosphate, nitrogen and potassium) as well as water scavenged from the soil (Smith and Read, 2008; Jonhson, 2010). In addition to increasing the plant growth and productivity (van der Heijden et al. 2008; Wagg et al. 2011), AMF are also reported to increase their resistance to several biotic and abiotic stresses (Lendzemo, 2005; Avis et al. 2008; Bennett et al. 2009; Veresoglou and Rilig, 2011).

AMF develops into the roots (i.e. the intraradical phase) and within the soil (i.e. the extraradical phase). Both phases develop as a continuum from soil to plant. The intraradical phase is composed of arbuscules, hyphae and, for some genera, vesicles (i.e. they are present in species belonging to the *Rhizophagus* genus, the largest genera of AMF)**.** The extraradical

phase is composed of hyphae, spores and, for some genera *(i.e. Gigaspora***,** *Pacispora***,** and *Scutellospora***)** auxiliary cells (Fig. 2).

The arbuscule is the most important structure involved in carbon-minerals exchanges between the plant and the AMF (Bago et al. 2000). It is formed within the cells of the inner root cortex (Mosse, 1973).

Intracellular hyphae play a major role in the transfer of nutrients from fungal cell to plant cell, while the extraradical hyphae are involved in the uptake and translocation of nutrients from the soil (Bieleski, 1973). Intraradical hyphae may develop straight or form H- or Y-shaped branches. They may also form coils, which are more abundant at fungal entry points.Vesicles are storage organs rich in lipids and glycolipids (Mosse, 1981). They may be inter- or intracellular and are present in both the inner and the outer layers of the cortical parenchyma. The extraradical hyphae are structures involved in the uptake and translocation of minerals and water from the soil to the plant (Zhu et al. 2007). The uptake of nutrients is mostly located at the level of branched absorbing structures (BAS) that are highly branched hyphal structures formed numerously by second and higher order runner extraradical hyphae (Bago et al. 1998). These structures are the preferential sites for nutrients acquisition by the extraradical hyphae (Bago et al. 1998, 2004). The extraradical hyphae also support de production of spores. These structures contain numerous lipids and nuclei. They act as reserve and propagation structures. Sometimes, spores also appear inside the roots on the intraradical hyphae. Spores are the most important structure used in the identification and classification of AMF. Auxiliary cells are believed to be involved in propagation and storage (Declerck et al. 2004).

Extraradical mycelium spread from the roots into the soil forming a hyphal network able to interconnect plant from the same species or from

different species. These hyphae may also interconnect neighbour hyphae by a mechanism of anastomosis. Giovannetti et al. (2004) and Croll et al. (2009) suggested that anastomoses are involved in the maintenance of genetic diversity in the absence of sexual recombination by migration of nuclei via the fusion bridge between mycelia.

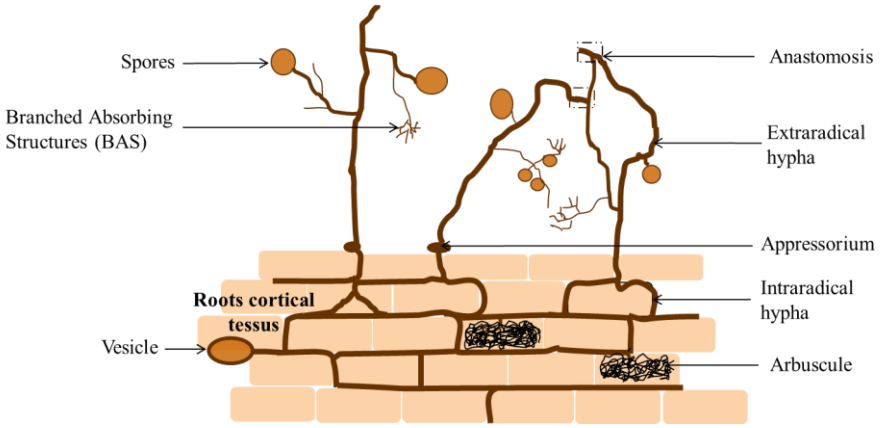

Figure 2 Schematic simplified representation of an arbuscular mycorrhizal fungus belonging to the *Glomeraceae*.

1.1 AM fungal life cycle

AMF are obligate root symbionts that cannot complete their life cycle in the absence of a suitable host plant. In soil, these fungi produce asexual spores, and, for some genera, vesicles and auxiliary cells. These structures are named propagules because they are able to germinate and colonize new roots.

Context of the study

The AMF life cycle is separated in three phases involving a series of complex morphogenetic changes in the fungus: the (1) asymbiotic, (2) pre-symbiotic and (3) symbiotic phase (Akiyama, 2007) (Fig.3)

a. Asymbiotic phase

The development of the AMF starts with the germination of propagules (i.e. spores, vesicles or colonized root fragments). These structures produce a limited amount of branched, coenocytic hyphae, which are capable of anastomosis. In absence of host plant, their growth is arrested. The hyphae septate, with an intense vacuolisation and retraction of nuclei, cytosol and mitochondria.

In presence of root signals (i.e. strigolactones – Akiyama, 2007), the growth and branching of hyphae is strongly stimulated and the fungus switch its development from the asymbiotic to the pre-symbiotic phase.

b. Presymbiotic phase

This phase is initiated by the exudation of plant signalling molecules and their perception by the fungus. This leads to the activation of specific fungal genes and the secretion of Myc factors (i.e. lipochitooligo-saccharides). Physiological and morphological changes occur in the fungus with an increased hyphal growth and branching (Kosuta et al. 2003; Song and Kong, 2012). In return, the host plant discriminates the Myc factors by superficial receptors located on the epidermal cells, and then initiates the symbiotic cascade by the expression of specific genes to prepare the intracellular environment for fungal colonization (Kosuta et al. 2003; Genre

et al. 2005; Song and Kong, 2012). In contact with the root, the fungus develops an appresorium also named hyphopodium on the surface of the epidermal cells (Genre and Bonfante, 2007) before penetration of the root.

c. Symbiotic phase

Four to five hours after the formation of appressorium, plant root cells' establish a pre-penetration apparatus that consists of a thick cytoplasmic bridge across the vacuole of the host cell (Genre and Bonfante, 2007). Microtubules, actin filaments and endoplasmic reticulum allow the fungus to spread within the epidermal cells (Parniske, 2004; Harrison, 2005; Genre et al. 2005; Sicilliano et al. 2007). Once the fungus crosses the epidermal layer, it grows inter- and intracellularly. The fungus develops preferentially inside the inner tissues of the host, commonly described as 'cortical tissues' (Bonfante and Genre, 2008). The fungus then develops arbuscules that lead to modifications in the host cell architecture. The nucleus moves from the periphery to the centre of the cell, the vacuole is fragmented, plastids change their morphology and a new apoplastic space is produced by the proliferation of the cytoplasmic membrane around the fungus (Reinhard, 2007).

After root colonization, the fungus produces in the soil extraradical hyphae and propagules to start a new life cycle.

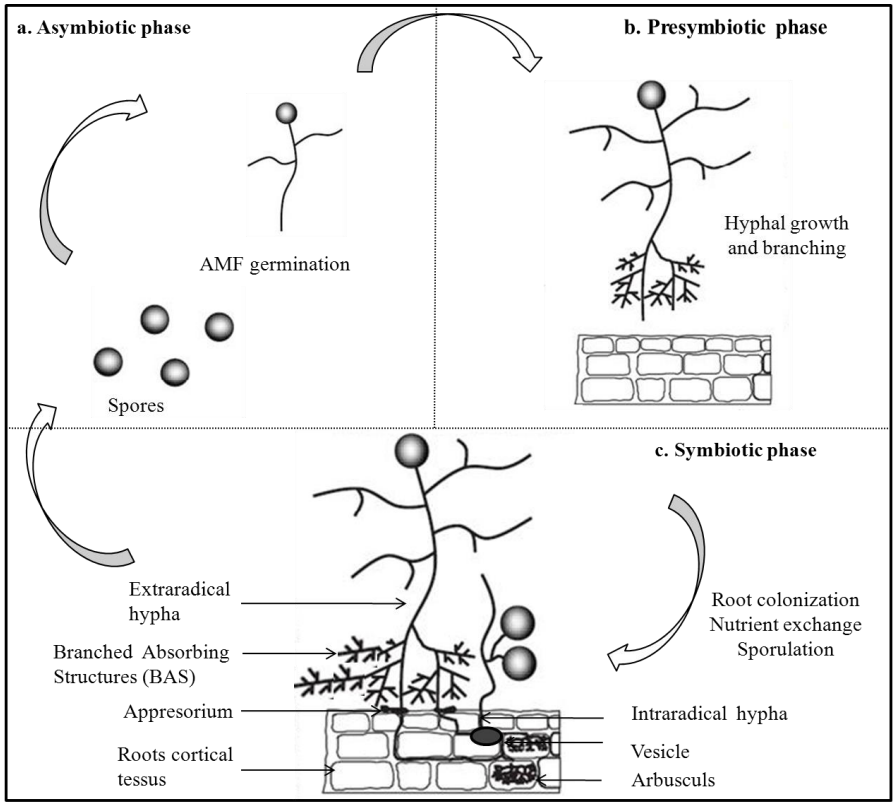

Figure 3 Simplified description of the life cycle of an arbuscular mycorrhizal fungus belonging to the *Glomeraceae* (adapted from Akiyama, 2007).

1.2 Classification of AMF

Arbuscular mycorrhizal fungi are an ancient asexual group of eukaryotes. Initially classified in the Zygomycota, they have been reclassified in the Glomeromycota (Schüβler et al. 2001) based on phylogenetic analyses of the sequence of the small subunit (SSU) rRNA gene.

The phylogenetic affiliation and identification of some AMF still remains unclear because of the complexity of morphological identification

based on spore characteristics. These structures have limited morphological characteristics and some species may form dimorphic spores (Krüger et al. 2009, 2012). Therefore, the use of molecular tools represents a promising alternative. However, for very closely related species, intraspecific variability of (SSU) rRNA marker region is not suited to separate them. Thus other molecular markers are needed (Krüger et al. 2012).

Due to these difficulties, frequent taxonomic changes occur within the *Glomeromycetes*. Recently, a major revision of the *Glomerales* was published by Schüßler and Walker (2010). Currently, 246 AMF species has been described (Table 1) and this list is constantly updated (http://schuessler.userweb.mwn.de/amphylo/).

Table 1 Orders, families, genera in the Glomeromycota and species distribution per genus (http://schuessler.userweb.mwn.de/amphylo/)

Order	Family	Genus	Number of species
Paraglomerales	*Paraglomeraceae*	*Paraglomus*	3
Archaeosporales	*Ambisporaceae*	*Ambispora*	9
	*Archaeospora*ceae	*Archaeospora*	2
	*Geosiphon*ceae	*Geosiphon*	1
Diversisporales	*Acaulosporaceae*	*Acaulospora*	41
	Diversisporaceae	*Diversispora*	7
		Otospora	1
		Redeckera	3
	Entrophosporaceae	*Entrophospora*	3
	Gigasporaceae	*Dendiscutata*	4
		Gigaspora	8
		Racocetra	13
		Scutellospora	26
	Pacisporaceae	*Pacispora*	7

(continued)

Glomerales	*Glomeraceae*	*Funneliformis*	9
		Glomus	84
		Rhizophagus	10
		Septoglomus	2
		Sclerocystis	10
	Claroideoglomeraceae	*Claroideoglomus*	6
Total 4	11	18	246

Updated, 17th August 2013 (http://schuessler.userweb.mwn.de/amphylo/)

2- Preservation methods of fungi

2.1 Periodic serial sub-cultivation

This method is largely used for the preservation of most filamentous fungi. It consists in growing the fungus on agar or in liquid medium at temperature within the range of 10-35°C (most of fungi are mesophilic) and their transfer at regular intervals onto fresh medium. The time separating successive sub-cultures depends on different factors such as the fungal isolate and the temperature of storage. For example, AMF are most often sub-cultured every 6 months. For ectomycorrhizal (ECM) fungi, the intervals of sub-culture are generally shorter (between 1 to 3 months depending on the isolate). Storage could be at low temperature (i.e. 4-8°C) to extend the interval between two successive cultures and thus to reduce the number of transfers required. This method is simple, does not require specialized equipment and is consumable-cheap. However sub-culture is labor-intensive, time-consuming and therefore not adequate for large culture collections. This method could present some risks, such as contamination by mites or other microorganisms, loss of viability or alteration of morphological and/or physiological characters following long periods of sub-cultivation. Genetic variation can also occur due to the continuous selection pressure in an artificial growth environment (Freitas et al. 2011; Houseknecht et al. 2012).

2.2 Preservation methods reducing cell growth and metabolism

Preservation in water

This method consists in placing agar discs supporting the fungus on an agar slant in sterile tubes and to cover the organism with sterile deionized water before storage at 4-25°C (Smith and Onions, 1994). This method has been used (and is still used) in large culture collections (e.g. MUCL) for the preservation of several fungal species for decades. Jones et al. (1991) stored more than 4000 cultures, mostly Hyphomycetes and Zygomycetes, for 2 years. Burdsall and Dorworth (1994) preserved 151 different fungal species belonging to the Basidiomycetes for 7 years at 4-5°C. Richter and Bruhn (1989) stored 135 Basidiomycetes isolates, represented by 83 species in 38 genera in sterile cold water at 5°C. After 20 years of cold storage, 69 isolates remained viable, from which 57 grew vigorously when revived. Richter, (2008) reported that the ECM genus *Laccaria* was well-adapted for sterile cold-water storage. However other ECM genera such as *Boletus*, *Lactarius*, *Paxillus*, *Scleroderma*, and *Thelephora* appeared less adapted for this type of storage (Richter, 2008).

The advantages of storage in water are the low cost and the easy application. Nevertheless, this mode of storage is not successful for all fungi and present some disadvantages. The viability is reduced after long storage. Marx and Daniel (1976) reported that the viability of ECM fungi stored in sterile cold water decreased after 2 years of storage. This method is considered useful for short-term preservation (2-5 years) and should be backed up by longer-term storage methods (Smith and Onions, 1994).

Preservation under mineral oil

This method consists in placing agar discs supporting the fungus on an agar slant, in sterile tubes and to cover the organism with sterile mineral oil or liquid parafin followed by their storage at room or refrigerated temperature (i.e. 4-8°C). Mineral oil reduce the effects of dehydration and decrease the metabolic activity due to the reduced availability of oxygen. The level of oil covering the fungus should not be too hight to allow the fungus to access oxygen. This method is generaly used as reserve reference collection in addition to serial sub-cultivation maintenance on agar (Perrin, 1979). It is appropriate for mycelium or non-sporulating fungi that do not survive other preservation methods. Perrin, (1979) reported that several isolates belonging to Ascomycetes and Basidiomycetes wood-inhabiting fungi remained viable after 27 years of storage in mineral oil. Around 1000 Basidiomycetes, Ascomycetes and Deuteromycetes isolates were also successfuly preserved for 10 years using this method (Johnson and Martin, 1992). Fungal isolates belonging to four *Botryosphaeria* species were recovered from 6 months oil storage after their transfer on agar medium (Baskarathevan et al. 2009). The advantages of storage under mineral oil are the low-cost of the method and the possibility to keep the cultures for several years at room temprature. However, to the risk for contaminations is not excluded Considerable loss of viability was reprtoed after 15 years of storage (Braverman and Crosier, 1966). In addition, oil storage may lead to the selection of mutants adapted to grow under difficult conditions (Smith and Onions, 1994; Houseknecht et al. 2012)

Preservation on silica gel

Usually this method consists in the suspension of fungal propagules in skimmed-milk, their inoculation onto anhydrous silica gel in bottles and their storage at 4-25°C. This method is used for the preservation of sporulating fungi in the absence of other preservation facilities. Preservation on silica gel enables the dehydration of culture and the storage without growth. Perkins (1962) developed this technique and successfully preserved for 4-5 years species of *Neurospora*. Other fungi such as species belonging to *Aspergillus*, *Chromelosporium* (Sharma and Smith, 1999) were stored successfully with this method for periods exceeding 20 years. This method is relatively simple, rapid and inexpensive. However, the risk of contamination as well as the loss of capacity to sporulate is not excluded. Therefore, the time of storage with this method is quite short (between 2 and 4 years) (Smith and Onions, 1994; Sharma and Smith, 1999).

Soil and sand preservation

Generally, this method consists in placing the fungus in sterile water or protectant solutions into sterile bottle containing double autoclaved (121°C for 15 min, with 12 h of interval) soil or sand. The bottles are stored at 4°C. Preservation in soil or sand is generally used for fungi that can withstand desiccation. This method was very successful for some fungi belonging to *Fusarium* (Smith and Onions, 1994), *Rhizoctonia* (Sneh et al. 1991), *Septoria* (Shearer et al. 1974), and *Pseudocercosporella* (Reinecke and Fokkema, 1979). Different studies reported the use of this technique for the storage of arbuscular mycorrhizal fungi (AMF) at room or refrigerated temperatures (Ferguson and Woodhead, 1982; Douds and Schenck, 1991;

Kuszala et al. 2001). However, some studies detected the presence of mutation after storage (Booth, 1971).

Others preservation methods are based on the storage of fungi on organic subtrate such as wood ships, cereal grains and plant, animal or insect tissus. In most cases, organic supports are placed on colony of growing fungi in agar medium. When the fungus starts to colonize the organic carrier, the carrier is transferred into sterile tubes containing sterile solid or liquid medium. The tubes are subsequently sealed and stored at 4°C. This method was reported appropriate for fungi belonging to *Pseudocercosporella* (Reinecke and Fokkema, 1979) and *Rhizoctonia* (Sneh et al. 1991).

2.3 Preservation methods arresting cell growth and metabolism

Freeze-drying (Lyophilization) preservation

This technique consists in the dehydration of frozen water in cells under reduced pressure, by sublimation of ice. A freeze-drying protocol involves (1) the cooling of the sample, (2) the conversion of freezable water into ice, (3) the sublimation of the water by low pressure under vacuum, (4) the evaporation of the gazes and (5) the drying of residual non-freezed water (Adams, 2007; Tan et al. 2007; Ryan and Smith, 2007). This method is one of the preferred fungal long-term preservation methods in culture collections (Tan et al. 1995). For long time it was reported that only fungi producing spores and conidia were able to survive freeze-drying (Tan et al. 1991; Smith and Onions, 1994). However a number of studies reported the successful

preservation of mycelium using freeze-drying (Croan, 2000; Voyron et al. 2009).

During freeze-drying, spores and/or mycelium are suspended in a protectant solution (lyoprotectant) and transferred into ampoules before being freeze-dried. The lyoprotectant used, rate of cooling, final temperature, rate of heat input during drying, residual moisture and storage condition are factors that may affect the viability and stability of the fungus (Smith and Onions, 1994; Ryan and Smith, 2007).

A variety of lyoprotectant solutions are used depending on the fungal species (Toegel et al. 2010). Skimmed milk is a suitable protectant for fungi which is sometimes used in combination with other lyoprotectants such as sugars (e.g. trehalose) and sugar-alcohol (e.g. inositol) (Tan et al. 1995; Croan et al. 1999; Croan, 2000). Other sugars such as fructose and glucose are also very effective as lyoprotectant (Toegel et al. 2010).

The rate of cooling applied is normaly $1°Cmin^{-1}$ (Smith and Onions, 1994). Organisms should be maintained at a temperature below -15°C until the water content is reduced to 5% (Sundari and Adholeya, 1999). The recommended final moisture content following drying is between 1 and 2% (w/v) to prevent damages of cell structure due to dehydration and the intracellular solute concentration. Once dried, the ampoules containing dried material can be stored at temperature between -70 to 4°C for a long period of time, theoretically without modification (Smith and Onions, 1994).

Dermatophytes fungi and fungi belonging to *Paecilomyces* survived 8-14 years storage following lyophilization (Rybnikar, 1995). Sundari and

Adholeya (1999, 2000a) reported that mycelium of ECM fungi retained their viability and stability after freeze-drying. Some AMF (Dalpé, 1987) have been revitalized successfully after lyophilization. Yeasts of the genus Candida spp. were preserved for 35 years. The isolates retained their viability and the phenotypic characteristics typical of their respective species (Freitas et al. 2011).

Another advantage of this preservation method is that ampoules can be stored easily in small space without special requirements. The ampoules are sealed and the samples totally protected from contaminants (Smith and Onions, 1994).

Cryopreservation

Cryopreservation consists in the storage at ultra-low temperatures of the fungus following various cooling rate, cryoprotectants and methods. It is considered as the most reliable method for fungal preservation especially for the species that are not adapted to freeze-drying (Smith, 1998; Ryan, 2001; Homolka et al. 2007). It is used since decades on a vast range of sporulating and non-sporulating fungi belonging to saprophytes, symbionts or parasites (Homolka et al. 2001, 2007; Chetverikova, 2009). At ultra-low temperature, the metabolism is stopped. Between 0 and -25°C the enzymatic activities are only slowed-down. Little metabolic activities take place below -70°C and no activity is noticed below -130°C (Mazur, 1984). Thus, to achieve long-term preservation of cells, the temperature should be ideally ≤ -130°C (Mazur, 1984; Shaw and Jones, 2003). Below this temperature, all chemical reactions, biological processes and intra- and extra physical interactions are stopped (Mazur, 1984).

The advantages of this technique are that the cell material can be stored without alteration or modification for a theoretically unlimited period of time. Moreover, cultures are stored in a small volume, protected from contaminations and require very limited maintenance. However, the main problem to reach temperatures below -130°C during the cryopreservation process is to keep the entire cellular content safe from damages, thus avoiding intra and extracellular water freezing and ice nucleation during cooling and storage.

a- Water Freezing during cryopreservation process

Living cells are water-dependent. Thus, liquid or solid forms of water influence the structure and functions of cells (Mazur, 1984). Physical properties of water are due to its atomic structure: central oxygen forms covalent bonds with two hydrogen atoms (H_2O) (Benson et al. 2006; Benson, 2008). These hydrogen bounds are responsible for the abnormally high boiling and melting points of water compared to other molecules of similar size (Mazur, 2004; Benson et al. 2006; Benson, 2008). In pure water the highest temperature at which ice can form at normal pressure is 0°C called the equilibrium freezing point. Water rarely freezes at this temperature, water is only super-cooled and the ice nuclei do not form until the temperature falls at around -40°C, called the temperature of homogenous ice nucleation (Zakariassen and Kristiansen, 2000; Wilson et al. 2003; Sakai, 2004; Meryman, 2007). Nevertheless, in nature, water is rarely pure. The presence of impurities, foreign particles and other molecules than water undergoes the heterogeneous nucleation at temperature higher than -40°C (Langham and Mason, 1958; Zakariassen and Kristiansen, 2000; Hienola et al. 2009). Temperature of heterogeneous nucleation varies markedly due to

the varying volume and varying purity of the water used. Once an ice nucleus is formed, the ice crystal grows. The growth rate of ice crystals will depend of the temperature, the lower is the temperature, the slower the ice growth rate.

b- Physical and biological detrimental effects of cryopreservation

During all the cryopreservation process, cryopreservation could present disadvantages as the other preservation methods, if cells are not protected and the water crystallization is not prevented. Below are the most frequently reported detrimental effects of cryopreservation on cells.

In cells, water molecules are intimately hydrogen-bonded to the atoms within molecules such as proteins, RNA, DNA or membrane phospholipid groups to maintain their structure and function. Sudden temperature decrease can cause thermal shock or cell water freezing and thus cell injury. At low temperature, water ionic dissociation constant decreases. As a result the pH increases, resulting in an irreversible protein denaturation (He, 2011). The dielectric constant of water increases at low temperature, which reduces ionic attractions. This change can have a distorting effect on the molecules and biopolymers (Mazur and Cole, 1989; He, 2011). The viscosity of water also increases and this can affect the flow of water through biological membranes.

During freezing, water volume increases by about 9%. This expansion can cause irreversible physical damages to cells. Intracellular ice crystals formation could cause irreversible damages to membranes and organelles (Ryan et al. 2001). Freezing of extracellular water causes changes of anionic components of the extracellular medium. In particular, acetate,

chloride, nitrate, iodide and sulfate can cause changes in membrane permeability, integrity and function and even, in some cases, can result in the extrusion of membrane components (Mazur and Cole, 1989).

It has been reported in several studies that fungi respond differently to freezing depending on cooling rate whether slow or fast (Coulson et al. 1986; Smith and Onions, 1994; Smith, 1998; Lalaymia et al. 2012).

Slow cooling

Slow cooling, also called controlled cooling, consists in cooling the organisms at a rate between 0.5 and 10°Cmin^{-1} (Mazur, 1984). This rate differs depending on the size of the cells, the water content and composition of cells and the cells' water permeability (Mazur, 1984). Samples are cooled until the temperature reach -30 to -80°C, after which the samples are transferred to lower temperature, generally below -130°C for final storage (Smith, 1993; Shawl and Jones, 2003).

During slow cooling, ice initially nucleate in the extracellular medium between -5 and -15°C. The first consequence of the external ice formation is the increase of the concentration of the solute surrounding the cells. By osmotic effect, the water is transported out of the cells. As a result, the cells will dehydrate, decrease in volume and shrink (Lovelock, 1953; Mazur, 2004; Meryman, 2007). In addition to mechanical damages, irreversible denaturation of proteins and membranes' constituents could occur due to the pH changes after water freezing. This slow cooling damage is called "solution effects" injury (Mazur, 2004). Smith and Thomas (1998) observed by cryogenic light microscopy that at slow cooling rates, hyphae of different fungal isolates shrink because of water loose from the cytoplasm.

Unless this drawbacks, the controlled cooling rate is the most recommended for fungi cryopreservation.

Fast cooling

Fast cooling rate consists in cooling the organisms at a rate higher than 10°Cmin^{-1} and can reach 20,000°Cmin^{-1} (Mazur, 1984; Shaw and Jones, 2003). As for slow cooling, fast cooling rate differs depending on the size of the cells, their content in water and composition and their water permeability (Mazur, 1984). However, to the contrary of slow cooling rates, fast cooling rates tend to encourage the formation of intracellular damaging ice crystals (Fuller, 2004a; Mazur, 2004). In fact, too rapid cooling does not allow the cell to have enough time to sufficiently dehydrate. Ice will form very rapidly in the intracellular compartment (Fuller, 2004a). Under the mechanic action and growth of ice, plasmic membrane and organelles could be perforated, resulting in cell death (Fuller, 2004a; Mazur, 2004; He, 2011). In addition, intracellular water crystallization increases the intracellular solute concentration that contributes to the cellular proteins and lipids denaturation. For each type of organism there is an optimum cooling rate and the optimum protocol can be found using the cryo-light microscope that allow to follow the direct ice nucleation during cooling.

Ice formation and crystal growth can be slowed-down during fast cooling by lowering the freezing point via the addition of solutes at high concentration (Meryman, 2007). This results in the increase of cell viscosity during cooling and subsequent decrease in ice formation. At the so-called glass transition temperature (Tg) The temperature at which a solutions

change to from a solid amorphous glass - the viscosity is sufficiently high to effectively prevent molecular diffusion, thus, stopping all phase transitions (Shaw and Jones, 2003; Meryman, 2007). The unfrozen solution remains in a metastable state, with an amorphous, vitrified, non-crystalline structure. This process is called **vitrification.**

Another stress caused by the ice formation during cryopreservation and storage is oxidative stress. The only reactions that can occur in frozen aqueous systems at -196°C are photo-physical events such as the formation of reactive oxygen species (ROS) (Mazur, 1984; Benson and Bremner, 2004). ROS with their free electron disrupt the cellular metabolites and damage the cell constituents. These phenomena enhance the production of toxic molecules and new ROS that will deteriorate molecules riche in electron, like proteins and DNA with the risk of metabolic arrest and cell death because of the non-occurring of enzymatic repair at low temperature.

Following freezing, the organisms must be returned to normal temperature. **Thawing** of frozen material to physiological temperature can cause further damages. The effects of thawing rate depend on the rate of temperature decrease (i.e. cooling rate) during freezing (Mazur, 2004). As a general rule, rapid thawing is preferable to slow thawing (Mazur, 1984, 2004; Shawl and Jones, 2003; Day et al. 2008). If cells cooled fastly are thawed slowly, the intracellular small ice crystals formed during cooling will tend to grow by recrystallization (Mazur, 2004). The slow thawing gives a sufficient time for small ice crystals to increase in size, and to form ice aggregation. This may result in mechanical injuries and cell death (Mazur, 1972, 1984, 2004; Shawl and Jones, 2003). The structure of cells rapidly coolled could also be fragilized during slow thawing, due to the gradual

relaxation of vitreous glasses, or due to the newly formed ice crystal (Shawl and Jones, 2003; Day et al. 2008).

The effect of slow thawing on cells cooled slowly is generally associated with the large time of exposure to subzero temperature during the time of slow thawing and to the potential injurious event that have been activated during cooling (Mazur, 2004).

Even if the fast thawing is the most recommended, in some cases it can causes more injuries than slow thawing, especially for cells that have been frozen at a slow rate (Mazur, 2004). It is suggested that, during slow cooling, large ice crystals are formed in the extracellular medium and cryoprotectant solution can be driven into the cells. When the cells are rapidly thawed, ice crystals melt rapidly with no sufficient time for the cryoprotectant to diffuse back out from the cell. As a result, cell osmotic shock, swelling and cell lyses may occur (Mazur, 2004).

c- Cryoprotectants

To resist low-temperatures and ice crystal formations in nature, some plants, animals, algae and microorganisms change the chemical composition of their membrane, synthesize natural cryoprotectants (e.g. sugars, amino acids, glycol-protein…) and soluble proteins (Rubinsky et al.1992; Zakariassen and Kristiansen, 2000). To protect living cells from being injured during cooling, storage and thawing processes, cryoprotective agents are often added to the cellular suspension prior to freezing. The presence of suitable cryoprotectants usually increases considerably survival (Fuller, 2004b). In most cryopreservation protocols developed for fungi, the

addition of cryoprotectant prior to freezing seems necessary (Mata and Pèrez-Merlo, 2003).

The cryoprotective substances generally reduce shrinkage effect by penetrating the cells and replacing water, enhancing the viscosity of the cellular medium, decreasing the nucleation temperature and reducing ice crystal size (Smith, 1998; Zakariassen and Kristiansen, 2000; Fuller, 2004b; Benson et al. 2006). The chemical nature of the cryoprotectants is variable. They could have alcoholic, aldehyd, amine, acid or thiol functions. They could also have saturated or unsaturated bounds. The common criteria of these substances are that they dissolve in water and are able to form hydrogen bounds with water molecules, proteins and hydrophilic groups of phospholipid. For some cryoprotective substance, more the hydrogenic bounds are high, more the protective effect is important (Fuller, 2004b).

Cryoprotective solutions can be classified on their molecular weight. A more traditional division of the cryoprotectant solution depends upon the rate of penetration. They are classified into two categories; penetrating cryoprotectants and non-penetrating cryoprotectants.

Penetrating cryoprotectants

Penetrating cryoprotectants are small molecules, with a molecular weight lower than 400, able to cross cell membranes (Hubålek, 2003). By entering and remaining inside cells, the role of penetrating cryoprotectants is to reduce ice growth and cell dehydration, and protecting the cell components during freezing (Hubålek, 2003; Fuller, 2004b). In vitrification, the role of penetrating cryoprotectants is to lower the freezing point and

completely prevent ice formation. Penetrating cryoprotectants are the major ingredients of vitrification solutions. The most used penetrating cryoprotectants in fungi cryopreservation are Dimethyl Sulfoxid (DMSO) and glycerol.

Non-penetrating cryoprotectants

Non-penetrating cryoprotectants are large molecules, usually polymers (Hubålek, 2003). They have extra-cellular action by protecting the membranes and inhibiting ice growth by the same mechanisms as penetrating cryoprotectants. They have a potential to cause intracellular dehydration. Trehalose and Ethylene glycol (EG) are examples of non-penetrating cryoprotectants. However, these cryoprotectants could be toxic.

The toxic effect of cryoprotectants on cells depends on their chemical nature and concentration. In general, the toxicity of cryoprotectants is lower at low temperature, and may even become negligible if the cryoprotectant is introduced at a sufficiently low temperature (Fuller, 2004a; Mazur, 2004). For example DMSO cryoprotecant is generally applied at 4°C. At higher temperature 30°C it was reported as toxic on certain microorganisms such as yeast (Hubålek, 2003).

DMSO, **Glycerol** and **trehalose** are the most common cryoprotectants used in fungi cryopreservation (Jong, 1981; Corbery and Le Tacon, 1997).

DMSO is a very effective cryoprotectant. During freezing, as ice crystals grow, cells are exposure to hypertonic solution. DMSO has the

capacity to interact with cell membrane phospholipids layers and stabilise the membrane preventing the dehydration during freezing (Fuller, 2004b). It has also the ability to sequester the harmful free radical that could be produced during cryopreservation (Benson and Bremner, 2004). DMSO is generally adequate at concentration of ~10% (Corbery and Le Tacon, 1997). It was widely used at different concentrations for the cryopreservation of a large range of fungi (Dahman, 1983; Sundari and Adholeya, 1999; Houseknecht et al. 2012). Using a cryogenic light microscope, Smith and Thomas (1998) observed that DMSO (10%) reduced the shrinkage and the temperature of nucleation of hyphae of different fungal isolates belonging to *Aspergillus, Achilya, Pleurotus* and *Thanatephorus* species.

Glycerol is highly soluble in water. It can interact by forming hydrogen bound with water and penetrate the plasma membrane of different type of organisms (Fuller, 2004b). This cryoprotectant penetrates less rapidly than DMSO and is usually applied at concentration between 5 and 20% (Corbery and Le Tacon, 1997) and temperatures between 4 and 22°C. It has a lower toxicity when compared to other cryoprotectants such as DMSO (Fuller, 2004b; Colauto et al. 2011). A variety of fungi were successfully preserved using this protectant. At the international mycological institute (IMI; Eghal, UK), over 4000 fungal species have been successfully frozen in 10% glycerol (Kolkowski and Smith, 1995). Crahay et al. (2013a) successfully cryopreserved 98 ECM fungal isolates belonging to different genera with glycerol as cryoprotectant.

Trehalose is the only disaccharide that has two water molecules in its crystal (Dawson et al. 1968). These molecules confer to trehalose the capacity to interact with phospholipid of the cell membrane to maintain their fluidity during freezing and desiccation (Crowe et al. 2001). This

disaccharide forms a stable glass with a relative high Tg compared to other cryoprotectants (Crowe et al. 2001; Tan and van Ingen, 2004). It was more effective on the cryopreservation of an AMF *Rhizophagus sp.* isolate as compared to other cryoprotectants such as glycerol, sucrose and mannitol (Declerck and Van Coppenolle, 2000). Trehalose has been used at concentrations lower than 2M for the preservation of different microorganisms. For the cryopreservation of AMF, Lalaymia et al. (2012) used trehalose at 0.5M. ECM fungi *Cantharellus cibarius* were successfully preserved using 10% trehalose as cryoprotectant (Danell and Flygh, 2002).

d. Methods of cryopreservation

There are three basic approaches for the cryopreservation of cells and tissues:

Slow-Freezing cryopreservation method

This method is also called controlled freezing or cooling. The principle of this method is to remove most of the water from the cells before it freezes intracellulary. It is commonly based on the slow cooling of the cells to a defined pre-freezing temperature (i.e. between -30 and -80°C) in the presence of a cryoprotectant solution, followed by a rapid immersion in the freezer, at temperature ≤ -130°C or in liquid nitrogen (Sakai, 2004). During slow cooling, the extracellular medium is firstly supercooled, followed by the formation of ice crystal (Mazur, 1984). As the temperature is further decreased, an increasing amount of the extracellular solution is converted into ice, thus resulting in the concentration of solutes. Cells progressively dehydrate to maintain the osmotic equilibrium. Depending

upon the rate of cooling and the pre-freezing temperature, different amounts of water will leave the cells before the intracellular content solidify. Under optimal conditions, most intracellular freezable water is removed, thus reducing or avoiding detrimental intracellular ice formation before biological material storage at final temperature preservation (≤ -130°C). Thawing should be as rapid as possible to avoid the phenomenon of recrystallization. Cryopreservation using slow freezing and rapid thawing is the most widely used method for fungi (Hwang, 1966; Dahman et al. 1983; Kolkowski and Smith, 1995; Colauto et al. 2011). Hwang (1966) preserved with success 162 fungal strains belonging to different family, such as *Pythiceae*, *Mucoraceae,* Basidiobolacea, Sclerotiniaceae, Thelephoraceae…, using a controlled cooling rate of approximately 1°Cmin^{-1} from ambient temperatures to -35°C and fast transfer at -165 to -196°C. Identically, Dahman et al. (1983) observed that controlled freezing (1°Cmin^{-1}) from ambient temperature to -40°C followed by a quick immersion in liquid nitrogen was successful for the preservation of 31 phytopathogenic fungi such as *Plasmodiophora brassicae*, *Phytophthora infestans*, *Podosphaera leucotricha*, *Uromyces phaseoli* and *Penicillium digitatum*. These fungi kept their germination, hyphal growth and their pathogenic ability. Whatever the conditioning and the medium culture, is was shown that slow freezing is the most effective method for fungi (Colauto et al. 2011).

Encapsulation-dehydration

This method was developed for the cryopreservation of plants by Fabre and Dereuddre (1990). It consists in the encapsulation of biological material in alginate beads. The encapsulated material is treated with an adequate cryoprotectant, and then partially desiccated under a laminar air

flow cabinet or on silica gel, to water content around 20% (moisture content in alginate beads, fresh weight basis). The beads are then frozen rapidly in a freezer or in liquid nitrogen. The beads are thawed and placed on culture medium for recovery (Block, 2003; Engelman 2004; Sakai, 2004; Reed et al. 2005). Osmotic treatment with cryoprotectant and the dehydration of the alginate beads reduce the water content of the cell material, and thus, decrease or prevent ice damages during freezing (Sakai, 2004; Smith and Ryan, 2012). During rapid cooling, in liquid nitrogen for example, the cryoprotectant that was concentrated due to beads dehydration could vitrify and be amorphous, which could prevent the intracellular ice formation due to rapid cooling (Sakai, 2004). Alginate beads are easy to handle and manipulate during a cryopreservation protocol. Beads may increase the protection of dried material from mechanical stress during storage (Suzuki et al. 2005; Sakai and Engelman, 2007) and could also promote cell regrowth after thawing (Panis and Lambardi, 2005). This method is not frequently used for fungal preservation and is poorly documented. Mycelium of *Serpula lacrymans* immobilized in alginate beads survived after one month storage at -20°C (Ryan, 2001). For AMF, Lalaymia et al. (2012) used with success this technique for the preservation of several AMF isolates belonging to *Rhizophagus*, *Glomus*, *Claroideoglomus*, *Septoglomus*, *Paraglomus* and *Gigaspora* genera.

Vitrification

Vitrification is defined as the solidification of intra and extracellular liquid solution to amorphous glass without crystallization (Taylor et al. 2004; Benson et al. 2006). Vitrification is achieved via the extreme elevation of viscosity during ultra-rapid cooling by the addition of a high concentrated

cryoprotectant or a mixture of cryoprotectants as vitrificant solution (Sakai, 2004; Taylor et al. 2004; Benson, 2006; Sakai and Engelman, 2007). Prior to cooling, cells dehydrate when they are exposed to concentrated vitrificant solutions. During rapid cooling, the vitrification solutions as well as the cells content are rapidly transformed to a vitreous state (Taylor et al. 2004; Sakai and Engelman, 2007). As a result, intracellular ice formation is avoided (Taylor et al. 2004). Cooling and thawing should be rapid enough to prevent crystallization (Taylor et al. 2004). After thawing the probable toxic vitrificant solution must be immediately removed by suspending the sample in less concentrated cryoprotectant, to dilute the vitrificant solution and to take the cryoprotectant out from the cells with less cell's osmotic damages (Tan and Staplers, 1996; Sakai and Engelman, 2007; Smith and Ryan, 2012). Vitrification technique does not require the use of controlled freezer and eliminate the risk of damaging effect of intra and extracellular ice formation. Temperature of vitrification - glass transition temperature (Tg) - of cryoprotectant solution can be detected using differential scanning calorimetory (DSC) (Sakai, 2004; Benson et al. 2006; Day et al. 2008). For some recalcitrant fungi that do not survive slow cooling, vitrificant protocols were developed (Smith, 2012). The technique has been successful applied for fungi belonging to the genera *Flammulina, Boletus,* and *Mycena* with no effect on fungal morphology (Smith, 2012).

Conclusion

There is no standard cryopreservation protocol that can be applied universally to all fungi. It has been shown that there is no taxonomic link between fungi responses to freezing and thawing even at the species level

(Dahman et al. 1983; Morris et al. 1988; Smith and Thomas 1998; Ryan et al. 2000; Bardin et al. 2007; Gleason et al. 2007).

For the success of long-term preservation, the cooling rate, storage temperature, thawing and other intrinsic factors (e.g. growth conditions, physiological states and age of culture) should be taken into consideration and require to be handled at species level. In Chapter I we discuss these parameters that are critical for ECM fungi and AMF maintenance/preservation.

3- Preservation methods of AMF fungi

Several preservation methods have been developed for mycorrhizal fungi. These methods are reviewed in Chapter I.

Chapter I

Maintenance and preservation of ectomycorrhizal and arbuscular mycorrhizal fungi

Submitted to Mycorrhiza journal

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My contribution to this chapter was approximately 90% and involved the literature study itself and the writing of the manuscript. Co-authors of the chapter have been involved in revising the manuscript.

Maintenance and preservation of ectomycorrhizal and arbuscular mycorrhizal fungi

Preface

Before developing a long term preservation protocol for arbuscular mycorrhizal fungi (AMF) it is essential to make a thorough review of the literature on the subject. This chapter was thus oriented towards an overview of the different methods that have been developed until now for the short to long-term preservation of ECM fungi and AMF. The information gathered from this literature study was used for the development of a cryopreservation protocol for AMF propagules belonging to different species.

Maintenance and preservation of ectomycorrhizal and arbuscular mycorrhizal fungi

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. Whatever the method applied, the viability and genetic/physiological stability are key parameters that should be considered to evaluate the effectiveness of the procedure. 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. Here we report and describe the principal methods developed so far for ectomycorrhizal and arbuscular mycorrhizal fungi, the most widely-studied and economically-important groups of mycorrhizal fungi. We present the factors which are the most important for their storage and propose a protocol applicable, although not generalizable, to a large panel of these organisms. Finally, we suggest some novel avenues for the long-term preservation of these root fungal symbionts.

Keywords: Cryopreservation, Lyophilization, Oil and water storage, Alginate bead, Culture collection, Genetic Stability.

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

Maintenance and preservation of ectomycorrhizal and arbuscular mycorrhizal fungi

Introduction

The importance of microbial culture collections as source of germplasm for basic as well as applied research and for the white, green, grey and red biotech sectors is undisputable (Smith and Ryan, 2012; Houseknecht et al. 2012). However, their use often presuppose that the microorganisms are correctly identified and maintained under conditions that preserve their original properties over long periods (Smith, 2012; Smith and Ryan, 2012). It is therefore common or desirable to preserve isolates under conditions that slow-down/arrest their metabolism and by 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, 2012). 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 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), Centre for Mycorrhizal Culture Collection (CMCC), the International Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM) and the International Bank of Glomeromycota (IBG)).

Mycorrhizal fungi are important microorganisms for plant production (e.g. they increase yield and improve plant resistance against a/biotic stresses), bioremediation, afforestation and ecosystem functioning (van der Heijden and Scheublin, 2007; Duponnois et al. 2007, 2011; Siddiqui and Kataoka, 2011). 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, this method is 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).

In the present review, we report on the several methods developed so far for the maintenance and preservation of ectomycorrhizal (ECM) fungi and arbuscular mycorrhizal fungi (AMF), which are the most abundant and diverse groups among the mycorrhizal fungi, playing key roles in soil-plant interactions and presenting many potentials for biotechnology applications (Smith and Read, 2008). We discuss the advantages and disadvantages of these methods and conclude on the most effective ones. Finally, we propose some innovative avenues for the long-term preservation of these microorganisms.

Maintenance and preservation of ectomycorrhizal and arbuscular mycorrhizal fungi

1- 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). This method is typically used for the short-term storage of fungi. Cultures are most often grown on gelled medium and stored at room temperature or at temperatures close to 25°C. The cultures may also be stored at lower temperatures (e.g. 4°C) to increase the interval between sub-cultures (Smith and Onions, 1994). This method is simple, widely-used and relatively cheap because specialized equipment is not required. However, this method is also time-consuming, labor-intensive and is 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).

Preservation of fungi in sterile water or under oil, by drying on silica gel or encasing in glass beads and stored in soil at room or cold (e.g. 4-8°C) temperatures are alternatives to sub-cultivation (Smith and Onions, 1994; Abd-Elsalam et al. 2010). These methods are simple and do not require expensive equipment. They are adapted for short-term preservation (2-5 years) but, similarly to sub-cultivation, may present the risk of unwanted contaminations and could favor the selection for mutants able to grow under difficult conditions (Smith and Onions, 1994; Houseknecht et al. 2012).

Maintenance and preservation of ectomycorrhizal and arbuscular mycorrhizal fungi

Methods that arrest growth and metabolism are the most reliable for long-term preservation. Lyophilization (also termed freeze-drying) and/or cryopreservation (i.e. storage at ultra-low temperature) are mandatory in international culture collections storing large number of fungi for extended periods (Smith and Onions, 1994; Abd-Elsalam et al. 2010). Lyophilization consists in the removal of water by freezing, volatilization and drying at low pressure and temperature under vacuum (Tan et al. 1995, 2007). Although adequate equipment is necessary for the freeze-drying process, no specific infrastructure is needed to store the organisms following lyophilization (Tan et al. 1995, 2007). Cryopreservation consists in the freezing of 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 periods of time (Mazur, 2004).

Viability of the lyophilized and cryopreserved fungi depends mainly on the freezing rate, the use and the nature of lyo- or cryoprotectants and the thawing rate (Tan et al. 1995; Mazur, 2004), which may cause injuries to the fungi if they are not correctly applied (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 fungal 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. Below we review the methods used for ECM fungi and AMF, the most economically-important groups of mycorrhizal fungi.

2- Methods of maintenance and preservation of ECM fungi

The maintenance/preservation of ECM fungi is rather delicate due to their intrinsic characteristics (Homolka at al. 2006). Under *in vitro* culture conditions, these organisms do not produce conidia and sexual structures are rarely formed (Sundari and Adholeya, 1999, 2000a; Homolka et al. 2006). Their dominant form *in vitro* is hyphae that generally grow slowly in axenic culture (Molina et al. 2002; Homolka et al. 2006). Hyphae 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 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 le Tacon, 1997; Siddiqui and Kataoka, 2011). The time between sub-cultures can be extended by reducing the storage temperature. For instance, Tibbett et al. (1999) stored a wide geographical range of *Hebeloma* isolates during 3 years at 2°C.

Although the 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 the ability of the organism to colonize plant roots (Marx and Daniel, 1976; Thomson et al. 1993; Di Battista et al. 1996) and may impact some essential properties (e.g. capacity of the fungi 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.

The preservation under water is also reported, although less frequently, for ECM fungi.This method consists in placing agar discs supporting the ECM fungus on an agar slant in sterile tubes and to cover it with sterile deionized water before storage at 4-25°C (Smith and Onions, 1994). Richter (2008) reported that the genus *Laccaria* (Table A2– Annex II) was well-adapted to cold storage (i.e. 5°C) in sterile water. Conversely, *Boletus, Lactarius, Paxillus, Scleroderma,* and *Thelephora* appeared less adapted to this type of storage (Richter, 2008). Marx and Daniel (1976) showed that the survival of ECM fungi stored in sterile water at 5°C was 100% (Table A2– Annex II) after 1 year but was reduced for some isolates to 95% and 64% after 2 and 3 years of storage, respectively. This method is considered only useful for short-term preservation (2-5 years) and should be backed up with long-term storage methods (Smith and Onions, 1994).

Preservation of fungi on agar slant, in sterile tubes, covered with sterile mineral oil at room or refrigerated temperature (i.e. 4-8°C) is also reported (Smith and Onions, 1994). This method is appropriate for mycelium or non-sporulating fungi (Homolka and Lisá, 2008; Perrin, 1979) and as such should be adapted for ECM fungi. However, little is known on ECM fungi preservation under mineral oil. Perrin, (1979) and Johnson and Martin, (1992) reported that hundred of isolates belonging to Ascomycetes and Basidiomycetes fungi remained viable up to 27 years of storage under mineral oil. This method prevent the dehydration of fungi and slows down their methabolic activity and growth. However, the risk of contaminations

and the selection of mutants adapted to grow under difficult conditions is not excluded (Smith and Onions, 1994; Houseknecht et al. 2012).

The preservation of hyphae within alginate beads is often used to produce ECM fungal inoculum for plant inoculation. The hyphae are fragmented, mixed into a sodium alginate solution and dropped into a solution of calcium chloride (Paloschi de Oliveira et al. 2006). An ionic exchange between Ca and Na takes place, 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 expand outside. Maupérin et al. (1987) maintained liquid-produced hyphae of *Hebeloma crustuliniforme* in alginate beads for at least 5 months at 4°C (Table A2– Annex II). Kuek et al. (1992) preserved for 7 months, eleven eucalypt ECM fungi belonging to *Paxillus*, *Laccaria* and *Hebeloma* (Table A2– Annex II) encapsulated 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* (Table A2– Annex II), although viability significantly decreased after 60 days of storage. Paloschi de Oliveira et al. (2006) preserved during 18 months at 8°C, hyphae of *Rhizopogon nigrescens* (Table A2– Annex II) immobilized in calcium alginate gel.

Because of their characteristics (i.e. absence of conidia and few or no spores produced *in vitro*), it is often reported that ECM fungi are not adapted to lyophilization (Tan et al. 1991; Smith and Onions, 1994). However, a few studies have reported the lyophilization of these microorganisms. Sundari and Adholeya (1999) succeeded in the

lyophilization of *Laccaria fraterna*. These authors tested different parameters (i.e. the physiological growth conditions and age of culture, the lyoprotectant and its concentration, the pre-freezing procedure, the freeze-drying as well as the rehydration programme) to optimize the lyophilization process. 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 the 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* (Table A2– Annex II). With the same protocol Sundari and Adholeya (2000a, 2000b) investigated the stability of 7 of these ECM fungal isolates by qualitative enzyme assays. All isolates survived lyophilization and showed stable enzymatic activity. To the contrary, Obase et al. (2011) reported in their study 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 (see Obase et al. 2011).

Cryopreservation is the most reliable long-term preservation method for fungi. A multitude of protocols have been developed for Basidiomycetes, Ascomycetes and Zygomycetes. Curiously, only a few studies focused strictly on ECM fungi. This is probably because these organisms only produce hyphae *in vitro*, 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*, and *Hebeloma sp.* and *Cortinarius multiformis*, *Russula densifolia*, *Suillus granulatus*,

Lactarius deliciosus) was around -7 and -14°C (see list of isolates in the paper of Lehto et al. (2008); Dalong et al. (2011)). Smith (1998) recommended slow-cooling rate (1°Cmin^{-1}) and application of glycerol as cryoprotectant for Basidiomycetes and Ascomycetes. Although this author confirmed that there is no obvious link between taxonomic group and response of fungi to freezing and thawing. This is consistent with the findings of Corbery and Le Tacon (1997). These authors tested the cryopreservation of different ECM fungal isolates (i.e. *Hebeloma crustuliniforme*, *Laccaria bicolor*, *Paxillus involutus*, *Pisolithus tinctorius*, *Scleroderma flavidum*, *Rhizopogon luteolus*, *Thelophora terrestris* and *Cenococcum geophillum*) sampled from the margin of the colony, in 15% glycerol (v/v) following different cooling rates (i.e. samples were directly plunged in LN, directly placed in the freezer at -80°C, or slowly cooled (~1°Cmin^{-1}) before freezing in LN or at -80°C). The 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°Cmin^{-1}) (Table A2– Annex II), while the hyphae were injured if the cooling rate was faster. *C. geophillum* was not affected by freezing, whether the cooling rate was slow, fast (i.e. in LN) or uncontrolled. *T. terrestris* and *P. involutus* did not survive any freezing method. The authors suggested that, among the different species of ECM fungi, the response to freezing was dependent on the physiology and water relationships of the hyphae.

Danell and Flygh (2002) cryopreserved successfully *Cantharellus cibarius* (Table A2– Annex II) using slow (0.3°Cmin^{-1}) controlled cooling and storage in LN followed by rapid thawing. Sorbitol (4M) and DMSO (1M) were used as cryoprotectants. The cryopreservation of the same isolate

was ineffective using trehalose (10%) and glycerol (10%) and cooling rates of 0.5, 1 and 10°Cmin^{-1}. Homolka et al. (2003) tested the cryopreservation of *Entoloma clandestinum, Scleroderma citrinum and S. verrucosum* (Table A2– Annex II) among 250 Basidiomycetes isolates following 2 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 (Hoffmann, 1991). The isolates were grown on medium supplemented with 5% glycerol (v/v) at 24°C. Sterile plastic straws, open at both ends, were used to sample the agar colonized by the fungal hyphae. The straws were then transferred into sterile cryovials, sealed and frozen in a programmable freezer to -70°C, with a controlled 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 three ECM fungi survived the preservation with the OP. To the contrary, with the CP, *E. clandestinum* presented 100% survival rate and *S. citrinum and S. verrucosum* isolates 50%. These fungi kept their extracellular laccase production unchanged (Homolka at al. 2003). As for the other isolates tested in this study, the CP using a very slow cooling (i.e. 1°Cmin^{-1}) rate yielded the best results. This was consistent with several other studies (Corbery and Le Tacon, 1997, Smith, 1998). Homolka et al. (2006) compared the OP to the Perlite Preservation (PP) protocol on 442 Basidiomycetes isolates among which some ECM fungi (i.e. *Clavariadelphus pistillaris, Entoloma clandestinum, Laccaria laccata, L. proxima, Scleroderma citrinum* and *S verrucosum* (Table A2– Annex II). In the PP protocol, the fungal colony was grown directly on 200 mg of perlite

in cryovials moistened with 1ml of wort and enriched with glycerol (final concentration 5%). The cryovials were incubated 14 days at 24°C before freezing at -70°C following a slow-cooling rate of 1°Cmin^{-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. To the contrary, Kitamoto et al. (2002) successfully preserved different ECM fungal isolates (e.g. *Boletus pulverulentus, Entoloma sp.* and *Hebeloma* spp.) for 10 years (Table A2– Annex II) with rapid cooling, by direct transfer in the freezer at -85°C. Obase et al. (2011) tested the cryopreservation of several ECM fungal isolates (i.e. *Amanita ibotengutake*, 2 isolates of *Aminata sp*., *Cenococcum geophilum*, *Laccaria amethystina*, 2 isolates of *Lactarius sp. Lepista nuda, Paxillus involutus*, *Pisolithus tinctorius*, 4 isolates of *Rhizopogon sp*., 3 isolates of *Russula sp*. *Scleroderma sp.*, *Suillus granulatus, Suillus luteus*, *Suillus pictus*, *Suillus placidus*, and 2 isolates of *Tomentella* sp.), using 10% skimmed milk as cryoprotectant. This solution is habitually used for the freeze-drying of microorganisms. Hyphae agar disks of 30-45 days old cultures were freezed in cryovials after preconditioning in skimmed milk for 1h at 4°C. The organisms were then stored in a freezer for 3 h at -20°C, and transferred immediately to -70°C. Isolates were thawed rapidly at 37°C. Variation in revival was observed 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 the controls. Only 6 out of the 23 fungal isolates did not differ from the controls (i.e. *C. geophilum*, *L. nuda*, 2 *Rhizopogon sp.* isolates and *Suillus granulatus* and *Suillus luteus*) (Table A2– Annex II). Stielow et al. (2011) tested another

protocol for the cryopreservation of 18 isolates of ECM fungi (i.e. *Hysterangium stoloniferum, Geastrum triplex, Mutinus elegans, Gautieria morchelliformis, Serpula lacrymans, Serpula himantioides, Melanogaster broomeianus, Paxillus involutus, Rhizopogon luteolus, Tapinella panuoides, Boletus edulis, Clitocybe gibba, Gymnomyces xanthosporus* and *Tuber borchii*). The protocol involved the growing of the fungal isolates on charcoal filter paper strips (CFS) placed on the surface of a culture medium for 3-5 weeks. The CFS were collected in a sterile Petri plate, incubated in 10% (v/v) for 1-2 min and transferred into cryovials by layering each of the CFS on top of each other. The closed cryovials were placed 24 h in the gas phase of a LN tank (i.e. at a cooling rate of approx. 1-10°Cmin^{-1} until -120 to -140°C), before direct transfer into LN. This protocol was compared to the conventional straw preservation protocol. This method consist in pushing hyphae plugs, grown on medium flooded with a cryoprotectant (glycerol, 5% w/v), within straws, before transfer in cryovials and subsequent freezing in the gas phase of LN for 24h before transfer into LN. Fungi were revived by rapid thawing to 25-30°C. Viability was 100% for 13 isolates with the CFS protocol (Table A2– Annex II). With the exception of *Clitocybe gibba* and *Boletus edulis* none of the isolates survived freezing with the conventional straw technique.

Recently, Crahay et al, (2013a) developed a preservation protocol (Fig. 4) efficient for nearly one hundred ECM fungal isolates belonging to 8 species (*Cortinarius sp., Hebeloma crustuliniforme, Laccaria bicolor, Lactarius rufus, Paxillus involutus, Suillus bovinus, Suillus luteus* and *Suillus variegatus*) (Table A2– Annex II). These authors compared the survival of the isolates using the straw protocol (SP) of Hoffman (1991) and

the cryovial protocol (CP) of Voyron et al. (2009). The differences between these protocols were in the preparation of the isolates and their conditioning before cryopreservation. In both protocols, 10% glycerol (v/v) was used and the same rate of decrease in temperature was followed: 8°Cmin^{-1} from +20°C to +4°C; 1°Cmin^{-1} from +4°C to -50°C; 10°Cmin^{-1} from -50°C to -100°C. The isolates were then directly transferred into a freezer at -130°C.

With the SP (Hoffman,1991), the cultures were grown on medium for 2 or 4 weeks, depending on the isolate, submerged with the cryoprotectant and incubated for 1-2 hours. Hyphal plugs were then pushed into straws and transferred in cryovials. With the CP (Voyron et al. 2009), the cultures were grown directly in cryovials for 7-9 weeks. The hyphae were then covered for 1-2 hours with glycerol before cryopreservation. For revival, cryovials were directly thawed at +38°C. Greater survival rate of the cryopreserved ECM fungal isolates was obtained with the CP as compared to the SP. 89% of the isolates survived with the CP suggesting its adequacy to a large set of ECM fungi. Contrarily to the study of Obase et al. (2011), 2 out of 3 *Paxillus involutus* isolates survived this preservation protocol.

Crahay et al. (2013a) suggested that the preparation of the culture was the key factor for survival to cryopreservation. In the CP, as in other studies (Homolka et al. 2003, 2006; Stielow et al. 2011), the ECM fungi were grown directly in the cryopreservation containers (i.e. cryovials) avoiding manipulations and damages to the hyphae before freezing. The density of hyphae was also greater than with the SP and the whole colony rather than the margin of the colony was cryopreserved. The ECM fungal cultures were older with the CP (7-9 weeks) than with the SP (2-4 weeks). This is consistent with the general statement that filamentous fungi from the

late exponential phase and stationary phase survive freezing better than actively growing cultures (Tanghe et al. 2003; Tan and van Ingen, 2004).

Crahay et al. (2013b) further tested the ability of eight ECM fungal isolates cryopreserved for 6 months at -130°C using the CP protocol to colonize *Pinus sylvestris* roots and to transport inorganic phosphate and NH_4^+ from the substrate to the plant. Overall, this mode of preservation had no significant effect on the colonization rate of *P. sylvestris*, the concentration of ergosterol in the roots and substrate, and the uptake of Pi and $NH4^+$as compared to the non-cryopreserved controls.

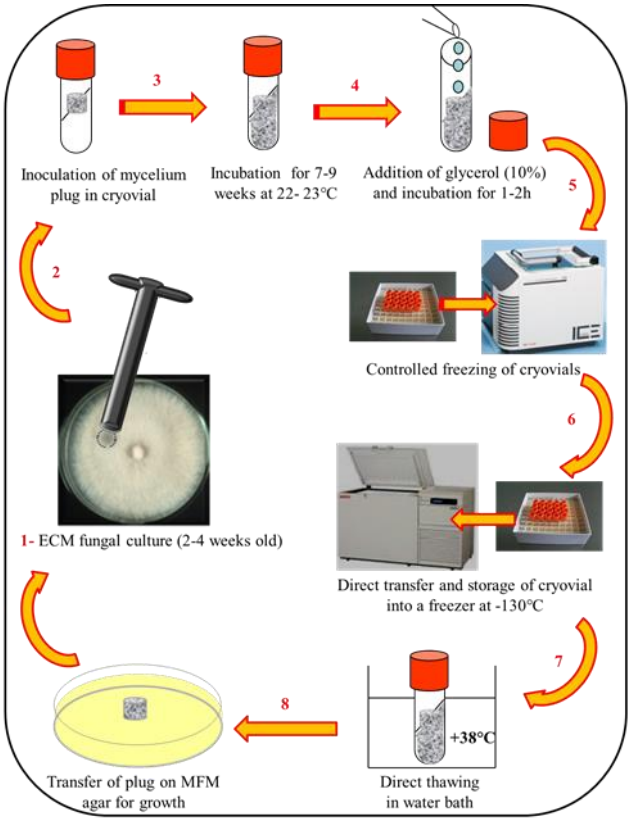

Figure 4 Protocol for the cryopreservation of ectomycorrhizal fungi (from the method developed by Crahay et al. 2013a): (1) ECM fungal isolates are grown on agar medium in Petri plates and incubated in the dark at 22-23°C for 2 to 4 weeks. (2) A mycelium plug of ~ 4 mm diameter is sampled from the margin of the growing colony and inoculated into a 2ml 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 hours before cryopreservation. (5) The cultures are cryopreserved following a controlled decrease in temperature (8°Cmin^{-1} from +20°C to +4°C; 1°Cmin^{-1} from +4°C to -50°C; 10°Cmin^{-1} from -50°C to - 100°C). (6) The cultures are directly transferred into a freezer at -130°C. (7) For revival, the ECM fungal isolates are directly thawed in a water bath at + 38°C for 2 minutes. (8) Plugs of cultures are transferred on the centre of a Petri plate on 30 ml MFM and incubated at 22-23°C.

3- Methods of maintenance and preservation of AMF

While ECM fungi can grow axenically, AMF should be associated obligatorily with a compatible host plant to complete their life cycle. Their *in vitro* cultivation is limited to a number of species and therefore most short to long-term maintenance/preservation methods are based on pot cultures. Whatever, the mode of cultivation (*in vitro* or *in vivo*), the propagules (spores, vesicles, colonized root pieces) needs to be re-associated to a host. This characteristic is thus of major importance for the maintenance/preservation of these organisms. They should survive the preservation process and be able to re-associate a plant root. Contrarily to ECM fungi, AMF produce spores both in and outside the roots, while the mycelium is often less abundant. Some genera also produce vesicles, which are intraradical structures involved in storage and reproduction (Smith and Read, 2008). Both structures (i.e. spores and to a lesser extend vesicles) are adequate for the preservation, while the mycelium, alone, is probably inappropriate.

AMF are mostly cultured *in vivo* and for a limited number of species *in vitro*. Several preservation methods, using propagules produced either *in vivo* or *in vitro*, have been developed and tested on AMF isolates.

i) In vivo maintenance/preservation of AMF

AMF are most often maintained on plants in the greenhouse or under growth chamber conditions. The first mention of a pot culture used to produce spores of a single member of AMF was by Mosse (1959). Currently,

in vivo cultures are used for research purposes, mass-propagation (see review by IJdo et al. 2011) and, in culture collections (e.g. IBG, INVAM), for germplasm conservation, distribution and/or taxonomic and molecular identification.

Different methods are used to initiate *in vivo* cultures (see review by IJdo et al. 2011). Soil containing AMF or propagules (i.e. spores, colonized roots) isolated from the field soil or from pot culture are mixed or layered in pots with sterilized substrate. A suitable plant is used as host and newly-produced propagules are obtained after several weeks or months according to the species. The propagules are then used as starter inoculum to initiate pot cultures in adequate, preferably sterilized, substrate. Cultures could also be started with single spores, which should be the preferred in germplasm collections. 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 commonly used for the maintenance of AMF. They are excellent plant hosts for a wide range of isolates. At INVAM (http://invam.wvu.edu/), sudangrass (*Sorghum sudanense* (Staph.) Piper) and sorghum (*Sorghum vulgare* L.) are routinely used for AMF maintenance (Morton et al. 1993). The host plants used at IBG (http://www.i-beg.eu/) are leek (*Allium spp*.), clover (*Trifolium pratense* L.), parsley (*Petroselinum crispum* L.) and *Tephrosia sp*., a tropical woody legume (Redecker, personal communication, 2013).

International culture collections such as INVAM and IBG maintain their isolates on actively growing plants in pots. At IBG, the isolates are sub-cultured every 1 to 2 years to minimize the risk of

viability loss and contaminations (Redecker, personal communication, 2013). At INVAM, the isolates are sub-cultured after 6 to 18 months (Morton et al. 1993; http://invam.wvu.edu/methods/inocula-storage/refrigeration-storage).

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 necessitate the development of alternative methods to maintain AMF isolates viable, pure and stable over long periods.

Different short and long-term AMF preservation methods have been developed in the past. Most used *in vivo* produced AMF propagules (i.e. spores and vesicles). Tommerup and Kidby (1979) preserved spores of four AMF species (i.e *Glomus caledonium*, *Acaulospora lae*vis, *Glomus monosporum* and *Gigaspora sp.*, isolated from 6 months-old pot cultures, by L-drying under vacuum (Table A2– Annex II). . They reported that the spores preserved in soil survived L-drying better than those extracted from the soil before L-drying. They also reported that slow dehydration of spores before L-drying (Table A2– Annex II) increased their viability. This is consistent with the findings of Ferguson and Woodhead (1982), reporting that AMF could be successfully stored in soil for at least 4 years at 5°C following air-drying. Even kept at room temperature, Young (1982) reported

that spores of *Glomus***,** *Acaulospora*, *Gigaspora* and *Sclerocystis* species dried in soil were able to germinate and colonize roots after 2 to 8 years storage. Wagner et al. (2001) stored spores of *Glomus claroideum* (Table A2– Annex II) at 4°C in soil for a period of at least 5 years. They observed that spores survived better at 4°C than at 24°C. At INVAM, most of the AMF isolates produced in pot cultures are stored in a walk-in cold room at 4°C. Spores of *Gigaspora* species could not be stored longer than 8 months because of their rapid degradation. Spores of *Scutellospora* species degraded less rapidly than those of *Gigaspora*. Spores of *Acaulospora* and *Entrophospora* species were the most resistant to storage (see http://invam.wvu.edu/methods/inocula-storage/refrigeration-storage).

Finally, for *Glomus* species, dark spores, large or small, tend to lose viability faster than small pale or colourless spores. Kuszala et al. (2001) successfully preserved 6 Glomalean species belonging to *Glomus* and *Acaulospora* genus in osmosed water at room temperature and at 4°C for up to 20 months (Table A2– Annex II). The isolates were extracted from 6-8 months old *in vivo* pot cultures and dried at room temperatures for 3-4 days. In the same study, these authors reported the successful storage by lyophilization of 10 AMF isolates belonging to *Glomus* and *Acaulospora* for up to 28 months (Table A2– Annex II).

Encapsulation of AMF propagules in alginate beads was used by several authors for storage before field application. Encapsulated AMF propagules were often stored at 4°C prior to use (Strullu and Plenchette, 1991). However, long-term storage decreased the viability and propagules infectivity (Plenchette and Strullu, 2003). These authors reported that

intraradical vesicles/spores of *Glomus intraradices* encapsulation in alginate beads, and stored at 4°C had a reduced infectivity after 62 months of storage.

Mugnier and Mosse (1987) found that *in vivo* cultured sporocarps of *Glomus mosseae* stored under saturated salt solutions at 4°C kept their viability for at least 4 years. Single stage lyophilization was also effective for some AMF isolates having spores with a thin wall such as *Glomus* species (i.e. *G. clarum*, *G. macrocarpum* and *G. pustulatum*) (Dalpé, 1987). Tommerup (1988) successfully preserved *in vivo* cultured *Acaulospora*, *Glomus Scutellospora* species for eight years at 4°C after L-drying under vacuum (Table A2– Annex II). However, these methods seem only valid for a reduced period of time.

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 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, the preservation was restricted to 3 months (Table A2– Annex II) and most of the *Scutellospora* and *Gigaspora* isolates and *Glomus clarum* isolate had a very low rate of germination. These authors suggested that the water content of soil was a determinant parameter in the success of the method. Kuszala et al. (2001) tested the preservation by direct freezing at -18°C of 20 Glomalean isolates belonging to 16 species and 4 genera (*Glomus*, *Acaulospora*, *Gigaspora* and *Scutellospora*) (Table A2– Annex II). The soils containing the isolates were from 6-18 months-old pot culture and provided by the IBG. The soils were first dried at room

temperature for 3-4 days before cold treatment. All the isolates survived storage 26 months. In their study, some isolates of *Glomus, Acaulospora* and *Gigaspora* survived direct storage in the freezer at -80°C for several months (Table A2– Annex II).

Kuszala et al. (2001) further succeeded in the cryopreservation by immersion in LN of 15 AMF isolates belonging to *Glomus* and *Acaulospora.* The cultures were isolated from 6-18 months old pot-cultures of the IBG and dried at room temperature for 3-4 days before storage (Table A2– Annex II). Lalaymia et al. (2013b) also succeeded in the cryopreservation of AMF propagules belonging to *Claroideoglomus*, *Septoglomus* and *Paraglomus* (Table A2– Annex II). The AMF were isolated from soil substrate of at least 5 months old pot cultures and cryopreserved one month at -130°C. The isolates were cryopreserved following the encapsulation-drying method developed by Lalaymia et al. (2012) (see the protocol detailed below for the *in vitro* produced isolates and Fig. 5). After one month storage at -130°C the isolates were able to colonize plant roots in pots and to produce new spores (Lalaymia et al. 2013b).

***ii) In vitro* maintenance/preservation of AMF**

The *In vitro* cultivation of AMF 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 (i.e. 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). This system was developed for fundamental research (e.g. transport studies – de Jaeger et al. 2011, gene expression analysis – Gallou et al. 2011) but could also be used 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.

Addy et al. (1998) were the first to demonstrated that *in vitro* produced extraradical hyphae of *G. intraradices* were able to survive at temperatures below 0°C (i.e. at -12°C) when slowly cooled before freezing (Table A2– Annex II).

Declerck and Van Coppenolle (2000) succeeded in the cryopreservation at -100°C of *in vitro* produced spores of an AMF [i.e. *Rhizophagus sp*. MUCL 41835]. The method was based on the encapsulation of spores in alginate beads followed by their incubation in trehalose (0.5M) before cryopreservation at -100°C in a two-steps temperature decrease (slow

(1°Cmin^{-1}) from +20°C to -35°C and rapid (18°Cmin^{-1}) from -35°C to -100°C). The spores remained viable after 3h of cryopreservation at -100°C and were able to reproduce the fungal life cycle *in vitro*. However, storage was not tested for more than 3h and when storage temperature was decreased to -140 and -180°C, spore germination dropped or was even not observed. Based on some factors selected by Declerck and van Coppenolle (2000) (i.e. encapsulation in beads, trehalose as cryoprotectant and the rapid thawing after cryopreservation), Lalaymia et al. (2012, 2013b) extended the method to 19 isolates belonging to *Rhizophagus*, *Glomus*, *Claroideoglomus* and *Gigaspora* genus produced *in vitro*. They improved the method of Declerck and van Coppenolle (2000) by drying the beads containing the propagules before cryopreservation. The rate of cooling was also faster as compared to the study of Declerck and van Coppenolle (2000). The AMF isolates were preserved at -130°C for up to 6 months (Table A2– Annex II). This method comprises 5 steps (Fig. 5): (1) the encapsulation in alginate beads of propagules (i.e. spores and mycorrhizal root pieces) isolated from 5 month old cultures, (2) the incubation overnight in trehalose (0.5M), (3) the drying of the beads during 48h at 27°C (i.e. at 8.1 ± 4.6% of beads water content), (4) the cryopreservation in the freezer at -130°C following a two-step decrease in temperature: a fast decrease (~12°Cmin^{-1}) from room temperature (+20°C) to -110°C followed by a slow decrease in temperature (~1°Cmin^{-1}) from -110°C to -130°C, and (5) the direct thawing in a water bath (+35°C). These authors reported that the survival after cryopreservation using this cooling rate was better than preservation in LN or following the cooling procedure used by Declerck and van Coppenolle (2000). After one to six months storage at -130°C the isolates kept their capacity to associate an excised root *in vitro* and to produce new spores. The percentage of root

colonization, number of spores produced and the architecture of hyphae (e.g. length of hyphae, presence and number of branched absorbing structure and number of anastomosis) did not differ between the cryopreserved and non-cryopreserved isolates (Lalaymia et al. 2013a). Similarly, no differences were noted in the activity of alkaline phosphatase and acid phosphatase 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).

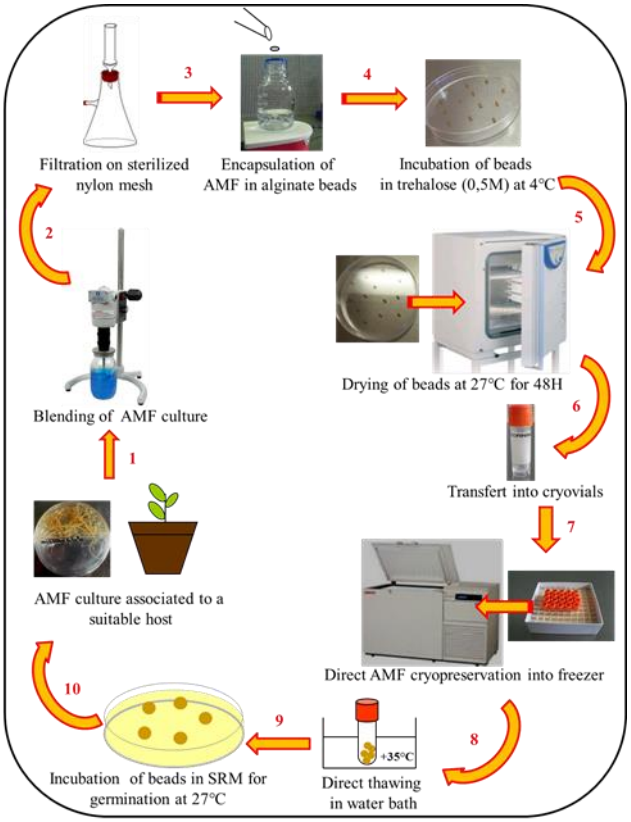

Figure 5 Protocol for the cryopreservation of AMF (from the method developed by Lalaymia et al. 2012): (1) Gelling medium, containing spores and roots of a 5 months old *in vitro* culture is extracted from the Petri plates and poured in 100 ml of sterilized (121°C for 15 min) deionized water and subsequently blended two times for 30 s at the 20,000 rpm of a sterilized (121 C for 15 min) mixer. (2) The mixture is filtered on a sterilized (121°C for 15 min) nylon mesh (40 mm). (3) The supernatant (i.e. spores and mycorrhizal/non-mycorrhizal root pieces) is encapsulated in 2 % (w/v) solution of sodium alginate (50 ± 5 propagules in each bead). (4) The encapsulated propagules are incubated in trehalose (0.5 M) overnight and (5) dried at 27°C for 48h (bead water content is approximately 8.1 ± 4.6%). (6) The beads are transferred in 2ml cryovials. (7) The cryovials are cryopreserved in a freezer at -130°C following a two-step decrease in temperature: a fast decrease (~ 12°Cmin^{-1}) from room temperature (+20°C) to -110°C followed by a slow decrease in temperature (~ 1°Cmin^{-1}) from -110°C to -130°C. (8) For reviving, the encapsulated AMF propagules are

directly plunged in a water bath at +35°C. (9) The beads are dropped in 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 a transformed root under *in vitro* culture conditions to reinitiate the fungal life cycle.
Lalaymia et al. (2013b) adapted this cryopreservation protocol to *in vivo* produced propagules, as follows: Pot-cultures of 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 plants in pots containing a sterilized (2x15min at 121°C, with 12h interval) substrate. The plants are grown for at least 8 weeks in a growth chamber before assessing the fungal viability following cryopreservation.

General discussion

Significant progress has been made over the last few years in the maintenance/preservation of mycorrhizal fungi. Several methods have been developed and factors tested with pot- and *in vitro*-cultured isolates. Some of them are adequate for the short-term storage under standard infrastructure and equipment (e.g. sub-cultivation, preservation in alginate beads or in dried soil), while others are adequate for the long-term storage (i.e. cryopreservation or lyophilization), requesting specific equipment and infrastructures.

Below we summarize the most common short and long-term methods of maintenance/preservation of ECM fungi and AMF and emphasize the factors which are essential for their storage over long periods.

Maintenance and preservation of ectomycorrhizal and arbuscular mycorrhizal fungi

We conclude with a proposal for a protocol applicable, although not generalizable, to a large panel of these organisms.

For ECM fungi, the maintenance via sub-cultivation at room temperature or preferably at refrigerating temperature (i.e. ~ 4°C), or under methods that slow down fungal growth (e.g. under water and oil) is adequate and easy-to-apply at the level of the laboratory. These methods are adequate to maintain some ECM fungi for periods up to 20 years (Richter, 2008). However, they present the disadvantages of being time-consuming, prone to contamination and potentially genetically unstable over time. Thus, methods arresting the fungal metabolism such as lyophilization and cryopreservation are the more adequate for the long-term preservation of ECM fungi. They should be the preferred if the infrastructure and equipment is available and if the objective is to keep the organisms under stable conditions for long periods (i.e. typically in culture collections).

From the various lyo/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, (2) the integrity of the hyphae, (3) the age of the culture, (4) the cryoprotectant used, (5) the cooling rate and (6) the thawing rate.

- **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. (2011) or directly into cryovials (Crahay et al. 2013a) on a growth medium (e.g. MFM) submerged with a suitable cryoprotectant (i.e. most often

glycerol) prior to cryopreservation, and eventually at refrigerating temperature (close to 4°C), greatly enhance the survival of ECM fungi to cryopreservation. Growing the fungi on a carrier or in a cryovial avoid the excessive manipulations of the organism that may damage the colony/hyphae before preservation (see below, the next parameter). Flooding the culture with a cryoprotectant (i.e. generally 1 to 2 hours before cryopreservation) or even growing the culture on a medium supplemented with a cryoprotectant (Homolka et al. 2006) enhances the cytoplasmic content with protective compounds and further increases the survival of the organism during cryopreservation (Smith and Onions, 1994; Tan et al. 1991). Finally, the pre-exposure of the fungal culture to refrigerating temperature (generally 4°C) and/or heat shock could also enhance resistance to freezing. Low and high temperatures pre-treatment induce the accumulation of trehalose, polyol and produce heat shock proteins (HSPs) that protect cells from preservation stresses and damages (De Viriglo et al. 1990; Neves et al. 1991; Neves and Francois, 1992; Tan and van Ingen, 2004; Tereshina, 2005).

- **The integrity of the hyphae prior to preservation**. It is essential that the culture is not physically damaged prior to cryopreservation to minimize leakage of cytoplasm content (Houseknecht et al. 2012) and to avoid the propagation of ice, formed in the medium during freezing, inside the hyphae (Smith and Onions, 1994; Smith, 1998). Growing the fungus on a carrier or into cryovials, as described above, avoid unnecessary manipulations of the organisms and as such prevent injuries to the mycelium.

Maintenance and preservation of ectomycorrhizal and arbuscular mycorrhizal fungi

- **The age of the culture.** Cultures in the late phase or stationary phase of growth 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 phase of growth, the fungal cells are stressed and thus produce and accumulate compounds, such as trehalose (van Laere, 1989), polyols and polysaccharides (Fuller et al. 2004a). These compounds are involved in the protection of membranes and proteins from denaturation (Fuller et al. 2004a; Tan and van Ingen, 2004).

- **The cryoprotectant.** Although, this is not a generality, glycerol seems the most reliable (and is the most used) cryoprotectant for ECM fungi as well as for the majority of filamentous fungi (Smith, 1998). This penetrating solution protects the intra-hyphae compounds during cooling and prevents the cells from excessive dehydration.

- **The cooling rate.** The slow cooling of the organism (i.e. ~1°Cmin^{-1}) seems the most appropriate for the majority of ECM fungi. Mycelium, which is the predominant form of ECM fungal colony 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)

- **The thawing of ECM fungi.** Fast thawing by direct immersion in a water bath (i.e. at a temperature between 35 and 38°C) is often reported to prevent the risk of ice recrystallization occurring during slow thawing.

As a conclusion, for most ECM fungi, we would recommend to grow the organisms on membranes or directly into cryovials until 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 ($1°Cmin^{1}$) until storage (ideally at temperatures below -130°C), while thawing should be fast by direct plunging in a water bath at 35-38°C.

For AMF, the maintenance/preservation via sub-cultivation, either *in vitro* on synthetic growth medium, in association with transformed roots, or *in vivo*, in association with plants in the greenhouse, is the most widely used method. Similarly to ECM fungi, sub-cultivation is time-consuming, present the risk of contaminations and do 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 temperature (i.e. ideally below -130°C).

From the lyo/cryopreservation protocols reviewed above, we noted that six factors are particularly important for the long-term preservation of

AMF: (1) the drying, (2) the culture age, (3) the carrier, (4) the cryoprotectant, (5) the rate of cooling, and (6) the thawing of the AMF.

- **Drying.** The drying of soil containing AMF propagules or the drying of propagules isolated either from *in vivo* or *in vitro* cultures and included or not in a carrier, before long-term preservation, is a key factor for the Lyo/cryopreservation (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 lyo/cryopreservation, depending on the soil moisture. For isolates produced *in vitro*, the drying of the carrier (i.e. 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 – i.e. several days before preservation) fungal stress and thus, the production of natural protectant such as trehalose (see explanation above - Tan et al. 1991; Smith and Onions, 1994; Tan and van Ingen, 2004, Ocon et al. 2007). The natural cryoprotectants produced during drying could help to reduce ice crystal size or to convert the fungus cytoplasm into glassy state during rapid freezing, thus improving the chances of survival (Tan and van Ingen, 2004).

- **The culture age.** Whatever their mode of production (i.e. *in vitro* or *in vivo*), AMF propagules isolated from cultures in the stationary phase of growth are the most suitable for the 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 phase of growth (frequently associated with stress conditions), the fungus accumulate cellular compounds such as trehalose (Van Laere, 1989), polysaccharide and glycoproteins to protect the intra and extracellular mycelium integrity.

- **The carrier.** AMF propagules cultured *in vivo* are usually preserved in the soil substrate in which they are produced. Tommerup and Kidby (1979) reported that the spores preserved in soil survived L-drying better than spores isolated from soil. The encapsulation in alginate beads was 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, protect them against the potentially toxic or osmotic effects of the cryoprotectant during treatment and from the mechanical and oxidative stress during cryopreservation (Suzuki et al. 2005; Sakai and Engelman, 2007).

- **The cryoprotectant.** Most AMF long-term preservation methods applied so far were achieved without cryoprotectant and with propagules produced *in vivo* (e.g. pot-cultured organisms). Interestingly, Declerck and Van Coppenolle (2000) and Lalaymia et al. (2012) reported that the use of cryoprotectants (i.e. trehalose)

with *in vitro* produced cultures, but also with pot-cultured isolates is a key factor to the success of cryopreservation. Trehalose has the capacity to interact with phospholipids of the cell membrane to maintain their fluidity during freezing and desiccation (Crowe et al. 2001).

- **The cooling rate**. Contrarily to ECM fungi, fast cooling seems the preferred for AMF. Kuszala et al. (2001) succeeded in the cryopreservation of different species produced *in vivo*, by direct storage in LN. Identically, Lalaymia et al. (2012) reported that preservation of *in vitro* produced AMF isolates was the most effective by direct cooling in the freezer. This could be explained by the fact that AMF propagules (i.e. spores and/or vesicles) are less rich in water as compared to hyphae. If the encapsulated propagules or soil containing propagules are cooled slowly, excessive drying will occur resulting in propagules shrinkage and subsequent death.

- **The thawing of the AMF.** Whatever the cooling rate (i.e. slow or fast – Mazur, 1984; 2004), fast thawing is generally the preferred to prevent the risk of ice recrystallization after cryopreservation. Declerck and Van Coppenolle (2000) and Lalaymia et al. (2012, 2013a, 2013b) succeeded to revive cryopreserved AMF propagules cultured either *in vitro* or *in vivo* after fast thawing in a water bath at +35°C. Noticeably, the slow thawing at room temperature seems to be successful in the reviving of cryopreserved *in vivo* cultured propagules (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 occurs or only occur weakly.

As a conclusion, for AMF, whatever cultured *in vivo* or *in vitro*, we would recommend the use of propagules from cultures in the late or stationary phase of growth, for their preconditioning to lyo/cryo-stresses. Lyo/cryopreservation of AMF propagules should be conducted in a carrier (i.e. soil or alginate beads) to protect them from preservation stresses and facilitate their manipulation. The carrier should be dried before lyo/cryopreservation to prevent the water crystallization outside and inside the propagules. For *in vitro* cultured AMF propagules we would recommend the use of trehalose (0,5M) as cryoprotectant, while the *in vivo* cultured propagules could be preserved in dried soil without cryoprotectant. Decrease in temperature should be fast, by direct immersion in LN (at -196 °C) or in the freezer until temperatures below -130°C. The fast thawing is recommended by direct immersion in a water bath at 35°C to prevent ice recrystallization.

Stability assessment after long-term preservation of fungi

Long-term preservation at ultra-low temperature or following freeze-drying may result in morphological, physiological and genetic modifications (Smith and Ryan, 2012). It is therefore essential to assess the biological stability of the organisms (ECM fungi and AMF) following preservation.

Maintenance and preservation of ectomycorrhizal and arbuscular mycorrhizal fungi

Growth rate, culture morphology, metabolic activity and genetic stability are the most frequently used parameters for fungi (Smith and Ryan, 2012).

The effects of freezing on the integrity of fungi can be checked instantly using cryogenic light microscopy (Smith and Thomas, 1998). Similarly, after preservation, some morphological characteristics (e.g. form and color of spores, conidia and hyphae) can be checked by microscopy. Growth rate of cultures, number and abundance of spores or conidia can be evaluated using counting methods. Architecture and ramification of hyphae and pigmentation of culture can also be assessed (Voyron et al. 2009; Lalaymia et al. 2012, 2013a). Detection of enzymatic activity before and after preservation using APIZYM (i.e. a system based on the simultaneous detection of different enzymatic activities using the appropriate substrate) may be applied (Ryan et al. 2001; Smith et al. 2001; Homolka et al. 2010; Smith and Ryan, 2012). Detection of secondary metabolites production using HPLC may be used to compare the organisms before and after cryopreservation (Ryan et al. 2001). The retention of fungal pathogenicity against insect, animal or plant (Hajek et al. 1995; Ryan and Ellison, 2003) or the fungal ability to establish symbiosis with the plant (Lalaymia et al. 2012, 2013a, 2013b; Crahay et al. 2013b) may also be compared before and after preservation.

It has been shown (Ryan et al. 2001) that even if fungi are viable after preservation and exhibit the same morphology and physiological characteristics as before preservation, their genetic stability could be affected. Damage to the DNA and mutations upon cryopreservation have been addressed in several studies (Ryan et al. 2001; Voyron et al. 2009; Homolka et al. 2010). 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 for genetic stability assessment 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. Randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism analysis (AFLP) which could reproducibly detect larger number of polymorphic loci have provided evidence for stability after cryopreservation in fungi belonging to different phylum (Voyron et al. 2009; Camelini et al. 2012; Lalaymia et al. 2013a).

Future direction

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 the long-term preservation, notwithstanding the extended list of mycorrhizal fungi reported in Table A2 (Annex II). The research for straightforward conservation methods is most often empirical but remains essential to increase the number of mycorrhizal fungi kept under stable condition for long-term periods.

On a methodological point of view, several, easy-to-apply pre-conditioning methods should be assayed. For instance, it is advisable to grow fungi on poor medium before freezing or freeze-drying to stimulate their entry into the stationary phase of growth and the subsequent production of natural protectants such as trehalose and glycerol (Smith and Onions,

1994). It is also conceivable to test light to stimulate the fungal growth before freezing and freeze-drying (Tan and van Ingen, 2004). In fact, Tan and van Ingen, (2004) reported that the exposure to light enhance the sporulation with the production of large quantities of disaccharide and polyols in spores and conidia and thus could enhance the resistance of the fungi to low temperature storage.

When trying to improve or adjust the lyo/cryopreservation method, the light cryomicroscope and the differential scanning calorimeter could be useful. They can help in the visualization and measurements of the physical changes in water (i.e. freezing, melting and glass transition) during cooling and warming and thus the understanding of its effect on mycorrhizal fungi.

Regarding the *in vitro* yet-uncultivable ECM fungi and AMF and for those that produce insufficient propagules *in vivo* or *in vitro*, their preservation in association with a suitable host plant could be an alternative. The cryopreservation of dried fragments of mycorrhizal roots or their cryopreservation following encapsulation in alginate beads could be a good and practical method for mycorrhizal preservation. The cryopreservation of section of roots surrounded by ECM fungi mantle and section of AMF mycorrhized transformed root or plant host roots with root hairs may be an elegant approach to reproduce the fungal life cycle *in vitro* without the necessity to re-associate a novel root, 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 the 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*.

Acknowledgements

This work was supported by the European Community's Seventh Framework Program FP7/2007-2013 under grant agreement no. 227522, entitled "Valorizing Andean microbial diversity through sustainable intensification of potato-based farming systems.

Chapter II

Preservation at ultra-low temperature of *in vitro* cultured arbuscular mycorrhizal fungi via encapsulation-drying

Adapted from the article published in

Fungal Biology (2012) 116: 1032-1041

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My contribution to this chapter was approximately 85% and involved experimental design and work, data analysis and the writing of the manuscript. Statistical support was provided by Prof Xavier Draye from the Laboratory of Crop Physiology and Plant Breeding group at UCL. Co-authors of the chapter have been involved in revising the manuscript.

Preservation at ultra-low temperature of in vitro cultured AMF via encapsulation-drying

Preface

The preservation of biological material in a stable state is a fundamental requirement of most fungal collections. Among the various methods developed so far, ranging from serial sub-cultivation to methods that reduce/arrest growth and metabolism, cryopreservation is the most widely accepted method for achieving long-term storage. It is applied to an increasing range of fungal species. However, according to the literature, the long-term storage of *in vitro* cultured AMF using cryopreservation is challenging.

In order to develop a long-term cryopreservation protocol that guarantee the viability and stability of a large number of *in vitro* cultured AMF isolates, we initiated our study with a single AMF isolate, i.e. *Rhizophagus sp.* MUCL 43204. Once successfully established, the protocol was applied to other AMF isolates. *Rhizophagus sp.* MUCL 43204 was purchased from the Glomeromycetes *in vitro* collection (GINCO: http://emma.agro.ucl.ac.be/ginco-bel). This isolate is particularly adapted to the *in vitro* culture and produces large amounts of spores (i.e. hundred to thousands of spores are produced after 3 to 5 months). Different parameters were considered to develop a successful cryopreservation protocol: (i) the protection of AMF propagules in alginate beads, (ii) the water content of the beads, (iii) the age of the *in vitro* AMF culture, (iv) the rate of cooling used to reach the final ultra-low storage temperature and (v) the rate of thawing. The cryopreservation protocol was tested on 12 AMF isolates belonging to five species (i.e. *Rhizophagus sp.* MUCL 41833, *Rhizophagus sp.* MUCL 41835, *Rhizophagus sp.* MUCL 43195, *Rhizophagus sp.* MUCL 43196,

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Rhizophagus sp. MUCL 43204, *Rhizophagus sp.* MUCL 46239, *Rhizophagus sp.* MUCL 49424, *Rhizophagus irregularis* MUCL 43194, *Rhizophagus fasciculatus* MUCL 46100, *Glomus aggregatum* MUCL 49408, *Rhizophagus diaphanous* MUCL 49416, *Rhizophagus intraradices* MUCL 49410) After six months storage, the ability of the AMF isolates to germinate and reproduce their life cycle was assessed.

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Abstract

At present, over 250 species of arbuscular mycorrhizal fungi (AMF) have been identified, most of which being stored in international collections. Their maintenance is mostly achieved in greenhouse via continuous culture on trap plants or in *vitro* in association with excised root organs. Both methods are work-intensive and for the former present the risk of unwanted contaminations. The in *vitro* root organ culture of AMF has become an alternative preventing contamination. Nevertheless, the risk for somaclonal variation during the sub-cultivation process cannot be excluded. A method for the long-term conservation that guarantees the stability of the biological material is thus highly demanded to preserve the microorganisms and their genetic stability. Here, 12 AMF isolates cultured in *vitro* in association with excised carrot roots were encapsulated in alginate beads and subsequently cryopreserved. Several protocols were tested taking into consideration culture age, alginate bead pre-drying, and rate of decrease in temperature. The viability of the AMF isolates was estimated by the percentage of potentially infective beads (%PIB) that measure the % of beads that contain at least one germinated propagule. Thermal behaviour of alginate beads was analysed by a differential thermal calorimeter before and after drying to estimate the frozen and unfrozen water during the cryopreservation process. It was shown that the spore damage was directly related to ice formation during cryopreservation. The encapsulation and culture age were also determinant parameters for the successful cryopreservation. Irrespective of the AMF isolate, the optimal procedure for cryopreservation comprised five steps: (1) the encapsulation of propagules (i.e. spores and mycorrhizal root

pieces) isolated from 5 months old cultures, (2) the incubation overnight in trehalose (0.5 M), (3) the drying during 48 h at 27°C, (4) the cryopreservation in the freezer at -130°C following a two-step decrease in temperature: a fast decrease (~12°Cmin^{-1}) from room temperature (+20°C) to -110°C followed by a slow decrease in temperature (~1°Cmin^{-1}) from 110°C to -130°C, and (5) the direct thawing in a water bath (+35°C). The % PIB was above 70 % for all the isolates and even above 95 % for 11 out of the 12 isolates after several months of storage at ultra-low temperature. All the isolates kept their capacity to associate to an excised carrot root in *vitro* and to reproduce the fungal life cycle with the production of several hundreds to thousands of spores after 2 months. This method opens the door for the long-term maintenance at ultra-low temperature of AMF isolates within international repositories.

Keywords: Alginate bead, Arbuscular mycorrhizal fungi, Cryopreservation, Differential thermal calorimeter, Encapsulation-drying, Potentially infective beads.

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Introduction

Arbuscular mycorrhizal fungi (AMF) are root symbionts forming associations with an estimate of 80 % of land plants (Smith and Read, 2008). Their roles in nutrients uptake, plant growth improvement and biotic and abiotic stress alleviation have been widely documented (Requena et al. 2007) making these organisms important actors in plant production and ecosystem functioning (Van Der Heijden and Scheublin, 2007).

In the recent years, the taxonomy and phylogeny of AMF have received an increasing attention. The Glomeromycetes was proposed as a new fungal phylum (Schüßler et al. 2001) and several new families and genera were erected based on phylogenetic and molecular analyses (Schüßler and Walker, 2010; Oehl et al. 2011). Over 250 species are identified at present (Schüßler and Walker, 2010) and with the development of molecular tools for species identification (Oehl et al. 2011; Krüger et al. 2012; Young, 2012) it is expected that this species' list will markedly grow in the close future. It is therefore essential to preserve this diversity in collections to (1) increase the range of AMF available to the research community and industrial sector, (2) facilitate the access to AMF identified with the most up-to-date techniques, and (3) guarantee the purity and stability of the biological material.

Nowadays, the preservation of AMF within culture collections is mostly achieved via the continuous culture on trap plants under greenhouse facilities [e.g. the international bank of Glomeromycota (IBG) and the international culture collection of (vesicular) AMF (INVAM)]. This method

allows the conservation of an important number of AMF but is time, space and energy consuming and may also present the risk of unwanted contaminations (Douds and Schenck, 1990; Plenchette et al. 1996; Declerck and Van Coppenolle, 2000). Maintaining the AMF under *in vitro* culture conditions in association with excised, transformed or non-transformed roots (i.e. on root organ cultures (ROC)), is an alternative proposed by the Glomeromycota in *vitro* Collection (GINCO). With this system the risk of contaminations is circumvented. An increasing number of isolates are successfully grown in ROC (Cranenbrouck et al. 2005). However, this method is also work-intensive via the regular sub-cultivation necessary to maintain the isolates and the risk of somaclonal variation is not excluded (Plenchette et al. 1996; Cardenas-Flores et al. 2010). Therefore, a method for the long-term preservation that guarantees the stability of the biological material under minimum maintenance and restricted space is highly demanded to preserve the genetic resources and stability.

Several long-term preservation methods have been tested in the past on AMF isolates maintained in pot cultures. Douds and Schenck (1990) observed that drying of soil pot cultures containing AMF spores followed by freezing at -60°C to -70°C was satisfactory for several AMF isolates belonging to the genus *Rhizophagus*, *Gigaspora*, *Entrophospora*, *Acaulospora*, and *Scutellospora*. Single stage lyophilisation was also effective on some AMF having spores with a thin wall (Dalpé, 1987). Tommerup (1988) also successfully preserved a number of AMF species by L-drying. *Rhizophagus fasciculatus* (synonym *Glomus fasciculatum*) was preserved for 4 years at -4°C in a dried soil (Douds and Schenck, 1990). More recently, Declerck and Van Coppenolle (2000) were the first to

succeed in the cryopreservation of an AMF [i.e. *Rhizophagus sp.* Mycothèque de l'Université catholique de Louvain (MUCL) 41835] cultured *in vitro*. The technique was based on the encapsulation of spores in alginate beads followed by their incubation in trehalose before cryopreservation at -100°C in a two steps temperature decrease (slow ($1°Cmin^{-1}$) from +20°C to -35°C and rapid ($18°Cmin^{-1}$) from -35°C to -100°C). The spores remained viable after 3 h of cryopreservation at -100°C and were able to reproduce the fungal life cycle *in vitro*. However, the spores were only cryopreserved for a short period of time (3 h) and the method was only successfully reported for one isolate.

In recent years, the encapsulation-drying cryopreservation method was developed for the preservation of seeds and adapted to numerous plant species (Engelmann, 2004; Sakai and Engelmann, 2007; Engelmann et al. 2008) and to some algae (Hirata et al. 1996; Vigneron et al. 1997). In this technique, the biological material is encapsulated in alginate beads and osmotically dehydrated in sucrose. The beads are subsequently dried under sterile air or on silica gel to a water content of 20 % (fresh weight basis) before cryopreservation at ultra-low temperature (Fabre and Dereuddre, 1990).

In the present study, several experiments were conducted to test and adapt the encapsulation-drying cryopreservation method to several AMF species. In particular, the effects of culture age, drying, and cryopreservation cooling rate were tested on the germination potential of the spores after cryopreservation. In addition, the capacity of the preserved propagules to reproduce the fungal life cycle after association with a transformed carrot root was evaluated.

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Materials and methods

Biological material

Twelve AMF isolates originating from different biotopes were considered (Table 2). The isolates were purchased from the GINCO (http://www.mycorrhiza.be/ginco-bel/index.php) and provided in Petri plates in association with Ri T-DNA transformed carrot (*Daucus carota*, clone DC2) roots. The isolates were maintained on the modified Strullu-Romand (MSR) medium (Declerck et al. 1998), solidified with 3 gl^{-1} phytagel (SigmaeAldrich, USA). The Petri plates were incubated in an inverted position in the dark at 27°C. After 4-5 months, several hundred to thousand of spores were obtained in each Petri plate.

Table 2 AMF isolates tested for the encapsulation-drying cryopreservation method.

AMF isolates	Authorities	Synonymy	Local code	Other code	Origin	Biotope
Rhizophagus sp.	Schüßler and Walker 2010.	*Glomus sp.*	MUCL 41833	DAOM 233750	Canaries Islands	Tropical
Rhizophagus sp.	Schüßler and Walker 2010	*Glomus sp.*	MUCL 41835	DAOM 233751	Unknown (contaminant of a Danish isolate)	Unknown
Rhizophagus sp	Schüßler and Walker 2010	*Glomus sp.*	MUCL 43195	DAOM 212349	Wasaga beach, Outario, Canada.	Temperate
Rhizophagus sp.	Schüßler and Walker 2010	*Glomus sp.*	MUCL43196	DAOM 229456	Unknown (contaminant of a New Zealand isolate)	Unknown
Rhizophagus sp.	Schüßler and Walker 2010	*Glomus sp.*	MUCL 43204	DAOM 229457	Clarence-Creek, Ontario, Canada	Temperate
Rhizophagus sp.	Schüßler and Walker 2010	*Glomus sp.*	MUCL 46239	DAOM 234181	Cap-aux-Meules, iles-de-la Madeleine, Québec, Canada	Temperate
Rhizophagus sp.	Schüßler and Walker 2010	*Glomus sp.*	MUCL 49424	FTSR203	Martinique	Tropical
Rhizophagus irregularis	(Błaszk., Wubet, Renker and Buscot) Schüßler and Walker 2010 [as '*irregulare*']	*Glomus irregularis*	MUCL 43194	DAOM 181602 DAOM 197198	Pont-Rouge, Québec, Canada	Temperate

(continued)

Rhizophagus fasciculatus	Schüßler and Walker 2010	*Glomus fasciculatum*	MUCL 46100	NA	Unknown	Tropical
Glomus aggregatum[a]	N.C. Schenck and G.S. Sm, 1982.	NA	MUCL 49408	10573	Brittany, France	Temperate
Rhizophagus diaphanus	(J.B. Morton and C. Walker) Schüßler and Walker 2010	*Glomus diaphanum*	MUCL 49416	STR05-130A	Eschikon-lindau, Switzerland.	Temperate
Rhizophagus intraradices	(N.C. Schenck and G.S. Sm.) Schüßler and Walker 2010)	*Glomus Intraradices*	MUCL 49410	DAOM197198	Florida, USA	Temperate

NA= not applicable.
a = Species of uncertain position [Schüessler and Walker, (2010)]

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Encapsulation procedure

For each experiment described below, the AMF isolates were encapsulated in alginate beads following the procedure described by Declerck and Van Coppenolle (2000). Briefly, spores were isolated from the Petri plates by solubilisation of the MSR medium (Doner and Bécard, 1991) and further separated from roots with forceps, filtered on a sterilized (121°C for 15 min) nylon mesh (40 mm) and suspended in a 2% (w/v) sterilized solution (121°C for 15 min) of sodium alginate (acid sodium salt from brown algae, SigmaeAldrich, UK). Groups of 50 ± 5 spores were recovered with a micropipette and dropped into a sterilized (121°C for 15 min) solution of 0.1M $CaCl_2$ maintained under agitation during polymerization. After 30 min, the beads (22.16 ± 2.8 mg fresh weight, n = 10) were rinsed with sterilized deionized water (121°C for 15 min) and stored in Petri plates.

Experimental procedures

Experiment 1: impact of AMF culture age on the percentage of potentially infective beads (%PIB) following cryopreservation

Spores were isolated from 4, 4.5, and 5 months old cultures of *Rhizophagus sp.* MUCL 43204 and encapsulated in alginate beads as described above. The beads were subsequently stored overnight at 4°C in trehalose (0.5M) or in sterilized (121°C for 15 min) deionized water. The beads were then placed in 2 ml cryotubes and cryopreserved for 3 h at -100°C following a two steps decrease in temperature: a slow decrease ($1°Cmin^{-1}$) from room temperature (+20°C) to -35°C followed by a fast

decrease in temperature ($18°Cmin^{-1}$) from -35°C to -100°C. After cryopreservation, the beads were thawed by immersion for 15 min in a water bath set at 35°C. The beads were then dropped in sterilized (121°C for 15 min) MSR medium cooled in a water bath to 40°C. In addition, three controls were considered for each culture age and treatment: (i) non-encapsulated non-cryopreserved spores, (ii) encapsulated non-cryopreserved spores, and (iii) non-encapsulated cryopreserved spores. Twenty beads (i.e. replicates) containing each 50 ± 5 spores were considered per treatment. Data were expressed as the %PIB, i.e. containing at least one germinated spore (Declerck et al. 1996b). The %PIB was determined 4 weeks after cryopreservation and incubation on sterilized (121°C for 15 min) MSR medium at 27°C in the dark.

For each treatment, the ability of the encapsulated germinated spores to re-initiate a fungal life cycle following association with a transformed carrot root clone DC2 was tested on the MSR medium. The capacity of the AMF to produce new spores after association of one bead with carrot roots was checked after 5 weeks incubation in the dark at 27°C under binocular microscope.

Experiment 2: impact of drying on the %PIB and on the proportion of frozen water during cryopreservation

Spores of 5 months old cultures of *Rhizophagus sp*. MUCL 43204 were encapsulated in alginate beads, incubated at 4°C overnight in trehalose (0.5M) and dried for 24h in an incubator at 27°C. The beads were subsequently cryopreserved for 3 h at -100°C and thawed following the protocol described in experiment 1. In addition, three controls were

considered: (i) dried non-cryopreserved beads (ii) non-dried cryopreserved beads, and (iii) non-dried non-cryopreserved beads. Twenty beads (i.e. replicates), containing each 50 ± 5 spores, were considered per treatment. The experiment was repeated four times on independent AMF cultures. The %PIB was determined 4 weeks after cryopreservation and incubation on sterilized (121°C for 15 min) MSR medium at 27°C in the dark. The proportion of frozen water formed in dried and non-dried beads during the first step of cooling cryopreservation program as described in experiment 1 (i.e. a decrease in temperature of 1°Cmin^{-1} from +20 to -35°C) was determined by calorimetric analysis using differential scanning calorimetry (DSC) conducted with a Mettler-Toledo DSC 821 (Mettler-Toledo, Leicester, UK). Before placing in the DSC chamber, each alginate bead was weighted singly on an analytic balance (Mettler-Toledo AG245), placed in an aluminium pan (40 ml) and sealed. The analysis was repeated four times on a single bead for each treatment.

The proportion of frozen water in the beads was calculated from the DSC curves, that is the percentage of the ratio between the enthalpy of the frozen water in the sample (i.e. bead) during the temperature decrease and the enthalpy of a gram of water (334.5 Jg^{-1}).

Experiment 3: impact of the cooling rate on the %PIB

Spores of 5 months old cultures of *Rhizophagus sp.* MUCL 43204 were encapsulated in alginate beads and dried for 24 h as above. The beads were subsequently cryopreserved for 3 h at three different temperatures (i) -100°C following a two steps decrease in temperature (as in experiment 1): a slow decrease (1°Cmin^{-1}) from room temperature (+20°C) to -35°C followed

by a fast decrease in temperature (18°Cmin^{-1}) from -35°C to -100°C, (ii) -130°C by direct placement in the freezer following a two steps decrease in temperature: a fast decrease (~12°Cmin^{-1}) from room temperature (+20°C) to -110°C followed by a slow decrease in temperature (~1°Cmin^{-1}) from -110°C to -130°C (estimated by a Chromel- Alumel thermocouple probe (ANRITSU-BT-22K-TC1-ANP model) fixed to a digital thermometer (AOKTON-Thermo scientific) placed in the centre of the cryotube containing the encapsulated spores), and (iii) -196°C following a very fast temperature decrease (200°Cmin^{-1}) by direct immersion in liquid nitrogen. Non-cryopreserved dried beads were used as control. Twenty beads (i.e. replicates), containing each 50 ± 5 spores, were considered per treatment. The experiment was repeated four times on independent AMF cultures. The %PIB was determined 4 weeks after cryopreservation and incubation on sterilized (121°C for 15 min) MSR medium at 27°C in the dark.

Experiment 4: impact of long-term bead drying on the %PIB

Spores of 5 months old cultures of *Rhizophagus sp.* MUCL 43204 were encapsulated as described above. The beads were subsequently dried in an incubator at 27°C for 1, 2, 3 and 5 days. For each period of drying, 10 beads (i.e. replicates), containing each 50 ± 5 spores, were weighted singly on an analytic balance (Sartorius-TE214S model) to determine their water content (i.e. water content of the beads was expressed as the ratio between means of fresh and dry weights). After each period of drying, the beads were incubated on the MSR medium at 27°C in the dark. The %PIB was determined after 10 days of incubation on sterilized (121°C for 15 min) MSR medium at 27°C in the dark. The integrity of spores was evaluated

visually under optic microscopy (Olympus-SZ61 model). The experiment was repeated four times on independent AMF cultures.

Experiment 5: impact of encapsulation-drying cryopreservation method of blended AMF cultures on the %PIB of 12 AMF isolates

For each isolate (Table 2), the gelling medium, containing spores and roots of 5 months old cultures, was extracted from the Petri plates and poured in 100 ml of sterilized (121°C for 15 min) deionized water. The medium containing the culture was subsequently blended two times for 30 s at 20,000 rpm of a sterilized (121°C for 15 min) mixer (Omni mixer Homogenizer-Omni International). The mixture was filtered on a sterilized (121°C for 15 min) nylon filter (40 mm) and the supernatant (i.e. spores and mycorrhizal/non-mycorrhizal root pieces) was encapsulated in each bead (50 ± 5 propagules, incubated in trehalose (0.5 M) overnight at 4°C, dried at 27°C for 2 days and cryopreserved in the freezer at -130°C). The isolates were cryopreserved for 1 day, 1 month, 3 months and 6 months. Non-cryopreserved beads were used as control. Twenty beads were considered per treatment. For each time of preservation, the %PIB was determined 4 weeks after cryopreservation and incubation on sterilized (121°C for 15 min) MSR medium at 27°C in the dark. In addition, the ability of the encapsulated propagules cryopreserved for 6 months to re-initiate the fungal life cycle was evaluated. Beads containing germinated propagules were associated with a transformed carrot root clone DC2 (two beads $root^{-1}$) and spore production and root colonization estimated after 2 months. Spore production was evaluated under a binocular microscope at 10-40X magnification. A 10 mm grid was marked on the bottom of each Petri plate to facilitate spore counting

(Declerck et al. 2004). Root colonization was assessed by staining according to the protocol described by Phillips and Hayman (1970). Colonization was estimated under a bright-field light microscope at 50-250X magnification, following the method of McGonigle et al. (1990). For each replicate, 300-350 root intersections were assessed. Both parameters (i.e. spores number and root colonization) were evaluated on five replicates isolate^{-1}.

Statistical analysis

The data were analysed using the software package SAS System (2008). The effect of cryopreservation was analysed using a two-ways contingency and the %PIB was analysed using the logistic regression ($P < 0.05$) categorical independent variable. Data on the proportion of water content and the thermal analysis of alginate beads were submitted to one-way analysis of variance (ANOVA) ($P < 0.05$).

Results

Experiment 1: impact of AMF culture age on the %PIB following cryopreservation

Whatever the culture age, the encapsulation of spores had no detrimental effect on the %PIB as compared to the non-encapsulated spores, before cryopreservation. For each treatment, the %PIB was 100 % (Table 3). To the contrary, after cryopreservation, the %PIB was higher for the spores

encapsulated in beads as compared to the non-encapsulated spores, whatever the culture age. Indeed, in the absence of encapsulation, no germination was observed whatever the treatment (%PIB = 0 %). Conversely, with the exception of the spores encapsulated and incubated in deionized water, germination was observed whatever the culture age. The culture age significantly impacted the %PIB of encapsulated cryopreserved spores ($P < 0.0001$). The %PIB of beads containing spores sampled from the 4.5 and 4 months old culture was 50 and 25 % respectively. Interestingly the highest %PIB was observed with spores isolated from 5 months old cultures. The %PIB was 80 % and significantly differed as compared to the %PIB of encapsulated spores isolated from 4.5 and 4 months old cultures.

Table 3 %PIB of beads before and after cryopreservation at -100°C for 3 h of encapsulated and non encapsulated AMF spores of *Rhizophagus sp.* MUCL 43204 cultured *in vitro* and isolated from 4, 4.5 and 5 months old cultures after incubation in different cryoprotectant.

		%PIB			
		Before cryopreservation		After cryopreservation	
Cryoprotectant	culture age (months)	Non encapsulated(1)	Encapsulated	Non encapsulated(1)	Encapsulated
Deionized water	5	100*	100	0.0*	0.0
	4.5	100	100	0.0	0.0
	4	100	100	0.0	0.0
Trehalose (0.5M)	5	100	100	0.0	80[a]
	4.5	100	100	0.0	50[b]
	4	100	100	0.0	25[b]

%PIB was estimated after 4 weeks incubation on the MSR medium in the dark at 27°C of beads containing 50 spores ± 5. (1)Non encapsulated isolated spores (group of 50 spores ± 5). Twenty replicates were considered per treatment. Values in a column followed by an identical letter did not differ significantly (logistic regression categorical independent variable). *No statistical analysis were done for %PIB = 0 and 100 because of the impossibility to calculate the variance (logistic regression categorical independent variable). Values of %PIB > to 80% were not significantly different to %PIB of 100% (2-way contingency statistical analysis).

For each bead showing spore germination, profuse hyphal re-growth was observed within the beads, extending through the $CaCl_2$ coating in the MSR medium (Fig. 6). The protruding hyphae were able to colonize the carrot roots and to produce hundreds of new spores (data not shown) within a period of 5 weeks.

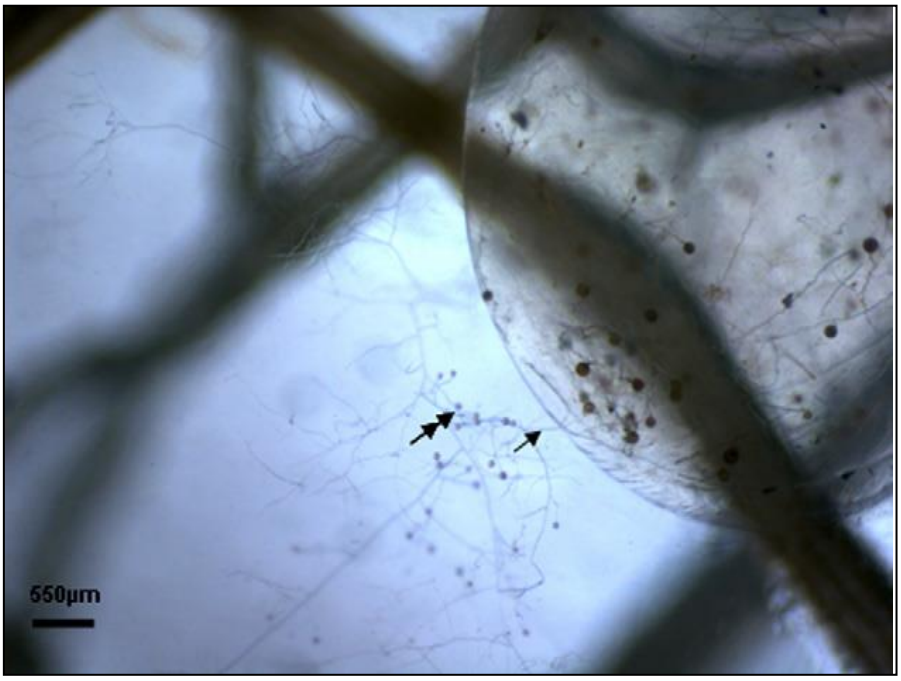

Figure 6 Encapsulated spores of *Rhizophagus sp.* MUCL 43204 incubated in trehalose (0.5 M) and cryopreserved for 3 h at -100°C, associated to a transformed carrot root. Details showing hyphal re-growth through the calcium chloride coating (arrow) and spore production (double arrow).

Experiment 2: impact of drying on the %PIB and on the proportion of frozen water during cryopreservation

The %PIB of encapsulated spores of *Rhizophagus sp*. MUCL 43204 isolated from four different cultures (i.e. considered as independent assays) and dried for 24 h were evaluated after cryopreservation at -100°C for 3h.

Whatever the assay, the drying of beads before cryopreservation had no detrimental effect on the %PIB. For each assay, the %PIB was 100 % (Table 4). To the contrary, after cryopreservation at -100°C for 3 h, the %PIB of the non-dried beads was zero for all the assays (Table 4). For the dried beads, the % PIB significantly differed after cryopreservation as compared to the %PIB of non-cryopreserved dried beads for all assays. Significant differences were observed between the independent assays ($P < 0.0001$) with %PIB varying from 5% to 75% (Table 4).

Table 4 %PIB after encapsulation-drying and cryopreservation at -100°C for 3h of beads containing spores isolated from 5 months old cultures of *Rhizophagus sp.* MUCL 43204

	%PIB			
	Before cryopreservation		After cryopreservation	
Replicates	Non dried	Dried	Non dried	Dried
1	100*	100	0.0*	70[a]
2	100	100	0.0	75[a]
3	100	100	0.0	10[a]
4	100	100	0.0	5[b]

%PIB was estimated after 4 weeks incubation on the MSR medium in the dark at 27°C of beads containing 50 spores ± 5. For each independent assay, twenty replicates were considered per treatment. Values in a column followed by an identical letter did not differ significantly (logistic regression categorical independent variable). *No statistical analysis were done for %PIB = 0 and 100 because of the impossibility to calculate the variance (logistic regression categorical independent variable). Values of %PIB > to 80% were not significantly different to %PIB of 100% (2-way contingency statistical analysis).

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The DSC data for beads osmohydrated with trehalose and dried or not are presented in Fig. 7. The non-dried beads showed an exothermic peak associated with ice formation at -7.96 ± 0.6°C, with an enthalpy ranging between 214.82 and 333.22 Jg^{-1} (Fig. 7) corresponding to a frozen water proportion of 79.9 ± 14.4 %. The beads dried for 24 h at 27°C had a significant impact ($P < 0.0001$) on the ice nucleation during freezing. The proportion of frozen water decreased to zero after 24 h drying which is supported by the disappearing of the exothermic signal during the DSC analysis (Fig. 7).

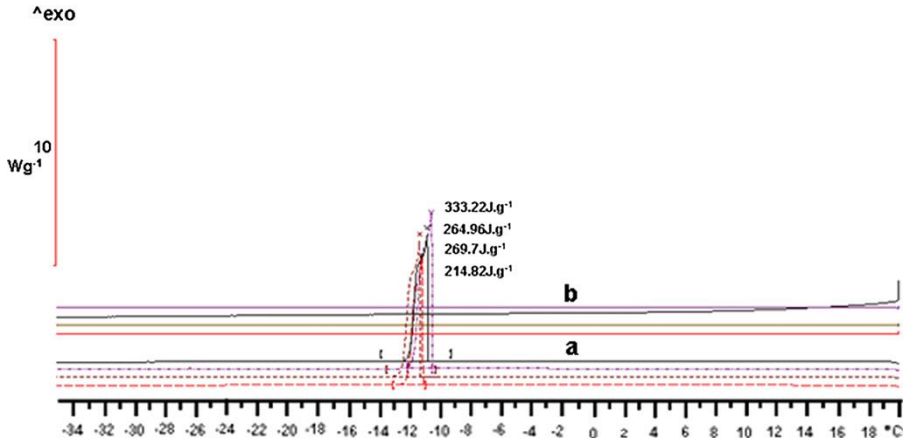

Figure 7 Thermograms resulting from thermal analysis (using DSC) of alginate beads containing spores of *Rhizophagus sp*. MUCL 43204. (a) Beads osmodehydrated with trehalose and not dried, (b) beads osmodehydrated with trehalose and dried for 24 h in an incubator set at 27°C. Each curve represents one bead (n = 4).

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Experiment 3: impact of the cooling rate on the %PIB

Beads, containing spores of *Rhizophagus sp*. MUCL 43204 sampled from 5 months old cultures were dried at 27°C for 24 h and cryopreserved 3 h at -100, -130, and -196°C following three different cooling rates as described above.

The encapsulation and drying (24 h at 27°C) of beads containing spores did not affect the %PIB before cryopreservation (Table 5). For each assay, the %PIB was 100 %. The three different cooling rates tested significantly affected the %PIB ($P < 0.0001$) after cryopreservation for 3 h. Irrespective of the assay, the highest %PIB was observed for the beads cryopreserved at -130°C, i.e. following a two steps decrease in temperature: a fast decrease (~12Cmin°C) from room temperature (+20°C) to -110°C and subsequent slow decrease in temperature (~1°Cmin^{-1}) from -110°C to -130°C (i.e. %PIB = 75 ± 13.5). Indeed, the %PIB of beads cryopreserved at this temperature was significantly higher as compared to the %PIB of beads cryopreserved at -100°C involving a two steps decrease cooling rate until -100°C (i.e. a slow decrease (1°Cmin^{-1}) from room temperature (+20°C) to +35°C followed by a fast decrease in temperature (18°Cmin^{-1}) from -35 °C to -100°C) (i.e. %PIB = 37.5 ± 25) and to the %PIB of beads cryopreserved with direct freezing in liquid nitrogen until -196°C (i.e. %PIB = 27.5 ± 25.6) (very fast temperature decrease of 200°Cmin^{-1}) ($P < 0.0001$ and 0.0001 respectively).

Table 5 %PIB after drying (24h) and cryopreservation for 3 h following (i) a two steps decrease in temperature (1st step: decrease from +20 to -35°C (1°Cmin^{-1}), 2nd step: decrease from -35 to -100°C (18°Cmin^{-1})) until -100°C, (ii) by direct preservation in the freezer at -130°C with 2 steps decrease in temperature: (1st steps: decrease from +20°C to -110°C (~12°Cmin^{-1}), 2nd step: from -110°C to -130°C decrease (~1°Cmin^{-1})) and (iii) direct immersion in liquid nitrogen at -196°C (very fast cooling: 200°Cmin^{-1}) of beads containing spores sampled from 5 months old cultures of *Rhizophagus sp.* MUCL 43204.

	%PIB			
Assay	Before cryopreservation	2 steps decrease cooling rate until -100°C	2 steps decrease cooling rate until -130°	In liquid nitrogen at -196°C
1	100*	75^{a}	70^{a}	60^{a}
2	100	25^{b}	65^{a}	45^{a}
3	100	25^{b}	95^{a}	0*
4	100	25^{b}	70^{a}	5^{b}
Mean	100	37.5±25^{ab}	75±13.5^{bc}	27.5±25.6^{ac}

%PIB was estimated after 4 weeks incubation on the MSR medium in the dark at 27°C of beads containing 50 spores ± 5. For each independent assay, twenty replicates were considered per treatment. Values in a column followed by an identical letter did not differ significantly, Values of the mean ligne followed by different letters differ significantly (logistic regression categorical independent variable). *No statistical analysis were done for %PIB = 0 and 100 because of the impossibility to calculate the variance (logistic regression categorical independent variable). Values of %PIB > to 80% were not significantly different to %PIB of 100% (2-way contingency statistical analysis).

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Experiment 4: impact of long-term beads drying on the %PIB

Beads containing spores of *Rhizophagus sp*. MUCL 43204 isolated from 5 months old cultures were dried at 27°C for 1, 2, 3, and 5 days. The bead water content significantly differed with duration of drying (P = 0.0013). The mean dry weight and water content of beads decreased from 26.0 mg to 3.8 mg and from 84.9% to 6.3% respectively after 5 days drying (Fig. 8).

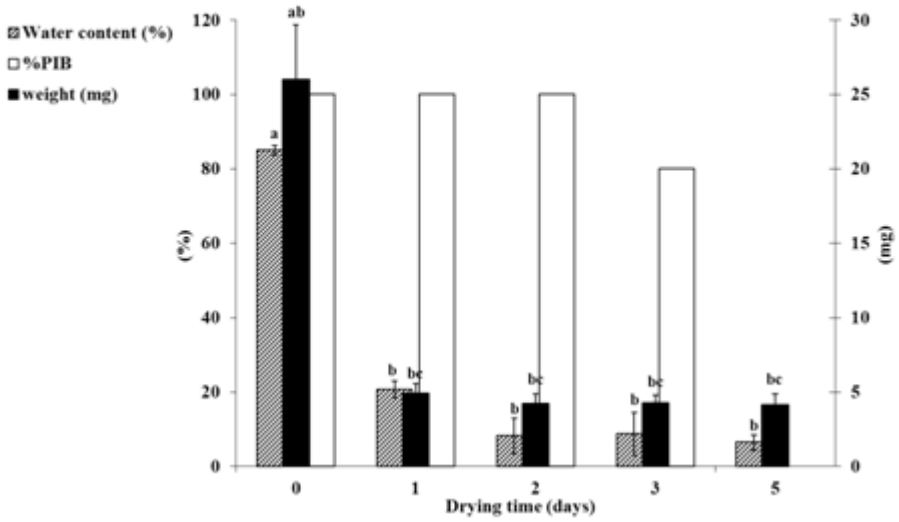

Figure 8 Effect of drying (days) on the weight of alginate beads (mg) containing spores of *Rhizophagus sp.* MUCL 43204 isolated from 5 months old cultures, the water content of alginate beads (%) and the %PIB. Beads contained 50 spores ± 5. Ten replicates were considered per treatment. The experiment was repeated 4 times on independent cultures. For each drying time, the data are a mean of 40 observations (± standard error). For each parameter bars with different letters are significantly different (one-way analysis of variance (ANOVA) (P < 0.05)).

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The % PIB measured after 10 days of incubation on the MSR medium (at 27°C in the dark) was 100 % for the beads dried 1 and 2 days (water content = 20.6 ± 2.1 and 8.1 ± 4.6%, respectively). After 3 days drying, the beads water content significantly decreased and the %PIB decreased to 80% (Fig. 8). After 5 days drying, germination was observed in the beads. The spores appeared empty (Fig. 9B).

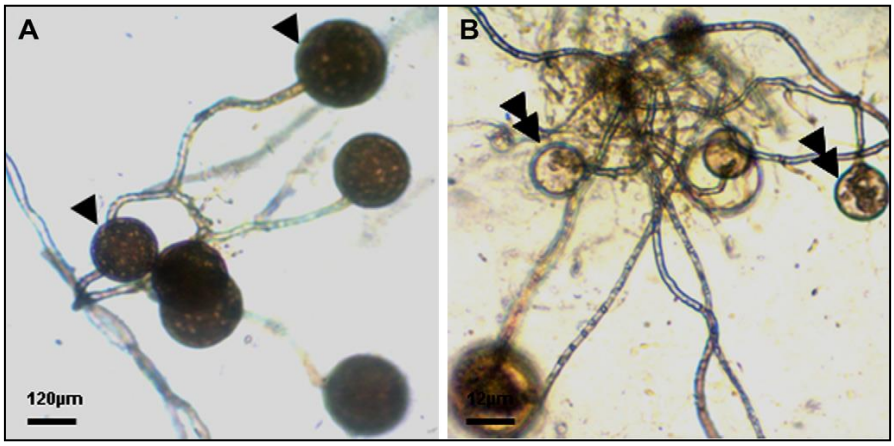

Figure 9 Spores of *Rhizophagus sp.* MUCL 43204 isolated from 5 months old cultures, encapsulated in beads and dried for 2 days (A) and 5 days (B) at 27°C. Details showing healthy spores (arrows) and damaged (empty) spores (double arrows).

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Experiment 5: impact of encapsulation-drying cryopreservation of crushed AMF cultures on the %PIB of 12 AMF isolates

The %PIB of encapsulated propagules (i.e. spores and non-mycorrhizal/mycorrhizal root pieces) of 12 AMF isolates (Table 2) isolated from 5 months old cultures, dried for 2 days before cryopreservation at -130°C for 1 day, 1 month, 3 months and 6 months is presented in Table 6.

Irrespective of the AMF isolates, the drying had no effect on the %PIB before cryopreservation. For each treatment, the %PIB was 100 % (Table 6). With the exception of encapsulated spores of *Rhizophagus fasciculatus* MUCL 46100 cryopreserved for 1, 3, and 6 months, no significant difference was observed in the %PIB whatever the isolate and the cryopreservation duration as compared with the non-cryopreserved ones. The duration of cryopreservation did not significantly affect the %PIB whatever the isolate ($P = 0.09$). Conversely, significant differences in the %PIB were noted between the AMF isolates ($P < 0.0001$). The %PIB of *Rhizophagus sp.* MUCL 41833, MUCL 41835, MUCL 43195, and MUCL 49424 and *Rhizophagus intraradices* MUCL 49410 remained unchanged (i.e. 100%), regardless of the cryopreservation duration. With the exception of *R. fasciculatus* MUCL 46100 (%PIB varied between 10% and 80% ($P = 0.0005$), the duration of cryopreservation did not affect the %PIB of the other isolates. Nevertheless, a high difference in the time of germination was observed between the cryopreserved and non-cryopreserved encapsulated spores of *R. fasciculatus* MUCL 46100. The germination of cryopreserved encapsulated spores of this isolate started 2 months later than the spores of the non-cryopreserved encapsulated treatment.

Table 6 %PIB after drying (2 days) and cryopreservation at -130°C for 1 day, 1 month and 3 months of beads containing AMF propagules (i.e. spores and mycorrhizal/non-mycorrhizal roots) sampled from 5 months old cultures of 12 AMF isolates. %PIB was estimated after 4 weeks incubation of beads containing 50 spores ± 5 on the MSR medium in the dark at 27°C.

	%PIB				
	Before cryopreservation	After cryopreservation			
AMF isolates		1day	1month	3months	6months
Rhizophagus sp. MUCL 41833	100*	100	100	100	100
Rhizophagus sp. MUCL 41835	100	100	100	100	100
Rhizophagus sp MUCL 43195	100	100	100	100	100
Rhizophagus sp MUCL 43196	100	95[a]	100	100	100
Rhizophagus sp. MUCL 43204	100	100	100	95[a]	100
Rhizophagus sp. MUCL 46239	100	100	95[a]	100	100
Rhizophagus sp. MUCL 49424	100	100	100	100	**
Rhizophagus irregularis MUCL 43194	100	85[a]	80[a]	100	90[a]
Glomus fasciculatus MUCL 46100	100	80[a]	45[b]	10[b]	70[b]
Glomus aggregatum MUCL 49408	100	95[a]	95[a]	100	100
Rhizophagus diaphanus MUCL 49416	100	100	100	95[a]	100
Rhizophagus intraradices MUCL 49410	100	100	100	100	100

%PIB was estimated after 4 weeks incubation on the MSR medium in the dark at 27°C of beads containing 50 spores ± 5. Twenty replicates were considered per treatment. Values in a row followed by an identical letter did not differ significantly (logistic regression categorical independent variable). *No statistical analysis was done for %PIB = 0 and 100 because of the impossibility to calculate the variance (logistic regression categorical independent variable). Values of %PIB > to 80 % were not significantly different to %PIB of 100 % (two-way contingency statistical analysis). ** There was not enough biological material of the same sets of culture to conduct the experiment until 6 months of cryopreservation.

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The ability of the encapsulated 6 months cryopreserved germinated spores to re-initiate the fungal life cycle was evaluated after 2 months of association (Table 7). Regardless of the isolate, spores production and root colonization (with hyphae, spores, vesicles, and arbuscules) was observed. The percentage of root colonization ranged from 4.5 ± 2.4 to 38.6 ± 17% in *Glomus aggregatum* MUCL 49408 and *Rhizophagus sp*. MUCL 41835 cultures, respectively. Spores production ranged from 615 ± 539 to 5359 ± 1733 in *Rhizophagus diaphanus* MUCL 49416 and *Rhizophagus sp*. MUCL 43204 cultures respectively.

Table 7 Estimation of spores production and root colonization of 12 AMF cultures from 6 months cryopreserved isolates after 2 months association with excised carrot roots in *in vitro* culture.

AMF isolates	Spore production (number)	Root colonization (%)
Rhizophagus sp. MUCL 41833	2655 ± 2255	13.8 ± 7.7
Rhizophagus sp. MUCL 41835	3291 ± 1620	38.6 ± 17
Rhizophagus sp. MUCL 43195	2356 ± 981	16 ± 3
Rhizophagus sp. MUCL 43196	3190 ± 828	15 ± 2
Rhizophagus sp. MUCL 43204	5359 ± 1733	33.7 ± 5
Rhizophagus sp. MUCL 46239	1472 ± 243	14.2 ± 3.6
Rhizophagus irregularis MUCL 43194	3013 ± 1367	13.2 ± 3.4
Rhizophagus fasciculatus MUCL46100	782 ± 536	14.8 ± 10.3
Glomus aggregatum MUCL 49408	644 ± 605	4.5 ± 2.4
Rhizophagus diaphanus MUCL 49416	615 ± 539	8.2 ± 2.2
Rhizophagus intraradices MUCL 49410	725 ± 494	9.2 ± 5.6

Discussion

Cryopreservation at ultra-low temperature is nowadays considered as the most suitable method to maintain the genetic stability of a large amount of filamentous fungi (Smith and Onions, 1994). This method was successfully applied to an *in vitro* cultured AMF, i.e. *Rhizophagus sp.* MUCL 41835, for a period of 3 h at -100°C (Declerck and Van Coppenolle, 2000). Several cryopreservation protocols were aasayed during the thesis and are appended in Annex I. Here we achieved the cryopreservation for several months at -130°C of twelve AMF isolates belonging to *Rhizophagus* genera (former *Glomus* Group Ab, '*Glomus intraradices* clade') using an encapsulation-drying procedure. Propagules (i.e. spores and mycorrhizal/non-mycorrhizal root pieces) isolated from 5 months old cultures, encapsulated in beads, incubated in trehalose and subsequently dried to ± 8.1% before cryopreservation at -130°C remained viable. The % PIB was above 70% after 6 months of cryopreservation for all the isolates and even above 95% for 11 out of the 12 isolates tested. The AMF were able to associate an excised carrot root *in vitro* and re-establish the fungal life cycle with the production of hundreds to thousands of new spores. This method opens the door for the long-term maintenance of AMF within international collections.

Whatever the treatment considered and experiment conducted, the encapsulation in alginate beads had no detrimental effect on the germination of spores, estimated before cryopreservation. Conversely, the cryopreservation was only successful for propagules encapsulated in beads and treated with trehalose. This corroborates earlier findings on the

cryopreservation of *Rhizophagus sp.* MUCL 41835 (Declerck and Van Coppenolle, 2000), conidia of several filamentous fungi (Chandler, 1994) and ectomycorrhizal fungi (Mauperin et al. 1987; Paloschi de Oliveira et al. 2006). Suzuki et al. (2005) and Martinez et al. (1999) suggested that alginate beads may protect plant shoot tips against the toxic effects of cryoprotectants during treatment and from mechanical and oxidative stress during cryopreservation. Alginate coating may also restrict cells' respiration and reduce cells' growth during storage (Brodelius et al. 1982). In our experiment, trehalose had no toxic effects on the AMF since the %PIB was 100% for non-cryopreserved non encapsulated spores. However irreversible damage was noted on the cryopreserved non encapsulated spores (in presence/absence of trehalose), consisting in the loss of cytoplasmic integrity and the release of lipid droplets out of the spores. This suggested that the encapsulation in beads in presence of trehalose is a determinant factor for the cryopreservation of AMF isolates.

Several cryoprotectants have been reported in the literature to protect cells from freezing-injury. In our experiment, no germination was observed in the absence of trehalose when encapsulated spores were cryopreserved. This cryoprotectant appeared effective whatever the age of the culture, even though its effectiveness was the highest with spores isolated from 5 months old cultures. This findings corroborated the earlier study conducted by Declerck and Van Coppenolle (2000). Trehalose is a natural disaccharide, non penetrating cryoprotectant. However, during cell freezing, the membrane becomes more permeable to its entry (Beattie et al. 1997). This cryoprotectant can with its hydroxyl groups substitute the cellular water molecules and interact with the polar residues of membrane and proteins,

which prevent ice formation and maintain membrane fluidity during freezing (Crowe et al. 1998).

In our experiment, it was clear that the age of the cultures was a critical parameter in the resistance of spores to cryopreservation. Spores isolated from 5 months old cultures had the highest %PIB. Spore production *in vitro* was shown to follow a sigmoid curve, with a lag, a log and a stationary phase (Declerck et al. 2000; Declerck et al. 2001). After 5 months culture, all the AMF isolates considered in our experiment were in the late stationary phase (data not shown). At this phase the AMF could be under stress, because of nutrients limiting conditions caused by medium depletion and excised roots growth arrest. The most important classes of fungi and yeasts react to cold-, heat-, drying-, osmo-stress and to the entry into a stationary growth phase by the production and accumulation of trehalose, polyols, glycoproteine, polysacharides and heat shock proteins, for membrane and proteins protection (Fuller et al. 2004a). In some algae, the accumulation of these compounds during the stationary phase was associated with a reduction in the vacuolar space rich in water susceptible to freeze during cryopreservation (Pyliotis et al. 1975). Smith (1998) demonstrated that old hyphae of some fungi showed lower ice nucleation during freezing that could be due to the high concentration of cytoplasmic content. In addition, Stürmer and Morton (1997) and Declerck et al, (2000) reported that mature spores of some *Rhizophagus* species developed thicker spore layers. This may increase the resistance to cryopreservation stress. Spore production is a dynamic process and it is obvious that the proportion of mature spores increased with the age of the culture. Therefore it is expected that the

proportions of mature spores isolated from 5 months old cultures are higher than 4.5 and 4 months old cultures.

Water content was another major factor impacting spore survival to cryopreservation in our experiment. Freeze-damage may be attributed to the extra and intra-ice crystals formation during the cryopreservation process. Even if the beads were osmodehydrated with trehalose, the water content remained high. The DSC analysis conducted in this study demonstrated that a high amount of water freezed (i.e. 79.87 ± 14.4%) in the alginate beads during cryopreservation, which could have damaged the spores. To the contrary, when beads were dried, no ice crystal formation was noted with the DSC thermogrames during cryopreservation. This may explain the higher %PIB observed after cryopreservation of dried beads containing AMF as compared with the %PIB of non dried cryopreserved encapsulated spores. In general the bead water content that ensures the highest survival after cooling is around 20%, which corresponds to the amount of unfrozen water in the samples (Panis and Lambardi, 2005; Engelman et al. 2008). However, this value may vary depending on the type of sample (Block, 2003). In our study, after 1 day of beads drying at 27°C, encapsulated spores were still viable and germinated. However, a variation in beads water content was observed between replicates. This variation was probably related to the bead size and to the air drying variation, because of the manual encapsulation by micropipette and of the airflow rate, temperature, and relative humidity variation. Actually, these variations affected the success of cryopreservation and reproducibility of the %PIB results after cryopreservation. Therefore, the optimal drying duration was shown to be 2 days (8.1 ± 4.6% of beads water

content). Beads treated with trehalose and dried for 2 days at 27°C had the highest and most homogenous %PIB.

The %PIB was the highest for encapsulated spores treated with trehalose, dried for 2 days and cryopreserved at -130°C in the freezer (by fast cooling (~12°Cmin^{-1}) until -110°C followed by a slow cooling (~1°Cmin^{-1}) until -130°C) as compared with the cryopreservation at -100°C with the controlled cooling (a slow decrease (1°Cmin^{-1}) from room temperature (+20°C) to -35°C followed by a fast decrease in temperature (18°Cmin^{-1}) from -35°C to -100°C) and with the cryopreservation in liquid nitrogen with the very fast cooling rate (i.e. 200°Cmin^{-1}). In common practices for fungal cryopreservation, fungi are cooled slowly at 1°Cmin^{-1} to dehydrate the cells and reduce the formation of the crystals within the cells. However, some fungi producing fragile thin-walled spores do not resist to slow cooling (Tan et al. 1998). In the slow cooling, at a rate lower than 10°Cmin^{-1} (Mazur, 1977), the bulk of extracellular water is slowly crystallized, leaving gradually a high extracellular concentrated solution (Mazur, 1984; Fuller et al. 2004a). As a result, the cells dehydrate gradually and the high dehydration could be lethal. Equally, when the cells are cooled very rapidly at a rate higher than 200°Cmin^{-1}, it may allow rapid intracellular and extracellular ice crystals production (Mazur, 1984; Meryman, 2007) which is lethal to the cells. During cooling, the response of the cells is determined largely by the water content of the cell. In our study, beads containing the spores were dried. Thus, a large proportion of osmotically active water that could damage the spores' membrane during cooling was removed.

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The encapsulation-drying cryopreservation protocol tested on the whole culture was particularly efficient. This method saved a considerable amount of time, used spores as well as mycorrhizal roots and was not harmful to the propagules integrity. The 6 months cryopreserved propagules germinated more or less after 4 weeks and newly produced spores were observed more or less 5 weeks after contact with the transformed carrot roots. The daughter spores were visually identical to the spores issued from non-cryopreserved mother spores. After 2 months of association the spores production and the percentage of roots colonization were close to the values reported in previous studies with species belonging to *Rhizophagus* genera (Declerck et al. 2000; Declerck et al. 2001; Gupta et al. 2002; Jaizme-Vega et al. 2003; de la Providencia et al. 2005; Voets et al. 2005; Fonseca et al. 2006).

In conclusion, the most optimal encapsulation-drying cryopreservation procedure developed in the present study consisted of five steps (1) the encapsulation in alginate beads of AMF propagules (i.e. spores and mycorrhizal root pieces) isolated from 5 months old cultures, (2) the incubation overnight in trehalose (0.5M), (3) the drying at 27°C for 2 days, (4) the cryopreservation in the freezer at -130°C with 2 steps decrease in temperature: 1) fast decrease (~12°Cmin^{-1}) from room temperature (+20°C) to -110°C followed by a slow decrease in temperature (~1°Cmin^{-1}) from -110°C to -130°C and (5) the direct thawing in a water bath set at +35°C. This protocol allowed the successful cryopreservation at -130°C for several months of 12 isolates belonging to 6 different AMF species.

Although numerous work reported that cryopreservation has no detrimental effects on morphological, physiological and on genetic stability

of plant, animal and filamentous fungi cells (Johnston et al. 2009); Labbe et al. 2001; Voyron et al. 2009), further experiments should be conducted on the genetic and morpho-physiological stability of AMF and on the adaptability of this method to species belonging to other genera.

Acknowledgements

The authors are grateful to Bart Panis (Laboratory of Tropical Crop Improvement, KUL; Belgium) for advice and to Jean-Jacques Biebuyck and Colette Douchamps (Unité de chimie et de Physique des hauts polymères, UCL; Belgium) for technical advices on the Differential Scanning Calorimetry. This work was supported by the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement N°227522, entitled "Valorizing Andean microbial diversity through sustainable intensification of potato-based farming systems".

Chapter III

Cryopreservation of *in vitro*-produced *Rhizophagus* species has minor effects on their morphology, physiology, and genetic stability

Adapted from the short note published in

Mycorrhiza (2013) DOI 10.1007/s00572-013-0506-y

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My contribution to this chapter was estimated at 90%. It was fully designed and conducted by myself. Co-authors of the chapter have been involved in revising the manuscript.

Cryopreservation of Rhizophagus species has minor effects on their stability

Preface

In **Chapter II**, the preservation at ultra-low (-130°C) temperature of *in vitro* cultured arbuscular mycorrhizal fungi (AMF) via encapsulation-drying, was reported for the first time. The isolates were able to germinate, reproduce the fungal life cycle and to sporulate abundantly following association with a carrot root *in vitro.* No significant differences were noted with the non-cryopreserved controls. However, no information is available on the possible modifications following cryopreservation. Therefore in **Chapter III** we investigated the morphology, metabolic activity and genetic stability of *in vitro* cultured *Rhizophagus* isolates cryopreserved for 6 months at -130°C by cryopreservation using the encapsulation-drying protocol. The results were compared to the same isolates that were not cryopreserved.

Cryopreservation of Rhizophagus species has minor effects on their stability

Abstract

Cryogenic storage is considered to be the most convenient method to maintain phenotypic and genetic stability of organisms. A cryopreservation technique based on encapsulation-drying of *in vitro* cultured arbuscular mycorrhizal fungi has been developed at the Glomeromycota *in vitro* Collection. In this study, we investigated fungal morphology (i.e. number and size of spores, number of branched absorbing structures (BAS), hyphal length, and number of anastomosis per hyphal length), activity of acid phosphatase and alkaline phosphatase in extraradical hyphae, and variation in amplified fragment length polymorphism (AFLP) profiles of *in vitro* cultured isolates of five *Rhizophagus* species maintained by cryopreservation for 6 months at -130°C and compared to the same isolates preserved at 27°C. Isolates were stable after 6 months cryopreservation. Comparing isolates, the number of BAS increased significantly in one isolate, and hyphal length decreased significantly in another isolate. No other morphological variable was impacted by the mode of preservation. Phosphatase activities in extraradical hyphae and AFLP profiles were not influenced by cryopreservation. These findings indicate that cryopreservation at -130°C of encapsulated dried and *in vitro* cultured *Rhizophagus* isolates (i.e. *Rhizophagus irregularis*, *Rhizophagus fasciculatus*, *Rhizophagus diaphanous*, and two undefined isolates) is a suitable alternative for their long-term preservation.

Cryopreservation of Rhizophagus species has minor effects on their stability

Keywords: Arbuscular mycorrhizal fungi, Cryopreservation, Encapsulation-drying, Genetic stability, Phosphatase activity, Amplified fragment length polymorphism (AFLP).

Cryopreservation of Rhizophagus species has minor effects on their stability

Introduction

Arbuscular mycorrhizal fungi (AMF) play an important role in plant biodiversity, variability of ecosystems, agricultural productivity, and plant protection against biotic and abiotic stresses (Van der Heijden and Scheublin, 2007). They are used in many national and international programs to improve crop yield and reduce the impact of environmental a/biotic stresses, while limiting the use of chemical inputs (Azcon-Aguilar and Barea, 1996). Preservation of their genetic, phenotypic, and physiological stability over long periods is thus required. Currently, AMF are maintained in collection in pot cultures and *in vitro* on root organs using serial sub-cultivation. These methods are time consuming and labor intensive. The risk of contamination is not negligible for the pot-cultured isolates, while the phenotypic stability and infectivity of the isolates cannot be formally guaranteed over years of routine maintenance for the *in vitro* material (Plenchette et al. 1996). An alternative method to the *in vitro* serial sub-cultivation is cryopreservation at ultra-low temperature. Cryopreservation is considered as the most reliable long-term storage method for most filamentous fungi (Smith, 1998). It requires limited space for storage and obviates the need for labor-intensive sub-cultivation. At ultra-low temperature, cell division and metabolic activities are arrested. The biological material may thus be preserved for an indefinite period of time.

Recently, Lalaymia et al. (2012) succeeded in the cryopreservation of 12 AMF isolates belonging to the genus *Rhizophagus* using encapsulation-drying for several months at -130°C. This method represents therefore a major breakthrough for culture collections. However, before

large-scale utilization, the impact of cryopreservation on morphological, physiological, and genetic stability of AMF isolates should be assessed. A number of studies have reported modifications in fungal characteristics after cryopreservation. For instance, Smith (1998) observed by cryomicroscopy, after cooling at -50°C, plasmolysis, and a 70% diameter reduction of hyphae of *Thanatephorus* species which are ectomycorrhizal (ECM) fungi. *Scleroderma flavidum*, another ECM fungus, survived cryopreservation but had weak regrowth capacity (Corbery and LeTacon, 1997). Obase et al. (2011) observed a reduced hyphal growth or complete inhibition of several ECM fungi after 6 months storage at -70°C. Finally, Ryan et al. (2001) detected genetic polymorphism in two isolates of *Metarhizium anisopliae* after storage at -196°C. From these examples, it is not excluded that similar problems may arise with AMF isolates following cryopreservation. In the present study, five AMF isolates belonging to different *Rhizophagus* species were cryopreserved for 6 months at -130°C following encapsulation-drying and compared to the same isolates maintained on root organ cultures (ROC) at 27°C. The morpho-anatomy (extraradical mycelium development, hyphal growth, production of branched absorbing structure (BAS), anastomosis, spore production and size, and root colonization), physiology (phosphatase activity), and genetic stability (evaluated by amplified fragment length polymorphism) are compared and discussed.

Material and methods

AMF species and isolates

Five *in vitro* cultured AMF isolates were considered: *Rhizophagus sp*. isolates MUCL 41835 and MUCL 43204 (Schüßler and Walker, 2010), *Rhizophagus irregularis* MUCL 43194 (B1aszk, Wubet, Renker and Buscot) (Schüßler and Walker, 2010), *Rhizophagus fasciculatus*MUCL 46100 ((Thaxt) Schüßler and Walker, 2010), and *Rhizophagus diaphanus* MUCL 49416 (Morton and Walker) (Schüßler and Walker 2010).

ROC of the five isolates were established in association with Ri T-DNA-transformed carrot (*Daucus carota)* roots clone DC2 on the modified Strullu-Romand (MSR) medium (Declerck et al. 1998) solidified with 3 gl^{-1} phytagel (Sigma Aldrich, USA), following the method detailed by Declerck et al. (1998). The Petri plates were incubated in an inverted position in the dark at 27 °C. After 5 months, several hundreds to thousands of spores were obtained in each Petri plate.

Cryopreservation procedure

For each AMF isolate, propagules (i.e. a mixture of extraradical spores and root fragments containing spores/vesicles - see Lalaymia et al. 2012) were cryopreserved at -130°C following the encapsulation-drying protocol developed by Lalaymia et al. (2012). Briefly, the propagules were isolated from 5 month old cultures and encapsulated in alginate beads. The beads were subsequently incubated overnight in trehalose (0.5 M), dried during 48 h at 27°C, and placed in a freezer at -130°C, following a two-step

decrease in temperature: a fast decrease (~12°Cmin^{-1}) from room temperature +20 to -110°C followed by a slow decrease in temperature (~1°Cmin^{-1}) from -110 to -130°C. Each alginate bead contained a mean of 50 ± 5 propagules. The beads were maintained for 6 months at -130°C. For each isolate, non-cryopreserved dried beads containing propagules were used as control and tested at the time the preserved isolates were frozen. Twenty replicates (i.e. beads) were considered per isolate and per treatment (i.e. cryopreserved or non-cryopreserved beads).

Regrowth after cryopreservation

For the non-cryopreserved treatment, the beads were placed directly after drying on 40ml sterilized (121°C for 15 min) MSR medium at 27°C in the dark for propagule germination.

For the cryopreserved treatment, the beads, following cryopreservation, were directly thawed in a water bath (+35°C) and placed on the MSR medium at 27°C in the dark as above for propagule germination.

After 4 weeks of incubation at 27°C, to evaluate the ability of the non-cryopreserved and cryopreserved encapsulated propagules to re-initiate the fungal life cycle, beads containing germinated propagules were placed in the vicinity of transformed carrot roots, clone DC2, (two beads per root) in Petri plates (90 mm diameter) containing 40 ml MSR medium. Ten Petri plates were set up per isolate and treatment. After 5 months, spores were extracted from three randomly selected *in vitro* cultures of each isolate and treatment and used to re-initiate 10 mono-compartmented and 10 bi-compartmented (St-Arnaud et al. 1996) *in vitro* cultures with transformed

carrot roots (clone DC2). After another 5 months, five mono-compartmented Petri plates were randomly selected per isolate and treatment for morphology and metabolic activity measurements. Similarly, five bi-compartmented Petri plate per isolate and treatment were used for genetic stability analysis. For each isolate, measurements were made at 6 month intervals, first on the isolates maintained on ROC at 27°C and then on the same isolates that had been cryopreserved for a period of 6 months at -130°C

Morphological stability

For each isolate and treatment, the total hyphal length, the number and size of spores, the number of BAS (Bago et al. 1998), and the number of anastomosis per hyphal length (de la Providencia et al. 2005) were evaluated on the five randomly selected mono-compartmented Petri plates (i.e. replicates). The total hyphal length was assessed using a 10 mm grid marked on the bottom of each Petri plate to form 10-mm squares, following the method described by Declerck et al. (2004). Spores were counted in each square formed by the gridlines (Declerck et al. 2004). BAS and anastomosis were recorded in each Petri plate and counted following the same process as described for spores. The measurements were conducted under a dissecting microscope at magnification 6.7X and 40X.

For each replicate, MSR medium was dissolved (Doner and Bécard, 1991). Twenty spores were randomly collected from each of the five replicates and their size was measured under stereomicroscope after mounting on a microscopic slide in polyvinyl alcohol, lactic acid, and glycerine (Koske and Tessier, 1983). Measurements were made with an eyepiece micrometer calibrated with a stage micrometer under a bright-field

light microscope (Olympus BH-2, Olympus Optical GmbH). Roots and extraradical mycelium were further collected from the dissolved MSR medium and percentage of root colonization was assessed following staining (Phillips and Hayman, 1970). Total colonization was estimated under a bright-field light microscope (Olympus BH-2, Olympus Optical GmbH) at 50-250X magnification using the method of McGonigle et al. (1990). For each replicate, 300–350 root intersections were assessed.

Enzymatic activity

For each replicate, hyphal samples were subjected to histochemical observations for the estimation of acid phosphatase (ACP) and alkaline phosphatase (ALP) activity according to Van Aarle et al. (2002). Hyphal samples stained for ALP or ACP activity were mounted on a microscope slide with lactoglycerol. Fast blue RR granular precipitation in the hyphae was assessed using a bright-field light microscope (Olympus BH-2, Olympus Optical GmbH) at 50X magnification. For each replicate, an approximation of 300-350 hyphal intersections was observed following the magnified intersects method (McGonigle et al. 1990). Intersections with hyphae were classified as active or nonactive, depending on whether they exhibited or not fast blue RR precipitation.

Genetic stability

Amplified fragment length polymorphism (AFLP) analysis was conducted on *Rhizophagus sp.* MUCL 41835, *Rhizophagus sp.* MUCL 43204, and *R. irregularis* MUCL 4319, from which sufficient DNA was

extracted for AFLP analysis. Spores and hyphae in the hyphal compartment from the five replicates of each isolate established from cryopreserved and non-cryopreserved encapsulated propagules were collected by gel dissolution and pooled. The samples were frozen in liquid nitrogen in 1.5 ml tubes and ground to a fine powder using a pestle. The DNA of each sample was extracted using DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The DNA was quantified by spectrophotometer (NanoDrop ND-1000, Isogen, USA). Genomic DNA was extracted twice from each non-cryopreserved isolate in order to detect variations in AFLP patterns due to differences in the DNA extraction process.

AFLP procedure

The AFLP analysis was conducted as specified by Cardenas-Flores et al. (2010) except that the restriction-ligation step for each isolate and treatment was performed on 200 ng DNA. Selective PCR was conducted with four primers couples: (1) EcoRI+AC-MseI+T, (2) EcoRI+TMseI+ CC, (3) EcoRI+AT-MseI+CC, and (4) EcoRI+GGMseI+TA (EcoRI primers labeled with D4 WellRED dye, Sigma-Proligo, Beckman Coulter license, USA). To detect the resulting DNA fragments, 2 µl of the selective PCR product was run on the CEQ™ 2000 XL DNA analysis system (Beckman Coulter, USA) under the conditions cited by Cardenas-Flores et al. (2010).

The reproducibility of the resulting AFLP patterns of the non-cryopreserved isolate was confirmed by three rounds of restriction–ligation–amplification reactions for each isolate on two different DNA extractions:

two independent rounds of reactions from the first and one from the second DNA extractions as described by Müller et al. (2007). A fragment position was counted as non-reproducible and excluded if it appeared in only one or two of the three replicated non-cryopreserved AFLP patterns. To estimate the technical error generated by the AFLP procedure, genomic DNA of *Rhizophagus sp*. MUCL 43204 was submitted to three independent rounds of restriction–ligation–amplification with all the primer combination described above.

AFLP data analysis

The fluorescently labeled amplified fragments were analyzed automatically with the fragment analysis software (CEQ 8000, Beckman Coulter, USA) and corrected manually. Polymorphic DNA fragments were scored by the program as 1 for presence and 0 for absence to generate a binary matrix. Only fragments with size between 80 and 400 base pairs with relative fluorescent units above 300 were scored. Fragments present in negative controls were excluded from the analysis.

In a binary matrix, the AFLP pattern of each cryopreserved isolate was compared with AFLP patterns of the three rounds of restriction–ligation–amplification reactions of its corresponding non-cryopreserved isolate with the Dice (Bonin et al. 2007) similarity coefficient and the unweighted pair group method of arithmetic averages using the XLSTAT software version 2012.6.02 (Addinsoft, 1995–2012). Fragment position that appeared to be non-reproducible in the three non-cryopreserved AFLP patterns resulting from the three rounds of restriction–ligation–amplification reactions of each isolate was excluded from comparison. The technical error

rate was then calculated in respective binary matrixes under simple match (m) (Bonin et al. 2007) similarities. Dissimilarities (i.e. the variation between two profiles) were calculated as d = (1 - m) × 100, where d=0 % corresponds to identical profiles and d=100 % to completely different profiles.

Statistical analysis

Statistical analysis was performed with the statistical software XLSTAT software version 2012.6.02 (Addinsoft, 1995–2012). The data were subjected to one- and two-way analysis of variance (ANOVA) and nested ANOVA. The Tukey's honest significant difference was used to identify the significant differences ($P < 0.05$).

Results

All five cryopreserved or non-cryopreserved AMF isolates were able to colonize roots, produce new spores, and extend hyphae within medium producing BAS and anastomosis (Table 8). Spores of each isolate were morphologically similar between both treatments. Treatments had no significant effect ($P = 0.681$) on the number of spores produced.

Table 8 Number of spores, number of branched absorbing structure, hyphal length, number of anastomosis per hyphal length, and spore diameter of five arbuscular mycorrhizal fungi grown in root organ culture following propagule encapsulation in alginate beads and drying-cryopreservation at -130°C as compared to non-cryopreserved encapsulated-dried isolates.

Isolate	**No. of spores**		**No. of branched absorbing structures**		**Hyphal length (cm)**	
	Non-cryopreserved (Control)	Cryopreserved	Non-cryopreserved (Control)	Cryopreserved	Non-cryopreserved (Control)	Cryopreserved
***Rhizophagus sp.* MUCL 41835**	9552 ± 3620 [a]	9861 ± 3546 [a]	4165 ± 672 [a]	4481 ± 973 [a]	4668 ± 1450 [a]	3215 ± 698 [a]
***Rhizophagus sp.* MUCL 43204**	10408 ± 1150 [a]	10685 ± 2061 [a]	4270 ± 819 [a]	3477 ± 517 [a]	4099 ± 1001 [a]	2828 ±681[b]
***Rhizophagus irregularis* MUCL 43194**	5931 ± 1395 [a]	6951 ± 2811[a]	2807 ± 469 [a]	3118 ± 574 [a]	2751 ± 665 [a]	2590 ± 1095[a]
***Rhizophagus fasciculatus* MUCL 46100**	6131 ± 1890 [a]	4053 ± 1555 [a]	2150 ± 526 [a]	2044 ± 607 [a]	3355 ± 870 [a]	2751 ± 650 [a]
***Rhizophagus diaphanus* MUCL 49416**	4647 ± 2026 [a]	3777 ± 1008 [a]	1133 ± 308 [a]	2945 ± 864[b]	3684 ± 377 [a]	3130 ± 449 [a]

Isolate	**No. of anastomosis per hyphal length (cm)**		**Spore diameter (µm)**	
	Non-cryopreserved (Control)	Cryopreserved	Non-cryopreserved (Control)	Cryopreserved
***Rhizophagus sp.* MUCL 41835**	0.17 ± 0.07 [a]	0.19 ± 0.05[a]	(30)-71-(116)[a]	(40)-72-(118)[a]
***Rhizophagus sp.* MUCL 43204**	0.19 ± 0.06 [a]	0.21 ± 0.06[a]	(32)-69-(116)[a]	(40)-72-(118)[a]
***Rhizophagus irregularis* MUCL 43194**	0.12 ± 0.02 [a]	0.18 ± 0.09[a]	(42)-81-(124)[a]	(40)-76-(116)[a]

(continued)

***Rhizophagus fasciculatus* MUCL 46100**	0.27 ± 0.06 [a]	0.33 ± 0.09[a]	(20)-41-(80)[a]	(18)-42-(76)[a]
***Rhizophagus diaphanus* MUCL 49416**	0.34 ± 0.08 [a]	0.44 ± 0.07[a]	(28)-49-(66)[a]	(32)-46-(74)[a]

For each parameter, values in the same line followed by identical letter did not differ significantly ($P \leq 0.05$, Tukey's HSD). Data represent the means of five replicates (mean ± SE). For spore diameter, values in the same line followed by identical letters did not differ significantly (nested ANOVA $P \leq 0.05$, Tukey's HSD). Data represent means and size distribution of diameter measurements based on 100 spores from five different replicates (i.e. 20 spores per replicate).

Cryopreservation of Rhizophagus species has minor effects on their stability

Overall, the treatment had no significant effect on the number of BAS (Fig. 10a) produced (P = 0.108). However, the number of BAS produced in cultures established from cryopreserved beads containing propagules of *R. diaphanus* MUCL 49416 was significantly higher (P = 0.002). Overall, the cultures established from the cryopreserved encapsulated propagules had a significantly lower hyphal length (P = 0.001). However, considering the isolates independently, this effect was only detected for the cultures established from the cryopreserved *Rhizophagus sp.* MUCL 43204 (P = 0.047). Whatever the isolate and treatment, anastomoses were observed in all the isolates (Fig. 10b). Overall, the cultures established from the cryopreserved encapsulated propagules produced a significantly higher number of anastomosis per hyphal length (in centimeter) (P = 0.019). Nevertheless, for each isolate considered independently, no significant difference was detected. Overall, the treatment had no significant effect on the spore size (P = 0.527) (Table 8). Similarly, color and shape of spores of each isolate were identical between the cultures established from cryopreserved and non-cryopreserved encapsulated propagules.

Cryopreservation of Rhizophagus species has minor effects on their stability

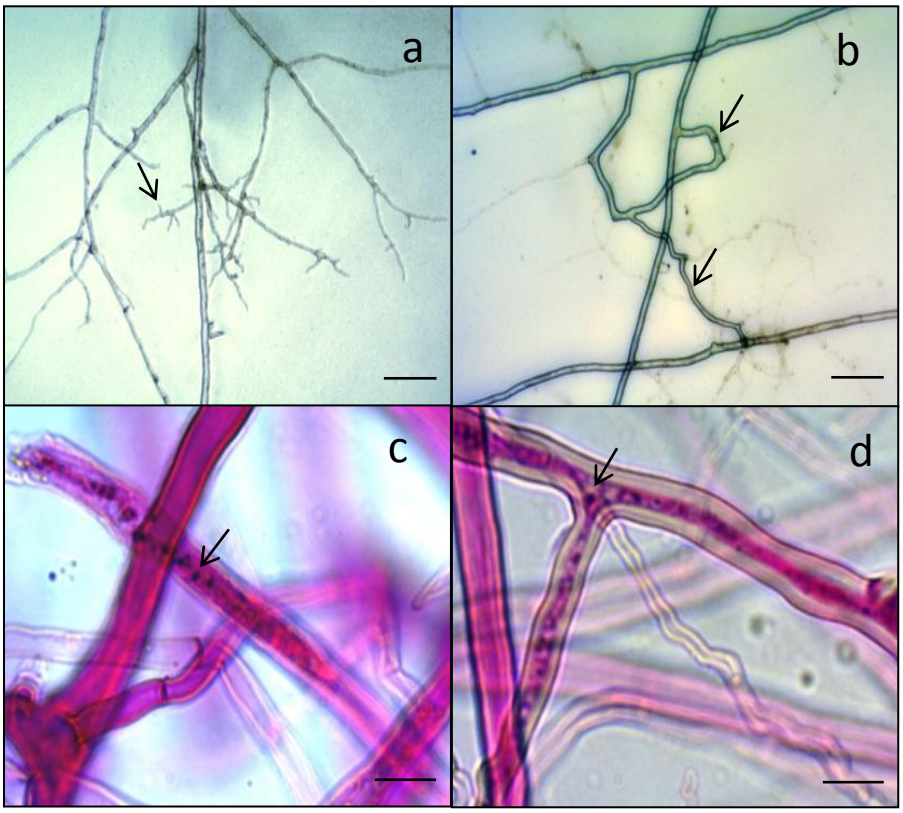

Figure 10 Branched absorbing structures of *Rhizophagus sp.* MUCL 41835 (a) (see black arrow), anastomosis of *Rhizophagus diaphanus* MUCL 49416 (b) (see black arrows), ALP-Active extraradical hyphae of *Rhizophagus irregularis.* MUCL 43194 (c) and ACP-Active extraradical hyphae of *Rhizophagus fasciculatus* MUCL 46100 (d) from root organ cultures (ROC) established from AMF isolates cryopreserved from 6 months at -130°C . Dark dots in c-d (see black arrows) show fast-blue-RR precipitation, thus, phosphatase active hyphae. Scale bar = 50µm in a-b, 10µm in c-d.

Cryopreservation of Rhizophagus species has minor effects on their stability

Whatever the isolate and treatment, intraradical structures (i.e. arbuscules, vesicles/spores, and hyphae) were observed. Overall, the percentage of roots colonization was not affected (P = 0.823) (Fig. 11a). For each isolate considered independently, no significant difference was observed in the percentage of roots colonization following cryopreservation.

Whatever the isolate and treatment, phosphatase activity was observed in the extraradical hyphae (subpanels c and d of Fig. 11). Overall, the treatment did not affect ALP (P = 0.771) or ACP (P = 0.809) activity of extraradical hyphae (subpanels b and c of Fig. 11, respectively). When each isolate was considered independently, no significant difference was found in the AMF metabolic activity (i.e. ALP and ACP staining) between cultures established from cryopreserved and non-cryopreserved encapsulated propagules.

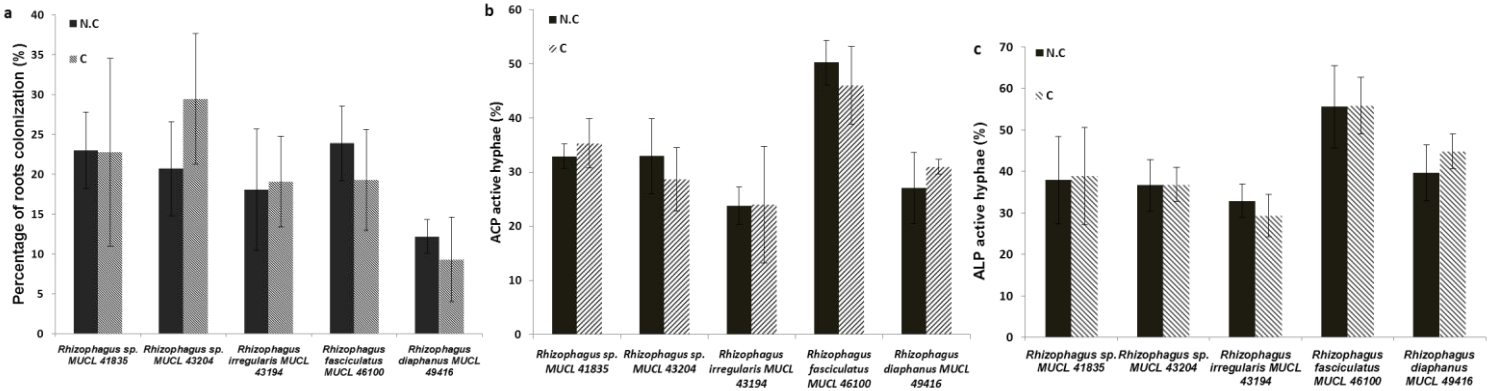

Figure 11 Percentage of carrot root colonization (a) proportion of alkaline phosphatase activity (b) and acid phosphatase activity (c) in the extraradical hyphae of five AMF isolates cryopreserved (C) for 6 months at -130°C or maintained on root organ culture (i.e. non-cryopreserved (NC) treatments) at 27°C. Mean value (± standard error, n = 5).

Cryopreservation of Rhizophagus species has minor effects on their stability

AFLP patterns contained between 156 (*Rhizophagus sp.* MUCL 41835) and 206 (*Rhizophagus sp.* MUCL 43204) DNA fragments. Isolates displayed a different AFLP pattern between cryopreserved and non-cryopreserved treatments but within the range of the technical error (i.e. 4.4 %). After cryopreservation, among the 206 fragments obtained with the four couples of primers, the *Rhizophagus sp*. MUCL 43204 pattern was similar at 98.8% to the non-cryopreserved pattern. The *Rhizophagus sp.* MUCL 41835 isolate presented a similarity of 98.5% between cryopreserved and non-cryopreserved patterns. For *R. irregularis* MUCL 43194, a similarity of 95.8% was obtained between the patterns of the two treatments (Fig. 12).

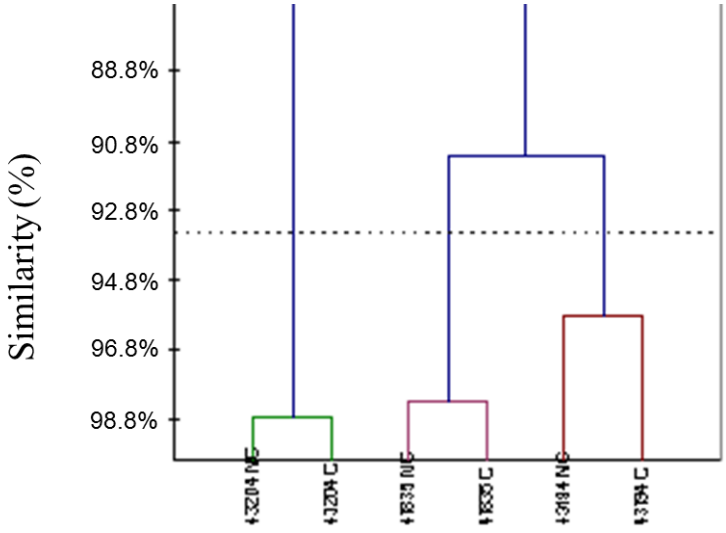

Figure 12 Dendrogram generated with UPGMA cluster analysis of the Dice similarity index based on the AFLP patterns of *Rhizophagus sp*. MUCL 43204, *Rhizophagus sp*. MUCL 431835 and *R. irregularis* 43194 before (NC) and after cryopreservation (C) respectively.

Discussion

Here we demonstrated that, with few exceptions, all the cryopreserved *Rhizophagus* isolates exhibited the same morpho-anatomy as the non-cryopreserved isolates. As previously reported by Lalaymia et al. (2012) for encapsulation-drying, subsequent cryopreservation for 6 months at -130°C of the five *in vitro* cultured AMF isolates did not impact their survival (i.e. germination within beads), root colonization, and spore production. Similarly, the daughter spores isolated from these cultures were able to re-associate a carrot root *in vitro* and to reproduce the fungal life cycle either in mono- and bi-compartmented Petri plates. Extraradical mycelium developed as compared to the cultures maintained at 27°C. The five AMF isolates were able to colonize the roots and produce typical intraradical structures (i.e. arbuscules, vesicles/spores, and intraradical hyphae). Similarly, an extensive extraradical mycelium was produced supporting spores, BAS, and anastomosis. With the exception of *Rhizophagus sp.* MUCL 43204, the hyphal lengths in cultures established from cryopreserved encapsulated propagules were identical to those obtained from non-cryopreserved encapsulated propagules and were consistent with previous data reported on *Rhizophagus* species (de Jaeger et al. 2011) but were larger as compared to the values reported by de la Providencia et al. (2005) with the same isolate. The reason for such a difference may be attributed to the intrinsic heterogeneous growth of AMF *in vitro*. Indeed, in several studies, it has been reported that growth dynamics may greatly vary from replicate to replicate within a same isolate (Declerck et al. 1996, 2001, 2004). Nevertheless, this difference did not affect the fungal growth and the

development of a profuse hyphal network supporting BAS, spores, and anastomosis. Whatever the isolate and treatment, spores were produced on hyphae of primary, secondary, and lower order as well as associated to BAS, and no significant difference was found in the number of spores produced between treatments. Identical values were reported by de la Providencia et al. (2005) and Lalaymia et al. (2012) for the same isolates maintained by regular sub-cultivation and following cryopreservation, respectively. BAS structures, reported as the preferential sites for mineral nutrient acquisition (Bago et al. 1998, 2004), were also produced in large quantities. BAS number was only significantly higher in cultures established from cryopreserved encapsulated propagules of the *R. diaphanus* MUCL 49416 isolate as compared with the control. As above, the difference may be related to the variability in BAS number between replicates of a same isolate (Declerck et al. 1996, 2001, 2004) or to the difficulty to distinguish BAS within the profusely branched fungal colony. Anastomosis, that is the process of fusion between branches of the same or different hyphae to produce a mycelial network (Kirk et al. 2001), was also observed in the extraradical hyphae for all the isolates and treatments under study. Whatever the isolate and the treatment, the number of anastomosis per hyphal length recorded here was much lower than the number observed by de la Providencia et al. (2005) and Voets et al. (2006). This difference could be influenced by cultivation systems due to the variation in physiological processes and/or gene expression (Purin and Morton, 2011). Whatever the isolate and treatment, ALP and ACP activity was observed in the extraradical hyphae as reported earlier in other systems (Joner and Johansen, 2000; Van Aarle et al. 2001, 2002; Ezawa et al. 2001; Olsson et al. 2002;

Aono et al. 2004; Zocco et al. 2011). Cryopreservation did not affect phosphatase activity.

Finally, when genetic stability of the AMF cultures was assessed using AFLP fingerprinting (Vos et al. 1995), a technique used to assess the stability of animal cells, plant and fungi after maintenance by cryopreservation (Hsu et al. 2008; Mikula et al. 2011; Voyron et al. 2009), no differences in AFLP patterns were observed within three isolates maintained for 6 months by cryopreservation or maintained on ROC at 27°C, outside the range of technical error. This suggested, that in the time frame of conservation, the genomic stability of the isolates was maintained. Using AFLP, Cardenas-Flores et al. (2010) also did not observe any genetic variation in different clonal lineages of *R. irregularis* MUCL 43194 originating from the same ancestor culture and maintained by serial sub-cultivation for at least 70 generations under different growth conditions. In conclusion, the present results demonstrate for the first time that *in vitro* cultured AMF isolates that are cryopreserved at -130°C for 6 months maintain their morphology, physiology, and genetic stability, as compared to the same isolates maintained on ROC at 27°C. Consequently, the cryopreservation protocol developed here for AMF isolates belonging to the genus *Rhizophagus* (i.e. *R. irregularis*, *R. fasciculatus*, *R. diaphanous*, and two undefined isolates) appears to be a suitable method for maintaining these obligate symbionts, decreasing the necessity for laborious sub-cultivations.

These results are of particular importance for culture collections, where cryopreservation can represent a preferred means to preserve the fungal organisms. The protocol of cryopreservation and subsequent effects on fungal characteristics needs to be extended/adapted to other species and

genera and for longer periods of time to validate this technique over time and for a large set of AMF.

Acknowledgments

The authors are grateful to Professor Bernadette Govaerts (Louvain School of Statistics, Biostatistics and Actuarial Sciences, UCL; Belgium) for advices on statistical analysis. This work was supported by the European Community's Seventh Framework Program FP7/2007-2013 under grant agreement no. 227522, entitled "Valorizing Andean microbial diversity through sustainable intensification of potato-based farming systems."

Chapter IV

Cryopreservation of arbuscular mycorrhizal fungi from root-organ and pot cultures

Published as a short note in

Mycorrhiza DOI: 10.1007/s00572-013-0525-8

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My contribution to this chapter was estimated at 70 %. For the practical work of this experiment, I received the help of Françoise Naveau (Technician at the laboratory of mycology). The experiment design, data analysis and writing of the paper were conducted by myself. Co-authors of the chapter have been involved in revising the manuscript.

Preface

The studies conducted in **Chapters II** and **III** revealed that the protocol developed for the cryopreservation of AMF using encapsulation-drying and storage at -130°C was effective for long-term preservation of *in vitro* cultured isolates belonging to the *Rhizophagus* genus. The cryopreservation at -130°C by encapsulation-drying for 6 months did not affect the morphology, metabolic activity and genetic stability of the AMF isolates. However, there are twenty AMF genera among the *Glomeromycetes* phylum (http://schuessler.userweb.mwn.de/amphylo/) and large differences exist in morphological characters as well as fungal life cycle. In addition, most AMF are yet unculturable *in vitro*. Therefore, in **Chapter IV,** we aimed to extend the cryopreservation method developed, to other AMF isolates belonging to other species and genera and to isolates cultured either *in vitro* or *in vivo*.

Cryopreservation of arbuscular mycorrhizal fungi from root-organ and pot cultures

Abstract

Long-term maintenance of arbuscular mycorrhizal fungi (AMF) by *in vitro* or *in vivo* subcultivation is of ten expensive and time-consuming and could present the risk of contaminations and possibly morphological, physiological, and genetic variations over time. Recently, *in vitro* cultured AMF isolates belonging to the genus *Rhizophagus* were successfully cryopreserved at -130°C following encapsulation-drying. Here, this method was tested on 12 single species cultures belonging to six different genera (i.e. *Rhizophagus*, *Glomus*, *Claroideoglomus*, *Septoglomus*, *Paraglomus*, and *Gigaspora*) produced *in vitro* or *in vivo*. Their viability was estimated, after 1 month of cryopreservation at -130°C, by the percentage of potentially infective beads (i.e. the percentage of beads that contained at least one germinated propagule) for the i*n vitro* produced species and the percentage of infective beads (i.e. the percentage of beads that contained at least one propagule able to colonize a new host plant in pot culture) for the *in vivo* produced species. With the exception of *Gigaspora* sp. MUCL 52331 and *Septoglomus constrictus* PER 7.2, no significant differences were observed in the viability of the single species cultures before and after cryopreservation. These results, thus, demonstrated the suitability of the cryopreservation method by encapsulation-drying for AMF species belonging to different genera and produced *in vitro* or *in vivo*. This method opens the door to the long-term preservation at ultra-low temperature of a large number of AMF species and for the preservation of species that are still recalcitrant to *in vitro* cultivation.

Cryopreservation of arbuscular mycorrhizal fungi from root-organ and pot cultures

Keywords: Arbuscular mycorrhizal fungi, Glomeromycota, Continuous culture, Cryopreservation, Encapsulation-drying.

Cryopreservation of arbuscular mycorrhizal fungi from root-organ and pot cultures

Introduction

At present, an estimated 250 arbuscular mycorrhizal fungi (AMF) have been identified (Schüßler and Walker, 2010). This number is expected to increase markedly in the future with the development of powerful molecular identification tools (Krüger et al. 2012; Young, 2012).

AMF are of economic value for agriculture, horticulture, and forestry. Therefore, adequate long-term storage protocols are needed to preserve this diversity in international repositories (e.g. Glomeromycota *IN vitro* Collection (GINCO)). Nowadays, most AMF species are maintained via continuous culture on plants under greenhouse facilities or *in vitro* in association with excised transformed roots. These methods are often expensive, time-consuming, and prone to contaminations and do not prevent genetic and physiological changes over time (Douds and Schenck, 1990; Plenchette et al. 1996; Declerck and Van Coppenolle, 2000).

Recently, Lalaymia et al. (2012) developed a long-term storage method for AMF based on the cryopreservation of encapsulated dried propagules. Twelve *in vitro* produced isolates belonging to the genus *Rhizophagus* were successfully cryopreserved for more than 6 months at -130°C. The morphology, physiological activity, and genetic stability, estimated on a selection of these isolates, were identical between the cryopreserved isolates and the non-cryopreserved controls (Lalaymia et al. 2013). At present, the number of AMF species produced *in vitro* remains limited. With a few exceptions (e.g. *Dentiscutata reticulata* (de Souza and Declerck, 2003) and *Gigaspora margarita* (Karandashov et al. 1999)), most

of the species cultured *in vitro* so far belong to the genus *Rhizophagus* (e.g. GINCO, http://www.mycorrhiza.be/ginco-bel/index.php), while the vast majority of species are maintained on plants in the greenhouse (e.g. the International Bank of Glomeromycota (IBG), http://www.i-beg.eu/, and the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM), http://invam.caf.wvu.edu/). Therefore, in the present study, we evaluated whether the encapsulation-drying cryopreservation method developed by Lalaymia et al. (2012) could be extended to the preservation of other AMF isolates belonging to different genera maintained either *in vitro* on root organs or in the greenhouse on plants.

Material and methods

AMF species

Eight *in vitro* (on root organs) and four *in vivo* (on plants) produced AMF single species cultures (i.e. cultures established via either a single spore or multiple spores or a single root piece containing several spores/vesicles from the same species) belonging to different genera were considered (Table 9). All the single species cultures were provided by GINCO (http://www.mycorrhiza.be/ginco-bel/index.php). An *in vitro* culture of *Rhizophagus sp.* MUCL 43204, which was previously cryopreserved at -130°C using the encapsulation- drying protocol (Lalaymia et al. 2012), was used as control to evaluate the success of the cryopreservation protocol.

The *in vitro* produced single species cultures were provided in Petri plates in association with Ri T-DNA-transformed carrot (*Daucus carota*,

clone DC2) roots. They were maintained on the modified Strullu-Romand (MSR) medium (Declerck et al. 1998) and solidified with 3 gl^{-1} Phytagel (Sigma-Aldrich, USA). The Petri plates were incubated in an inverted position in the dark at 27°C. After 5 months, hundreds to thousands of spores were obtained in each Petri plate.

The *in vivo* produced AMF single species cultures were isolated from pot cultures of *Plantago lanceolata*. They were maintained on an initially sterilized (2 × 15 min at 121°C, with a 12h interval) substrate composed of a mixture of TerraGreen (Agsorb 8/16 LVM-GA, Chicago, IL, USA), fine quartz, and coarse quartz (2:2:1). The plants were grown in sunbags (Sigma-Aldrich, USA), under greenhouse conditions (set at min 20°C during winter) with natural light intensity. Cultures were kept for at least 5 months with several thousands of spores produced within this period.

Table 9 Arbuscular mycorrhizal fungal single species cultures used for the encapsulation-drying cryopreservation method

AMF Culture	AMF Genus	AMF species	Synonymy	MUCL code/ intern code	Origin	Biotope
Produced *in vitro*	*Rhizophagus*	*Rhizophagus sp.*	*Glomus sp.*	MUCL 49422	Québec, Canada	Temperate
		Rhizophagus proliferus	*Glomus proliferum*	MUCL 41827	Guadeloupe	Tropical
		Rhizophagus sp.	*Glomus sp.*	MUCL 43204	Ontario, Canada	Temperate
		Rhizophagus clarus	*Glomus clarum*	MUCL 46238	Pinar del Rio, Cuba	Tropical
		Rhizophagus sp.	*Glomus sp.*	MUCL 45686	Finland	Temperate
	Glomus	*Glomus sp.*	NA	MUCL 43208	Québec-Canada	Temperate
	Gigaspora	*Gigaspora sp.*	NA	MUCL 52331	Virginia, USA	Temperate
	Claroideoglomus	*Claroideoglomus claroideum*	*Glomus claroideum*	MUCL 54351	Finland	Temperate

(continued)

Produced *in vivo*		*Claroideoglomus claroideum*	*Glomus claroideum*	PER 9.1	Tarma-Peru	Tropical
		Claroideoglomus claroideum	*Glomus claroideum*	PER 8.5	Tarma-Peru	Tropical
	Septoglomus	*Septoglomus constrictus*	*Glomus constrictum*	PER 7.2	Tarma-Peru	Tropical
	Paraglomus	*Paraglomus brasilianum*	*Glomus brasilianum*	Ecu 18.VF	Loja-Ecuador	Tropical

NA not applicable

Cryopreservation of arbuscular mycorrhizal fungi from root-organ and pot cultures

AMF propagule isolation

In vitro produced propagules (i.e. spores, vesicles, auxiliary cells, and mycorrhizal/non-mycorrhizal root pieces) were sampled from 5 month old cultures as follows: the gelling medium was blended twice in 100 ml sterilized (121°C for 15 min) deionized water for 30 s at 20,000 rpm in a sterilized (12°C for 15 min) mixer (Omni Mixer Homogenizer, Omni International). The mixture was subsequently filtered on a sterilized (121°C for 15 min) nylon filter (40 mm) before being encapsulated in alginate beads. Briefly, the encapsulation procedure consisted in the suspension of the mixture in a 2% (w/v) solution of sterilized (121°C for 15 min) sodium alginate (acid sodium salt from brown algae, Sigma-Aldrich, UK), followed by the polymerization of the alginate droplets (each containing an approximate of 50 ± 5 spores) into a sterilized (121°C for 15 min) solution of 0.1M CaCl2 maintained under agitation for 30 min. The polymerized beads were then rinsed with sterilized deionized water (121°C for 15 min) and stored in Petri plates (see Lalaymia et al. 2012, for details).

In vivo produced spores were collected from 50 to 200 g pot culture soil by wet sieving (i.e. a 38 μm diameter sieve) and decanting. The supernatant was centrifuged in water for 5 min at 2,000 rpm and then in sucrose (2M) for 15 s at 2,000 rpm. Spores were collected from the gradient interphase, rinsed with water, selected manually under a stereomicroscope with a micropipette, and filtered on a sterilized (121°C for 15 min) nylon filter (40 mm) to remove the excess of water. Roots were collected with forceps and crushed in 100 ml sterilized (121 °C for 15 min) deionized water for 30 s at 20,000 rpm in a sterilized (121°C for 15 min) mixer (Omni Mixer

Homogenizer, Omni International) and were filtered as above. Spores and crushed roots were mixed and encapsulated in alginate beads as above.

Cryopreservation procedure

For each AMF single species culture, propagules were cryopreserved at -130°C following the encapsulation-drying protocol developed by Lalaymia et al. (2012). Briefly, the isolated propagules were encapsulated in alginate beads (see Lalaymia et al. 2012). The beads were subsequently incubated overnight at 4°C in trehalose (0.5M) used as cryoprotectant (Douds and Schenck, 1990; Declerck and Van Coppenolle, 2000), dried during 48h at 27 °C, and placed in 2 ml cryotubes, before freezing at -130°C following a two step decrease in temperature a fast decrease (~12°Cmin^{-1}) from room temperature (+ 20°C) to -110°C followed by a slow decrease in temperature (~1°Cmin^{-1}) from -110 to -130°C. For each single species culture, the alginate beads contained a mean of 50 ± 5 propagules, except for *Gigaspora sp*. MUCL 52331 (each bead contained 5 ± 1 propagules). The encapsulated propagules were maintained for 1 month at -130°C. Non-cryopreserved dried beads containing propagules were used as controls and tested at the time the preserved AMF were frozen.

Evaluation of viability after cryopreservation

For the *in vitro* produced propagules, the controls were placed directly after encapsulation-drying on a 40 ml sterilized (121°C for 15 min) MSR medium in contact with Ri T-DNA transformed carrot (*D. carota*, clone DC2) roots (i.e. five beads per Petri plate) at 27 °C in the dark. The

cryopreserved, encapsulated-dried propagules were directly thawed in a water bath (+35°C) and similarly placed on the MSR medium in contact with Ri T-DNA-transformed carrot (*D. carota* , clone DC2) roots. Four Petri plates (each containing five beads, i.e. replicates) were set up per species and treatment. After 4 weeks of incubation, the germination of encapsulated propagules was evaluated under a stereomicroscope (6.7-40X magnification). For each single species culture and treatment, data were expressed as the percentage of potentially infective beads (%PIB), i.e. the percentage of beads containing at least one germinated propagule (Declerck et al. 1996). For each single species culture and treatment, root colonization and spore production were checked under a stereomicroscope after 8 weeks.

For the *in vivo* produced propagules, the viability test was conducted *in vivo* in association with trap plants. For each control, the dried beads containing the propagules were placed directly in contact with *P. lanceolata* (i.e. one bead per plant) in pots (5 cm diameter) containing a sterilized (2×15 min at 121 °C, with a 12-h interval) substrate (TerraGreen, Agsorb 8/ 16 LVM-GA, Chicago, IL, USA), fine quartz, and coarse quartz (2:2:1). Plants were incubated in a growth chamber set at 70 % humidity, with 22/18°C day/night temperature, and illuminated for 16 h day^{-1} under a photosynthetic photon flux of 300 mmol m^{-2} s^{-1} for 8 weeks. Plants were watered every 7 days with 400 ml of deionized water. The cryopreserved encapsulated propagules were directly thawed in a water bath (+35°C) and placed in contact with *P. lanceolata* roots (i.e. one bead per plant) as above. Twenty replicates were used per single species culture and treatment.

After 8 weeks of incubation, the viability of non-cryopreserved and cryopreserved, encapsulated-dried propagules was evaluated by the

percentage of infective beads (%IB), i.e. the percentage of beads that contain at least one propagule able to colonize the plant. This parameter was estimated either by the presence of newly produced spores 2 in the substrate or by root colonization. For each single species culture and treatment, root colonization and presence of new spores were checked. Root colonization was evidenced following the staining method of Walker (2005) slightly modified as follows: roots were cleared by heating (90°C for 30 min) in 10% KOH. The roots were then rinsed with tap water before staining at 90°C (15 min) in acidified blue ink solution (1% of HCl solution with 2% of blue ink (Parker)). The roots were subsequently mounted on slides in lactoglycerol (1:2:1 (v/v/v) lactic acid/glycerol/H2O) and colonization checked under a bright field light microscope (Olympus BH-2, Olympus Optical GmbH) at 50-250X magnification. The presence of newly produced spores was evidenced after wet sieving (i.e. a 38-µm-diameter sieve) and decanting of the soil substrates. The supernatant was then centrifuged in water for 5 min at 2,000 rpm, by sucrose (2M) for 15 s at 2,000 rpm. The supernatant was finally observed under a stereomicroscope at magnification 6.7X and 40X in the sucrose gradient interphase.

Statistical analysis

Statistical analysis was performed with the statistical software XLSTAT Software version 2012.6.02 (Addinsoft, 1995-2012). The data were subjected to binary logistic regression. The chi- square testwas used to identify significant differences ($P \leq 0.05$).

Results and discussion

With the exception of *Gigaspora sp.* MUCL 52331 (i.e. %PIB = 80%), the %PIB estimated before cryopreservation was 100% for the *in vitro* produced single species cultures (Table 10). Following cryopreservation, the %PIB of *Gigaspora sp.* MUCL 52331 dropped to 20% and was significantly lower ($P < 0.001$) as compared to that of the control. On the contrary, the %PIB of the other single species cultures remained above 90% and did not differ statistically from the controls. The results obtained for the *Rhizophagus* single species cultures were consistent with the study of Lalaymia et al. (2012) and demonstrated the suitability of the encapsulated-drying method for the cryopreservation of a large set of AMF species belonging to this genus.

Table 10 Percentage of potentially infective beads ((%PIB) for *in vitro* produced single species cultures) and percentage of infective beads ((%IB) for *in vivo* produced single species cultures) after drying (2 days) and cryopreservation at -130°C for 1 month of AMF propagules (i.e. spores and mycorrhizal/non-mycorrhizal roots) isolated from *in vitro* or *in vivo* cultures. For the *in vitro* produced singles species cultures, the %PIB was estimated after 4 weeks of incubation of the beads on the MSR medium in the dark at 27°C. For the *in vivo* produced single species cultures, the %IB was estimated after 8 weeks of incubation of the beads in pot culture with *P. lanceolata*

		%PIB or %IB	
AMF Culture	AMF single species culture	Before Cryopreservation	After Cryopreservation
Produced *in vitro*	*Rhizophagus sp.* MUCL 49422	100[a]	90[a]
	Rhizophagus proliferus MUCL 41827	100	100
	Rhizophagus sp. MUCL 43204	100	100
	Rhizophagus clarus MUCL 46238	100	100
	Rhizophagus sp. MUCL 45686	100[a]	90[a]
	Glomus sp. MUCL 43208	100[a]	100[a]
	Gigaspora sp. MUCL 52331	80[a]	20[b]
	Claroideoglomus claroideum MUCL 54351	100	100
Produced *in vivo*	*Claroideoglomus claroideum* PER 9.1 [(1)]	70[a]	45[a]
	Claroideoglomus claroideum PER 8.5 [(1)]	100[a]	95[a]
	Septoglomus constrictus PER 7.2 [(1)]	90[a]	55[b]
	Paraglomus brasilianum Ecu 18.VF [(1)]	100[a]	90[a]

Values in a row followed by the same letter did not differ significantly (binary logistic regression, $P \leq 0.05$; chi-square test)
[(1)] *In vivo* produced single species culture for which the %IB was determined

Interestingly, the cryopreservation by encapsulation-drying was also effective for single species cultures belonging to Glomus (i.e. *Glomus sp.* MUCL 43208), *Claroideoglomus* (i.e. *Claroideoglomus claroideum* MUCL 54351) and *Gigaspora* (*Gigaspora sp.* MUCL 52331). Eight weeks after association with transformed carrot root, all the germinated encapsulated *in vitro* produced single species cultures were able to colonize roots and to produce spores and auxiliary cells (i.e. for *Gigaspora sp.* MUCL 52331).

The results obtained with *Gigaspora sp.* MUCL 52331corroborated the studies of Douds and Schenck (1990) and Kuszala et al. (2001), showing a low viability of *Gigaspora* species after cold storage (~60 and -80°C, respectively). As compared to the other single species cultures used in our study (i.e. *Rhizophagus*, *Glomus*, and *Claroideoglomus*), *Gigaspora sp.* MUCL 52331 produced few large spores, which have often been reported as difficult to subculture *in vitro* (Dalpé et al. 2005). This factor, combined to the low number of propagules encapsulated, may explain the lower %PIB obtained. It is obvious that increasing the number of propagules in the beads would increase the %PIB.

With the exception of *S. constrictus* PER 7.2 ($P = 0.011$), no significant differences were noted in the %IB before and after cryopreservation for *the in vivo* produced single species cultures. For each single culture species and treatment, several spores produced singly, in isolated clusters or attached to the plant roots, were observed. Both *C. claroideum* single species cultures (i.e. PER 9.1 and PER 7.2) survived cryopreservation at -130°C. Encapsulated-dried spores of *P. brasilianum* Ecu 18.VF also survived cryopreservation at -130°C. The reason for the lower viability observed for *S. constrictus* PER 7.2 remains unresolved. It is

well known that the physiological status of the culture is a determinant factor in the success of cryopreservation (Lalaymia et al. 2012) and that AMF species exhibit different kinetics of growth (Declerck et al. 2001, 2004). After 5 months of culture, *S. constrictus* was still in the exponential phase of growth, producing a significant proportion of young spores which were probably less resistant to cryopreservation.

Lalaymia et al. (2012) showed that the duration of storage at -130°C following encapsulation drying did not affect the viability of *Rhizophagus* species. Therefore, it is expected that the AMF single species cultures cryopreserved at -130°C for 1 month in our study would also be viable over longer periods of storage, although this need to be evaluated.

In conclusion, we demonstrated that the cryopreservation of encapsulated dried AMF propagules (Lalaymia et al. 2012) is adequate for several species belonging to *Rhizophagus*, *Glomus*, *Claroideoglomus*, *Septoglomus*, *Paraglomus*, and *Gigaspora* produced either *in vitro* or *in vivo*. The method used in this study opens the door to the long-term cryopreservation of AMF which are difficult or still impossible to grow *in vitro*. It may also represent an innovative approach to preserve field-sampled spores that are recalcitrant to be maintained on plant cultures.

Acknowledgments

This work was supported by the European Community's Seventh Framework Program FP7/2007–2013 under grant agreement no. 227522, entitled "Valorizing Andean microbial diversity through sustainable intensification of potato-based farming systems." The authors wish to thank Valentine Potten, Ph.D., student at the Université catholique de Louvain (Belgium), for the isolation of the *in vivo* produced AMF species and Dr. Christopher Walker from the Royal Botanic Garden Edinburgh (UK) and Carolina Senés from the University of Munich (Germany) for the taxonomic and molecular identification, respectively, of the *in vivo* produced species.

General discussion

In response to the world's growing population and pressure for food production, the application of synthetic fertilizers and pesticides in conventional agriculture is since decades considered as a necessity. However, this has resulted in a progressive pollution of soils affecting human health and environment. The consequences of these agricultural practices combined with economical and societal preoccupations have led to a reflection on our modes of production and has paved the way for a sustainable agriculture more respectful for the environment. This is reinforced by international legislations more severe on the utilization of pesticides.

The shift to organically-grown food combined with soil-borne beneficial microorganisms (e.g. arbuscular mycorrhizal fungi (AMF)) is an alternative to the above-mentioned preoccupations. AMF form symbiotic associations with most plant species of agricultural concern. Their importance for improving plant nutrients uptake, yield, resistance/tolerance against biotic and abiotic stress has been widely reported (Finlay, 2008; Smith and Read, 2008). In addition, these soil microorganisms improve soil microbial and plant diversity and productivity and improve ecosystem functioning (van der Heijden et al. 1998; Jonsson et al. 1999, 2001; Smith and Read, 2008).

The increasing use of AMF in national and international agriculture programs and the regular discovery of new species have made the preservation of these microorganisms in culture collection essential. Appropriate and easy-to-apply modes of preservation, that insure morphological, physiological and genetic stability over unlimited period, are thus mandatory. Nowadays the preservation of AMF, either *in vivo* or *in vitro*, is mostly achieved via serial sub-cultivation in association with plants

or excised roots. However, this method is time-consuming, labour-intensive, present the risk of contaminations and loss of phenotypic stability and infectivity with time (Plenchette et al. 1996). Progress in maintenance/preservation is thus needed to preserve these essential organisms that help feed the world (Marx, 2004).

Within this thesis we developed a long-term preservation method for AMF, as an alternative to the routinely-used sub-cultivation procedure. This method is applicable to a large number of isolates and insures morphological, physiological and genetic stability over a theoretically indefinite period of time.

Over the last decades, many long-term preservation methods have been developed for AMF among other fungi (**see Chapter I**). The preconditioning treatment, the cryoprotectant (type and concentration), the rate of cooling and thawing, have been reported as key factors that contribute to the long-term preservation success. These parameters have usually been empirically adjusted to the target group of microorganisms to preserve.

Different methods, including cryopreservation, vitrification and lyophilisation, were assayed (see **Annex I**) to delineate the parameters that are essential to succeed in the long term preservation of AMF. If most of the tests were unsuccessful, a number of factors appeared essential. Indeed, the viability and integrity of AMF spores seemed to be influenced by the cryoprotectant and its concentration, the cooling rate, the drying and the finale temperature of storage.

Declerck and Van Coppenolle, (2000) were the first to succeed in the cryopreservation at ultra-low temperature (-100°C) of *in vitro* produced

AMF spores. They used the technology of encapsulation in alginate beads. This method was a major breakthrough but was only assayed with a single AMF isolate, for a short period of time and with *in vitro* produced spores.

This protocol and our attempts/errors findings (**Annex I**) led us to test different important key factors to develop a successful cryopreservation technique for AMF (**see Chapter II**).

The encapsulation of mycorrhizal fungi in alginate beads has been used as a tool in the production of inoculum (Kuek et al. 1992; Declerck et al. 1996b; Paloschi de Oliveira et al. 2006). Besides being an excellent protection for cells against many biotic and abiotic environmental stresses (Bashan, 1998), the encapsulation in alginate beads appeared essential to protect the AMF spores against the stresses of cryopreservation (**see Chapter II)**. These findings corroborates with earlier reports (Suzuki et al. 2005; Sakai and Engelman, 2007) on the possible protective effect of alginate beads against mechanical and oxidative stress during cryopreservation.

Being essentially composed of water, the drying of the beads before cryopreservation seemed also a determinant factor for the successful cryopreservation of *in vitro* produced AMF propagules. The cryopreservation by encapsulation-drying has thus been adopted in our study. This technique was formerly used in many cryopreservation protocols for a wide variety of plant germplasms (Fabre and Dereuddre, 1990). These authors were the first to use osmotic and air-drying evaporative treatments to reduce the ice formation during the freezing process. Indeed, too high water content in alginate beads causes water crystallization during freezing leading to the damage of AMF propagules. Similarly, too low water content causes propagules death by excessive dehydration. To find a good compromise

between these two extremes, differential scanning calorimetry is often used to optimise the encapsulation-drying protocol (Benson et al. 2006). In our study, twenty eight hours of drying at 27°C of the encapsulated propagules (i.e. 8.1 ± 4.6% of beads water content), prevented water crystallization in alginate beads and gave the highest rate of survival after cryopreservation (**see Chapter II**). In addition, the utilization of trehalose as cryoprotectant prevented ice formation and maintained the membrane fluidity during freezing, as shown earlier (Crowe et al. 1998).

As previously reported on different fungal cultures (Smith and Onions, 1994; Smith, 1998), we demonstrated that AMF propagules sampled from cultures in the stationary phase of growth (i.e. 5 months old cultures in our case) survived better the freezing process than younger cultures. This observation could be explained by the production and accumulation in AMF propagules of natural cryoprotectants such as trehalose, polyols and/or polysacharides in response to stress (Fuller et al. 2004a, 2004b). In root organ cultures, it is estimated that the excised roots stops their growth after 7 weeks (Declerck et al. 2004) because of nutrients limiting conditions caused by medium depletion. Such compounds are effective on the protection of AMF propagules membrane and proteins during the different steps of cryopreservation (Tan and van Ingen, 2004).

It is known that the rate of cooling used prior to storage at finale ultra-low temperature affect the response of living cells (Mazur, 1984; Smith and Onions, 1994; Smith, 1998). We demonstrated that cryopreservation at -130°C by direct storage in the freezer (i.e. a fast cooling procedure following a two steps decrease in temperature: a fast decrease (~12°Cmin^{-1}) from room temperature (+20°C) to -110°C followed by a slow decrease in temperature (~1°Cmin^{-1}) from -110°C to -130°C see **Chapter II**) gave a higher viability

than the cryopreservation at -100°C with the slow controlled cooling and the cryopreservation by direct immersion in liquid nitrogen using the very fast cooling rate (i.e. 200°Cmin^{-1}). This could be explained by the fact that the slow cooling procedure dehydrated significantly the spores and caused spores damages, mainly following beads drying. Identically, very fast cooling may result in intracellular and extracellular ice crystals production, which is also lethal to the cells (Meryman, 2007).

Besides the advantages to be very easy to handle, the AMF cryopreservation technique via encapsulation-drying and storage at -130°C (i.e. for at least 6 months) was effective for twelve *Rhizophagus* isolates produced *in vitro*. These isolates kept their ability to reinitiate the life cycle by the colonization of plant roots and the production of new spores (**see Chapter II**).

In this thesis, we observed that the duration of storage had no significant effect on the viability of the AMF propagules. This result suggested that the encapsulation-drying of AMF propagules is adequate to store AMF isolates at -130°C for a theoretically unlimited period of time.

The storage at -130°C is advised in cryopreservation methods. Below this temperature all chemical reactions, biological processes and intra- and extra physical interactions are arrested (Mazur, 1984), which theoretically allows the storage of AMF without biological changes for an unlimited period of time. However, the risks for morphological, physiological and genetic alterations during cryopreservation process and cryogenic storage due, among others, to cells cryo-injury and oxidative stresses are not negligible (Fuller et al. 2004a; Chetverikova, 2009).

Therefore, in **Chapter III** we investigated the morphology (i.e. hyphal growth, production of branched absorbing structure, anastomosis, spore production and size, and root colonization), physiological activity (i.e. alkaline and acid phosphatase enzymatic activity) and genetic stability (i.e. by the mean of amplified fragment length polymorphism (AFLP)) of five AMF isolates belonging to 4 species following their cryopreservation at -130°C for 6 months. The genetic stability was evaluated only on 3 isolates due to the impossibility to yield enough DNA with the 5 isolates. With a few exceptions, all the cryopreserved isolates exhibited the same morphology and physiological activity as compared to the non-cryopreserved isolates. Similarly, the genetic integrity of the cryopreserved isolates was not disrupted. Such results indicate that the different cryopreservation steps did not impact the AMF isolates characteristics. This suggested that the cryopreservation by encapsulation-drying is a stable preservation method for AMF isolates.

It has often been reported that there is no link between fungal taxonomic groups and their responses to cryopreservation (Morris et al. 1988; Smith and Thomas, 1998; Ryan et al. 2000), and that the mode of fungi production/cultivation could affect considerably their response to preservation (Smith and Onions, 1994). This statement opened the debate on the possibility/necessity to apply the encapsulation-drying protocol to the cryopreservation of other AMF isolates belonging to the same of other genus, and to isolates produced following different mode of culture (i.e. cultured *in vitro* or in the greenhouse).

In **Chapter IV**, we tested if the cryopreservation by encapsulation-drying and storage at -130°C was effective on other AMF isolates belonging

to different genera and if it was applicable on AMF propagules produced *in vivo.*

After one month storage at -130°C, isolates belonging to *Rhizophagus*, *Glomus*, *Claroideoglomus*, *Septoglomus*, *Paraglomus* and *Gigaspora* produced either *in vitro* or *in vivo*, were viable. Except for *Gigaspora sp.* and *Septoglomus constrictus* isolates, the viability of the cryopreserved isolates was not significantly different as compared to the non-cryopreserved controls. The lower survival observed with the *Gigaspora* sp. and *F. constrictus* could be attributed to the spore size and thickness of the spore wall (Schüßler and Walker, 2010). Besides the fact that some AMF isolates are difficult to subculture *in vitro* (Dalpé et al. 2005), the encapsulation of a low number of AMF propagules could also explain the low rate of germination of the *Gigaspora sp.* isolate after cryopreservation.

The cryopreservation protocol developed in our thesis used propagules from cultures older than 5 months (i.e. supposedly in the stationary phase). However, not all the AMF species and isolates exhibit the same growth kenetic's. Even if this protocol works on different isolates belonging to different genus, the protocol should be adapted according to the growth kenetics of each fungal species/isolate.

In this thesis we developed a cryopreservation protocol for AMF propagules comprising 5 steps: (1) the encapsulation of propagules (i.e. spores and mycorrhizal root pieces) isolated from 5 months-old cultures, (2) the incubation overnight in trehalose (0.5M), (3) the drying during 48h at 27°C (i.e. at 8.1 ± 4.6% of beads water content), (4) the cryopreservation in the freezer at -130°C following a two step decrease in temperature: a fast decrease (~12°Cmin^{-1}) from room temperature (+20°C) to -110°C followed by a slow decrease in temperature (~1°Cmin^{-1}) from -110°C to -130°C, and

(5) the direct thawing in a water bath (+35°C). We demonstrated that this cryopreservation method is adequate for a large set of AMF isolates belonging to *Rhizopahagus*, *Glomus*, *Claroideoglomus*, *Septoglomus*, *Paraglomus* and *Gigaspora* cultured either *in vitro* or *in vivo*. This method, therefore, opens the door for the preservation at ultra-low temperatures of a large set of AMF species within culture collections.

Conclusion

Conclusion

The long-term preservation of arbuscular mycorrhizal fungi (AMF) has been insufficiently explored so far, due partly to their complex life cycle (needing a host plant) and to the availability of simple pot-cultivation techniques. Here we developed a preservation method for AMF that guarantee their biological stability over the long-term. The method is efficient for AMF isolates belonging to different genera and cultured either *in vitro* or *in vivo*.

Three central questions were addressed during this thesis:

1. Is it possible to apply a long-term preservation method at ultra-low temperature applicable to AMF isolates belonging to different genera?

The cryopreservation protocol developed by Declerck and Van Coppenolle, (2000) and others (see **Annex I**) helped to understand and delineate the factors which are essential for the long-term preservation of AMF isolates. We developed in our study, a cryopreservation method based on the encapsulation-drying of AMF propagules and further storage in the deep freezer at -130°C. This method allowed the long-term cryopreservation of several *in vitro* cultured AMF isolates belonging to *Rhizophagus*, *Glomus*, *Claroideoglomus* and *Gigaspora* genus for periods, varyinig from 1 up to 6 months (**Chapter II and IV**). Moreover, this technique did not affect the viability and ability of the isolates to reinitiate the fungal life cycle.

2. Does the cryopreservation by encapsulation-drying affect the biological stability of AMF?

It is admitted that the different steps in the cryopreservation procedure could affect the biological stability of the cells. In **Chapter III,** it was demonstrated that the AMF isolates tested for their stability after 6 months storage at -130°C kept the same morphological and physiological characteristics and genetic stability as the isolates maintained at 27°C by sub-cultivation. Taken together, these results suggested that the cryopreservation using the encapsulation-drying protocol is a suitable method to preserve the stability of AMF isolate.

3. Is the cryopreservation method by encapsulation-drying applicable to greenhouse-produced AMF isolates?

Another method to maintain AMF is under *in vivo* (i.e. in the greenhouse) culture conditions in association with a suitable host plant. Nowadays, this method remains the most largely used, allowing the maintenance of a large number of AMF isolates. Our results (**Chapter IV**) demonstrated that the encapsulation-drying cryopreservation method was adapted to *in vivo* produced AMF isolates (i.e. *Claroideoglomus*, *Septogolus* and *Paraglomus* genus), wich may present an interesting alternative to the greenhouse maintenance method.

The results obtained in the thesis confirmed the possibility to preserve AMF over long periods. Cyopreservation at -130°C by encapsulation-drying of both *in vitro* and *in vivo* cultured AMF is a suitable preservation method, requiring few space and workload. It is complementary

to the conventional maintenance methods, but particularly adapted to culture collections. It is applicable for a long period and suitable to a large set of isolates. Our results are of particular importance for AMF culture collections providing them with a very effective method for the long-term preservation of AMF isolates.

Perspectives

Perspectives

Preservation of arbuscular mycorrhizal fungi (AMF) in culture collections is crucial to provide stable and authenticated AMF isolates for basic research as well as field applications.

Our research supported the long-term cryopreservation (at -130°C) of AMF using the encapsulation-drying method. Twenty three AMF isolates belonging to different genus (i.e. *Rhizophagus*, *Glomus*, *Claroideoglomus, Septogomus, Paraglomus* and *Gigaspora*), either cultured *in vitro* or *in vivo*, were successfully cryopreserved for a period of up to 6 months. The AMF isolates kept their biological stability following cryopreservation.

It is obvious that the number of isolates cryopreserved in our study only represent a fraction of the diversity maintained in culture collections (e.g. GINCO, INVAM, IBG). Therefore a first perspective is to extend/assay/adapt the method developed to a larger array of isolates cultured *in vitro* and within the greenhouse.

In the present thesis, some isolates such as *Gigaspora sp* MUCL 52331 had a low viability following cryopreservation. Therefore, a second perspective is to understand the reason for this low viability by exploring the different steps of the cryopreservation process. Investigating the direct effects of cryopreservation on the living cells could be achieved via different methods. Cryogenic light microscopy can help in determining the response of fungi to freezing and thawing, by the direct visualisation of the cellular ultra-structural changes during those processes. This may help to determine the most optimal protocol of cryopreservation adapted to specific AMF isolate/species/genera (Smith and Thomas, 1998). The differential scanning calorimetry (DSC) technique could also be used to monitor water crystallization and ice growth during the freezing process, as well as ice recrystallization following thawing. Thereby, this method may give

information on the optimum freezing and thawing rates to obtain the highest AMF survival

For the *in vitro* produced isolates, the survival was estimated via the percentage of infective beads (i.e. the percentage of beads showing at least the germination of one propagule). However, this parameter does not take into consideration the actual number of germinating propagules. This number may be higher (or important) and therefore may be decreased to save inoculum. Conversely, this number may be low (mean < 1 per bead) and thus necessitate to increase the number of propagules per bead. The third perspective is thus to determine for each AMF isolate, the minimum number of propagules per bead to obtain 100 % of potentially infective beads able to colonize a new host root. It is suggested to investigate a dose-response curve to estimate the optimal number of propagules to encapsulate.

The encapsulation is a time-consuming process at risk for contaminations and damages to propagules. It is conceivable to grow the AMF isolates *in vitro* in bi-compartmented Petri plates filled with MSR medium in the root compartment (RC) (i.e. where roots and an AMF grow) and with alginate beads in the hyphal compartment (HC) (i.e. where only the extraradical mycelium and spores are allowed to proliferate). Each bead may be regarded as a niche in which the AMF may proliferate and sporulate. The alginate beads containing spores could thus be collected, treated with trehalose and dried for cryopreservation.

The cryopreservation of AMF in their symbiotic phase (i.e. in association with their plant host) may be an alternative for the preservation of fungi that produce insufficient material *in vivo* or *in vitro.* The

Perspectives

Encapsulation-drying techniques have been used with success in the cryopreservation of several plants species including carrot (*Daucus carota*) (Dereuddre at al. 1991). The encapsulation-drying cryopreservation of the apexes of excised roots containing AMF is thus a fourth perspective. It could represent a good approach to reproduce the fungal life cycle without the necessity to re-associate a novel root.

Some AMF are non-culturable or recalcitrant to culture in pots. Therefore another perspective would be the direct extraction of field collected spores and their encapsulation in beads. This may help safeguarding the AMF diversity under stable conditions until adequate methods are developed for their cultivation *in vitro* or *in vivo*.

The viability and stability of AMF isolates cryopreserved at -130 °C following encapsulation-drying remained unchanged for periods up to 6 months. However, before large-scale application to AMF culture collections, we would recommend to extend the duration of observation to periods exceeding 6 months to ascertain the long-term stability of the method developed. Finally, AMF preservation in culture collection should be ideally based on two methods. We recommend the combination of basic methods of conservation such as *in vivo* and *in vitro* sub-cultivation as back up to the cryopreservation of the AMF isolates.

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Overview of the Scientific Achievement

Overview of the scientific achievement

I. Scientific publications

I.1 Research articles published

- **Lalaymia L**, Cranenbrouck S, Draye X, Declerck S (2012) Preservation at ultra-low temperature of *in vitro* cultured arbuscular mycorrhizal fungi via encapsulation-drying. Published in *Fungal Biology journal* 116: 1032-1041.

- **Lalaymia L**, Declerck S, Cranenbrouck S (2013) Cryopreservation of *in vitro* produced *Rhizophagus* species has minor effects on their morphology, physiology and genetic stability. Published in *Mycorrhiza journal* DOI 10.1007/s00572-013-0506-y.

- **Lalaymia L**, Declerck S, Naveau F, Cranenbrouck S (2013) Cryopreservation of arbuscular mycorrhizal fungi from root-organ and pot cultures. Published in *Mycorrhiza journal* DOI: 10.1007/s00572-013-0525-8.

I.2 Research articles submitted

- **Lalaymia L**, Cranenbrouck S, Declerck S (2013) Maintenance and preservation of ectomycorrhizal and arbuscular mycorrhizal fungi Submitted to *Mycorrhiza journal.*

II. Conference participation

- **Lalaymia I**, Potten V, Cranenbrouck S, Declerck S (2009).Valorizing Andean microbial diversity through sustainable intensification of potato-based farming systems. ***Poster*** at the conference COST 870, Leuven, Belgium, 14 September 2009.

Overview of the scientific achievement

- Lalaymia I, Cranenbrouck S, Declerck S (2010). Préservation à ultra-basse température des champignons mycorhiziens à arbuscules. ***Poster*** presented at the Secondes Journées Francophones Mycorhizes, Brussel, Belgium, 16 September 2010.

- **Lalaymia I**, Cranenbrouck S, Declerck S (2009) Preservation at ultra-low temperature of in *vitro* cultured *Rhizophagus* species with entrapment-dehydration. ***Poster*** at Rhizosphere 3 conference, Perth, Western Australia, 25 September 2011.

- Lalaymia I, Cranenbrouck S, Declerck S (2012) Préservation à ultra-basse température des champignons mycorhizien à arbuscule cultivés *in vitro* par encapsulation-déshydratation. ***Oral presentation*** at the 3ème Journées Francophones Mycorhizes, Nancy, France, 07 September 2012.

III. Teaching

- International Training on *in vitro* Culture of Arbuscular Mycorrhizal Fungi Louvain la Neuve, Belgium (2009-2012).

Annex

Annex I

Long-term preservation of *Rhizophagus* sp. MUCL 43204 following different preservations tests

The objective of this annex is to give an overview of all the preservation protocols tested during the thesis. These protocols helped us to understand which factors were the most essential for the cryopreservation of arbuscular mycorrhizal fungi (AMF). We used *Rhizophagus sp*. MUCL 43204 produced *in vitro* on root organ culture (ROC) as model organism.

Materiel and methods

Biological material

Rhizophagus sp. MUCL 43204 was used in this study. The isolate was purchased from the Glomeromycota *in vitro* collection (GINCO: http://emma.agro.ucl.ac.be/ginco-bel) and provided in Petri plates in association with Ri T-DNA transformed carrot (*Daucus carota*, clone DC2) roots. The isolate was maintained on the modified Strullu-Romand (MSR) medium (Declerck et al. 1998), solidified with 3 gl^{-1} phytagel (Sigma-Aldrich, USA). Petri plates were incubated in an inverted position in the dark at 27°C. After 4 months culture, several hundred to thousands of spores were obtained in each Petri plate.

Annex I: Long-term preservation of Rhizophagus sp. MUCL 43204 following different preservations tests

Cryopreservation procedures

1- Cryopreservation in alginate beads

This protocol was set up following the procedure described by Declerck and Van Coppenolle (2000). Spores were isolated from 4, 4.5 and 5 months old cultures of *Rhizophagus sp.* MUCL 43204 and encapsulated in alginate beads (See chapter 2 for detailed description). The beads were subsequently stored in a Petri plate overnight at 15°C. The beads were then immersed for 1 day in a cryoprotectant solution (trehalose (0.5M), DMSO (10% v/v), PEG 4000 (20% v/v) or a combination of different concentrations of DMSO and PEG 4000 (10:20%, 10:15% and 5:20%, respectively)) or in sterilized (121°C for 15 min) deionized water. The beads were then placed in 2 ml cryovials and cryopreserved for 3h at -100°C following a two steps decrease in temperature (Icecube 1600, SY Lab, Austria): a slow decrease ($1°Cmin^{-1}$) from room temperature (+20°C) to -35°C followed by a fast decrease in temperature ($18°Cmin^{-1}$) from -35°C to -100°C. After 3h cryopreservation, the beads were thawed by immersion for 15 min in a water bath set at 35°C. The beads were then dropped in sterilized (121°C for 15 min) MSR medium cooled in a water bath to 40°C. In addition, three controls were considered for each culture age and cryoprotectant: (i) non encapsulated non-cryopreserved spores, (ii) encapsulated non-cryopreserved spores and (iii) non encapsulated cryopreserved spores. Twenty beads (i.e. replicates) containing each 50 ± 5 spores were considered per treatment. Data were expressed as the percentage of potentially infective beads (%PIB), i.e. containing at least one germinated spore (Declerck et al. 1996b). The %PIB

was determined 4 weeks after cryopreservation and incubation on sterilized (121°C for 15 min) MSR medium at 27°C in the dark.

2- Cryopreservation in cryovials

Five agar plugs (3mm diameter) containing spores and hyphae were isolated from a 5 months old culture of *Rhizophagus sp.* MUCL 43204 and subsequently transferred into sterile cryovials (2ml) submerged with a sterilized (121°C for 15 min) cryoprotectant solution (glycerol (10% v/v) or trehalose (0.5M)). After 1h treatment with the cryoprotectant at 4°C, the cryovials were (1) directly immersed in liquid nitrogen, (2) placed in a freezer at -130°C following a two steps 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°C to -130°C. (3) placed at -100°C (Icecube 1600, SY Lab, Austria) following a tow steps decrease in temperatures: a slow decrease of 1°C per min from room temperature to -35°C followed by a rapid decrease of 18°C per min from -35°C to -100°C. In addition two controls were considered for each cryoprotectant and storage condition; (i) non-cryoprotected non-cryopreserved fungal plugs and (ii) protected non-cryopreserved fungal plugs. Ten replicates (i.e cryovials containing 5 fungal plugs) were considered per treatment. After 3 h in liquid nitrogen at -130°C or at -100°C, the cryovials were thawed by immersion for 15 min in a water bath set at +35°C. Cryovial were surface sterilised with ethanol (70%) and the fungal plugs subsequently spread onto sterilized (121°C for 15 min) MSR medium on Petri plates (i.e. 5 plugs per Petri plates) and incubated in the dark at

27°C for germination. Spore germination from plugs was checked after 2 weeks of incubation.

3- Cryopreservation in straws

This protocol was slightly modified from the protocol of Hoffmann (Hoffmann, 1991) as follow: Petri plates containing 5 months old cultures of *Rhizophagus sp.* MUCL 43204 were flooded with 5ml sterilized (121°C for 15 min) glycerol (10% v/v) or trehalose (0.5M) cryoprotectant solution and incubated for 1h. Sterile plastic straws (4 mm diam with 37 mm in length, open at both ends) were used. Agar plugs containing spores and hyphae were pushed into the straws. The straws were transferred into sterile cryovials (i.e. five straws per cryovial) and then frozen directly (1) in liquid nitrogen and (2) in a freezer at -130°C. In parallel, straws were cryopreserved (3) at -100°C (Icecube 1600, SY Lab, Austria) following the two steps decrease in temperature described above and (4) at -130°C (Icecube 1600, SY Lab, Austria) following a four steps decrease in temperature: a fast decrease (10°Cmin^{-1}) from room temperature (+20°C) to 5°C, followed by a slow decrease in temperature (1°Cmin^{-1}) from 5°C to -14°C, followed by a slow decrease in temperature (2°Cmin^{-1}) from -14°C to -35°C and finally a fast decrease (-18°C min^{-1}) from -35°C to -130°C. In addition two controls were considered for each cryoprotecatant and storage condition; (i) non-cryoprotected non-cryopreserved agar plugs and (ii) protected non-cryopreserved agar plugs. For each treatment ten replicates were considered. After 3 h cryopreservation, cryovials were thawed by immersion for 15 min in a water bath set at +35°C. Straws were surface sterilised with ethanol (70%) and then agar plugs from each straw were spread onto sterilized

(121°C for 15 min) MSR medium on Petri plates and incubated in the dark at 27°C for germination. Spore germination was checked after 2 weeks incubation.

4- Cryopreservation in fine straw

This protocol was slightly modified from the protocol of Kaidi (Kaidi et al. 2001) used for oocytes preservation. AMF spores were isolated from 5 months old culture of *Rhizophagus sp.* MUCL 43204 by solubilisation of the MSR medium (Doner and Bécard, 1991) and further separated from roots with forceps. Spores were treated for 1h at 4°C with sterilized (121 °C for 15 min) trehalose (0.5M) as cryoprotectant. Three groups of 15 to 20 spores were loaded in trehalose (0.5M) in fine straws (0.25-ml with 91mm in length, open at both ends). Each group was surrounded by two columns of trehalose (0.5M). Air bubbles separated each of the group.

In addition two controls were considered; (i) non-cryoprotected non-cryopreserved groups of spores and (ii) protected non-cryopreserved groups of spores. For each treatment ten replicates were considered. Straws were heat sealed and immersed directly (1) in liquid nitrogen or (2) in the freezer at -130°C or (3) cooled at -100°C (Icecube 1600, SY Lab, Austria) following the decrease in temperature described above. After 3 h storage, the straws were immersed for 15 min in a water bath set at +35°C for thawing. The straws were then surface sterilised with ethanol (70%) and then the spore groups of each straw were streaked onto sterilized (121°C for 15 min) MSR medium on Petri plates (i.e. 3 groups per Petri plate) and incubated in the dark at 27°C for germination. Spore germination was checked after 2 weeks incubation.

Annex I: Long-term preservation of Rhizophagus sp. MUCL 43204 following different preservations tests

5- Cryopreservation by encapsulation-vitrification

Vitrification protocol was slightly modified from the procedure described by Helliot et al. (2003) used for the cryopreservation of banana meristems. Spores were isolated from 5 months old culture of *Rhizophagus sp.* MUCL 43204 by solubilisation of the MSR medium (Doner and Bécard, 1991) and further separated from roots with forceps, encapsulated in alginate beads as explained by Declerck and Van Coppenolle (2000) and treated before vitrification as follows: Beads were kept for 20 min in a filter-sterilised loading solution (2 M glycerol and 0.4 M sucrose dissolved in MSR liquid medium (pH 5.5)) at room temperature. Then, the loading solution was replaced by ice-cooled and filter-sterilised PVS-2 solution containing 3.26 M glycerol, 2.42 M ethylene glycol, 1.9 M dimethylsulfoxide and 0.4 M sucrose dissolved in MSR liquid medium (pH 5.5) for dehydration at 0°C for 30 min. Beads where then transferred into cyovials (2ml) and immersed into liquid nitrogen for 3h. Thereafter, cryovials were rapidly thawed at 35°C for 2 min. The PVS-2 solution was replaced by the filter-sterilised deloading solution (1.2 M sucrose dissolved in SRM liquid medium (pH 5.5)) for 10 min at room temperature. Two controls were considered; (i) non-cryoprotected non-cryopreserved beads and (ii) cryoprotected non-cryopreserved beads. For each treatment twenty beads were used. Beads were then dropped in sterilized (121°C for 15 min) MSR medium cooled in a water bath to 40°C and incubation on sterilized MSR medium on Petri plates and incubated at 27°C in the dark for spore germination. The %PIB was determined 4 weeks after storage.

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6- Lyophilization

This protocol followed the routine procedure used at the Mycothèque de l'Université catholique de Louvain (MUCL) for the preservation of filamentous fungi and yeasts. Spores were isolated from 5 months old cultures of *Rhizophagus sp.* MUCL 43204 by solubilisation of the MSR medium (Doner and Bécard, 1991) and further separated from roots with forceps. Around 500 spores were collected and suspended in 3ml of a cooled sterile mix of skimmed milk (15%),trehalose (10%), sodium glutamate (5%), and further vortexed and distributed into glass ampoules (i.e. 0.3ml per ampoule). The ampoules were sealed with cotton wool cap. After 15 h of freeze-drying, the ampoules were removed from the freeze-drier, heat sealed and stored for 7 days. Non lyophilised spores were used as control. Twenty ampoules were considered per treatment. After storage, the ampoules were opened and spores were streaked onto sterilized (121°C for 15 min) MSR medium on Petri-plates and incubated at 27°C in the dark for germination.

7- Encapsulation-drying lyophilisation

Gelling medium, containing spores and roots of 5 months old cultures of *Rhizophagus sp.* MUCL 43204, was extracted and subsequently crushed and mixed as explained in Lalaymia et al. (2012). The mixture was filtered on a sterilized (121°C for 15 min) nylon filter (40µm). The supernatant (i.e. spores and mycorrhizal/non-mycorrhizal root pieces) was then encapsulated (50 ± 5 propagules per beads), treated with trehalose (0.5M) and dried at 27°C for 48h as explained in Lalaymia et al. (2012). Encapsulated-dried propagules were lyophilized following MUCL protocol in glass ampoules. After 15 h of freeze-drying the ampoules were removed from the freeze-

drier, heat sealed and stored for 7 days. Three controls were considered: (i) non lyophilized non dried encapsulated propagules, (ii) non lyophilized dried encapsulated propagules and (iii) lyophilized non dried encapsulated propagules. Twenty beads were considered per treatment. The beads were then dropped in sterilized (121°C for 15 min) MSR medium cooled in a water bath to 40°C and incubated at 27°C in the dark. The %PIB was determined 4 weeks after storage.

Results and discussion

In experiment1, the %PIB of encapsulated spores of *Rhizophagus sp*. MUCL43204 (Table A1) from different physiologic ages was evaluated after treatment in different cryoprotectants and following cryopreservation for 3h at -100°C.

Annex I: Long-term preservation of Rhizophagus sp. MUCL 43204 following different preservations tests

Table A1 Percentage of potentially infective beads (%PIB) after incubation in different cryoprotectants of beads containing spores isolated from 4, 4.5 and 5 months old cultures of *Rhizophagus sp.* MUCL 43204, before and after cryopreservation at -100°C for 3 h.

		Before cryopreservation		After cryopreservation	
Cryoprotectant	culture age (months)	Non-encapsulated	Encapsulated	Non-encapsulated	Encapsulated
Deionized water	5	100	100	0.0	0.0
	4.5	100	100	0.0	0.0
	4	100	100	0.0	0.0
Trehalose	5	100	100	0.0	80
(0.5M)	4.5	100	100	0.0	50
	4	100	100	0.0	25
20% PEG4000	5	100	100	0.0	85
	4.5	100	100	0.0	25
	4	100	100	0.0	0.0
10% DMSO	5	100	100	0.0	20
	4.5	100	100	0.0	0.0
	4	100	100	0.0	0.0
DMSO +	5	100	100	0.0	50
PEG4000	4.5	100	100	0.0	25
(10:20%)	4	100	100	0.0	0.0
DMSO +	5	100	100	0.0	65
PEG4000	4.5	100	100	0.0	0.0
(5:20%)	4	100	100	0.0	0.0
DMSO +	5	100	100	0.0	20
PEG4000	4.5	100	100	0.0	25
(10:15%)	5	100	100	0.0	0.0

DMSO, PEG and trehalose are cryoprotectants frequently used for the cryopreservation of microorganisms. Interestingly, none of these cryoprotectants, single or in combination (i.e. DMSO and PEG) were detrimental to spore germination. The %PIB was 100% with these cryoprotectants before cryopreservation. The %PIB was 0% for all the encapsulated cryopreserved spores from the 4 months old culture age, except

for those treated with trehalose (0.5M) (i.e. the %PIB was 20%). Furthermore, spores from the 5 months old cultures presented at least a %PIB of 20%, regardless of the treatment (Table A1). However, a difference was observed in the %PIB between spores of 5 months old culture, spores of 4.5 months old culture and spores of 4 months old cultures. At 5 months, all the cultures considered in our experiment were in the late stationary phase. Old cultures were more resistant to cryoprotectant treatment and cryoprotectant, which is consistent with most studies on fungi preservation (Smith and Onions 1994; Smith, 1998; Tan and van Ingen 2004), possibly due to the accumulation of disaccharide polyol and other natural protective compound when fungus is in the late phase or growth (Fuller et al. 2004a). The higher %PIB was observed for the encapsulated spores treated with 20%PEG4000 and with trehalose (0.5M) for spores isolated from 5 months old cultures. Trehalose (0.5M) appeared the most effective cryoprotectant whatever the age of the cultures, although its effectiveness was the highest with spores isolated from 5 months old cultures. This findings corroborates the earlier study conducted by Declerck and Van Coppenolle (2000). Trehalose is a natural disaccharide, non penetrating cryoprotectant. However, during cells freezing, the membrane becomes more permeable to its entry (Beattie et al. 1997). This cryoprotectant can with its hydroxyl groups substitute the cellular water molecules and interact with the polar residues of membrane and proteins, which prevent ice formation and maintain membrane fluidity during freezing (Crowe et al. 1998). In the cryopreservation by encapsulation-vitrification (experiment 5) as for experiment 1, the encapsulation did not affect the germination of encapsulated spores non-cryoprotected and non-cryopreserved (%PIB = 100%). However, even if the spores used where from the late phase of

growth (i.e. from 5 months old culture), these encapsulated spores did not resist to the vitrification solution effect. The %PIB decreased to 65% before cryopreservation and we observed that some spores were damaged and empty. It was often reported that the nature and concentration of cryoprotectant are determinant for the success of cryopreservation (Hubálek, 2003) depending on organisms and cells type (Fuller, 2004b). Here, the protocol of encapsulation-vitrification used was borrowed from plant preservation. It is obvious that the concentration of cryoprotectant, the nature and the duration of exposition should be adapted to AMF spores. The mode of cold preservation also affected the viability of spores. The %PIB decreased to 0% after 3h preservation in liquid nitrogen. Preservation in liquid nitrogen corresponded to very fast cooling rate. During fast cooling, if cells are not well dehydrated, all intracellular water crystalizes and damages the cells. Thus, the damages observed after encapsulation-vitrification cryopreservation could be related to the toxic effect of cryoprotectant, the excess of dehydration of the spores due to the high concentration of the cryoprotectant, the damage caused by fast cooling or the combination of all these parameters. Whatever the cryoprotectant used and the mode of conditioning in fine straws, cryovials or straw, all storages modes (i.e. liquid nitrogen, at -130°C and -100°C) affected the survival and the germination of *Rhizophagus sp.* MUCL 43204. No spore germination was observed after 3h storage and most of the spores appeared damaged. However, whatever cryoprotected or not and stored in cryovials, in straws or in fine straws all non-cryopreserved fungi germinated in the medium after 2 weeks incubation at 27°C on MSR medium in the dark. In these three preservation modes (experiment 2, 3 and 4), the protective solution did not affect fungal survival. Methods of cooling used were the main factor that could influence the fungal

survival. In the cryovials and the straws, the spores were in agar medium which is mostly composed of water. During fast freezing, this extra as well as intracellular water is rapidly transformed in ice crystals if cells are not well dehydrated by cryoprotectant treatment. Ice crystals will be very damaging to cells. During slow and controlled cooling a progressive dehydration of cell and death could occur. In fact, slow cooling could induce cells dehydration, denaturation of membranes' constituents and cells' toxicity due to the increase of concentration of the solute surrounding the cells and pH change since extracellular water freezes firstly and progressively. Cryoprotectant used here could be not protective enough or not at the adequate concentration to protect AMF during fast or slow cryopreservation. In addition to freezing, thawing could also affect the survival following cryopreservation. In all these tests we used fast thawing. Fast thawing is the most common procedure used to revive cells after freezing and is preferable to slow thawing. When cells are frozen fast, the aggregation of small crystals formed during cooling could be prevented during reviving using fast thawing. However, fast thawing could be harmful if cells are frozen slowly. Large ice crystals formed during slow cooling melt, and can cause osmotic shock and cell death.

Lyophilization also affected the %PIB of spores of *Rhizophagus sp*. MUCL 43204. No germination was observed after storage and incubation on MSR medium at 27°C. Most spores appeared damaged and empty. For all non lyophilized spores, lyoprotectant did not affect their germination. All the steps of lyophilisation may affect survival such as the rate of cooling and the rate of heat input during drying. In the encapsulation-drying lyophilisation experiment, before lyophilisation non dried encapsulated propagules as well as dried ones had a %PIB of 100%. However, when non dried encapsulated

propagules were lyophilized, the %PIB decreased to 0% as compared to the non dried non lyophilized spores (%PIB = 100%). This tends to prove that water contained in beads was the limiting factor for the success of lyophilization. During the first step of lyophilisation (i.e. cooling) water crystalizes, ice will be formed in the intra or extracellular compartment; that induces cell damages and/or dehydration and death. Drying of alginate beads prevented these events and insured revival after storage.

Conclusion

From this study, we concluded that the cryopreservation/lyophilisation protocols tested can not be applied universally on all organisms. Even if some of the protocols tested here are applied currently on other fungi, they were not successful for AMF preservation. Different authors (Morris et al. 1988; Smith and Thomas 1998; Bardin et al. 2007) reported that all cryopreservation factors and steps should be taken into consideration and require to be handled at the species level. These tests allowed us to study, investigate and understand in depth cryopreservation limiting-factors in AMF cryopreservation/lyophilisation for further development of an adequate AMF long-term preservation protocol.

Annex II

Annex II

Table A2 Details on the most successful mycorrhizal fungi preservation methods (the order of cryopreservation methods is following their citation in the review). This Table refers to chapter 1

Mycorrhizae	Isolates	Culture	Protectant	Mode of preservation	Rate of cooling	Duration of storage	Reactivation of culture	Reference
Ecto-mycorrhizal fungi	*Scleroderma bovista***	Plugs from colony grown on MMN for 2-10 weeks	NA	In distilled water in glace tube at 5°C	NA	1years	Mycelia disc placed on MMN agar medium	Marx and Daniel (1976)
	*Boletinus meruliodes***	Plugs from colony grown on MMN for 2-10 weeks	NA	In distilled water in glace tube at 5°C	NA	3years	Mycelia disc placed on MMN agar medium	Marx and Daniel (1976)
	*Cenococcum graniforme***							
	*Laccaria laccata***							
	*Lepiota nuda***							
	*Pisolithus tinctorius***							
	*poria terrestris***							
	*Rhizopogon reseolus***							
	*Suillus albidipes***							
	*S. grevillei***							
	*S. luteus***							

	*Thelephora terrestris***							
	Amanita citrina DR-35 *Amanita flavoconia* DR-94 *Amanita muscaria* DR-59 *Boletus hosenae* DR-28 *Hebeloma crustuliniforme* DR-32 *Hebeloma sp.* DR-11 *Laccaria bicolor* DR-64 *Laccaria bicolor* DR-72 *Laccaria bicolor* DR-91 *Laccaria bicolor* DR-100 *Laccaria bicolor* DR-112 *Laccaria laccata* DR-5 *Laccaria laccata* DR-95 *Laccaria laccata* DR-102 *Laccaria laccata* DR-113 *Laccaria laccata* DR-115 *Laccaria laccata* DR-133	Plugs from the margin of actively growing colony	NA	In distilled water in glace tube at 5°C	NA	20years	Mycelia disc placed on MMN agar medium	Richter, (2008)

Laccaria laccata DR-137 *Lactarius rufus* DR-71 *Rhizopogon rubescens DR-128* *Suillus luteus DR-37* *Suillus luteus DR-82* *Suillus neoalbidipes DR-9* *Suillus neoalbidipes DR-44* *Tricholoma populinum DR-149* *Tricholoma resplendens DR-79*							
*Hebeloma crustuliniforme***	Mycelium grown on malt extract liquid medium for 25 days	NA	Encapsulation in alginate beads and storage at 25°C	NA	5months	Beads plated on malt extract agar in Petri dishes	Maupérin et al. (1987)
Laccaria laccata E439 *Laccaria laccata E2013* *Laccaria laccata E2058* *Laccaria sp. E1045*	ND	NA	Encapsulation in alginate beads and storage at 25°C	NA	7months	Beads plated on MMN medium in Petri dishes	Kuek et al. (1992)

Pisolithustinctorius H53 *Pisolithustinctorius H1101* *Descolea maculata, E1115* *Elaphomyces sp. H4318* *Elaphomyces sp. H4142* *Hebeloma westraliense E2067* *Setchelliogaster sp. nov. H1023*							
*Paxillus involutus***	Plugs From the margin of a colony grown on MMN agar for 10-50 days	NA	Encapsulation in alginate beads and storage at 25°C in CaCl2 (0.07 M) or in sterile water	NA	2months	Beads plated on MMN medium in Petri dishes	Rodrigues et al. (1999)
*Pisolithus tinctorius***	Plugs From the margin of a colony grown on MMN agar for 20-40days	NA	Encapsulation in alginate beads and storage at 25°C in CaCl2 (0.07 M) or in sterile water	NA	1month	Beads plated on MMN medium in Petri dishes	Rodrigues et al. (1999)

	*Rhizopogon nigrescens UFSC-Rh90**	Mycelium suspension grown in 25m liquid culture grown in PGM medium for 20-day	NA	Encapsulation in alginate beads and storage at 8°C	NA	18months	Beads plated on MMN medium in Petri dishes	Paloschi de Oliveira et al. (2006)
	Lacaria fraterna EM-1083 *L. laccata*** *L.amethystina*** *Amanita muscaria*** *Tricholoma albobruneum*** *Thelephora terrestris*** *Pisolithus tinctorius*** *Scleroderma cepa*** *S. auranteium*** *S. flavidum***	Plugs from the margin of a colony grown on MMN for 3-7 weeks	DMSO (10%)	Lyophilization	1°C/min until -30°C and transfer in condenser chamber for freeze-drying	ND	At room temperature in liquid malt extract for a 10h	Sundari and adholeya, (1999)

	Laccaria bicolor S238 N *Rhizopogon luteolus KTP* *Cenococcum geophillum SIV*	Plugs from the margin of a colony grown on malt medium for 2 weeks	Glycerol (15%)	Cryopreservation	1°C/min (cooling from +20 °C to -60°C in 80 min and transfer in LN	1month	At 4°C during 60 min and then placed at 25°C	Corbery and Le Tacon (1997)
	Cenococcum geophillum SIV	Plugs from the margin of a colony grown on malt medium for 2 weeks	Glycerol (15%)	Cryopreservation	1,25°C/min (cooling from +20°C to -30 °C in 40 min and transfer in LN)	5min	At 4°C during 60 min and then placed at 25°C	Corbery and Le Tacon (1997)
	Cantharellus cibarius ATTC 74488	Small fractions of approx 1mm3 of gar mycelia gown of MFM for 25 days	Sorbitol (4M) / DMSO (1M)	Cryopreservation	0,3°C/min	6days	In plastic jar with 45°C sterile water for 3-4min	Danell and Flygh (2002)

	Entoloma clandestinum CCBAS 497 *Scleroderma citrinum CCBAS 637* *S. verrucosum CCBAS 830*	Agar mycelium punshed in straws from colony grown on MEYA medium	Glycerol (5%)	Cryopreservation	1°C/min until -70°C and transfer in LN	2years	In water bath at 37 °C until the ice was completely thawed	Homolka et al. (2003)
	*Clavariadelphus pistillaris*** *Entoloma clandestinum*** *Laccaria laccata*** *L. proxima*** *Scleroderma citrinum*** *S verrucosum***	fungal cultures grown directly in sterile cryovials on perlite moistened with wort for 2 weeks	Glycerol (5%)	Cryopreservation	1°C/min until -70°C and transfer in LN	3years	In water bath at 37 °C until the ice was completely thawed	Homolka et al. (2006)
	*Hebeloma spoliatum 592**	Fungal culture grown in test tube in sawdust medium with moisture content of 60-64% for 4-5 weeks	Glycerol (10%)	Cryopreservation	The cultures were put directly in a deep-freezer set 85°C	3months	For 3h at room temperature	Kitamoto et al. (2002)

	*Hebeloma radicosum 546** *Hebeloma vinosophyllum A0K1** *Boletus pulverulentus TD-792*	Fungal culture grown in test tube in sawdust medium with moisture content of 60-64% for 4-5 weeks	Glycerol (10%)	Cryopreservation	The cultures were put directly in a deep-freezer set 85°C	33months	For 3h at room temperature	Kitamoto et al. (2002)
	*Entoloma clypeatum TD-793**	Fungal culture grown in test tube in sawdust medium with moisture content of 60-64% for 4-5 weeks	Glycerol (10%)	Cryopreservation	The cultures were put directly in a deep-freezer set 85°C	60months	For 3h at room temperature	Kitamoto et al. (2002)
	*Paxillus involutus TD-795**	Fungal culture grown in test tube in sawdust medium with moisture content of 60-64% for 4-5 weeks	Glycerol (10%)	Cryopreservation	The cultures were put directly in a deep-freezer set 85°C	10years	For 3h at room temperature	Kitamoto et al. (2002)

	*Cenococcum geophilum G-11** *Lepista nuda 8-31** *Rhizopogon sp. 8-21** *Rhizopogon sp. 93-3** *Suillus granulatus 8-16** *Suillus luteus9-22**	Plugs in cryovials from the margin of colony grown on malt medium for 30-45 days	Skimmed milk (10%)	Cryopreservation	Cryovials incubated for 1h at 4°C, stored in a freezer for 3h at -20°C (1°C/min), and then transferred immediately to 70°C	6months	In water bath at 37 °C until the ice was completely thawed	Obase et al. (2011)
	Hysterangium stoloniferum DSM24279* *Hysterangium stoloniferum* DSM24280* *Geastrum triplex* DSM 24285* *Mutinus elegans* DSM 24286* *Serpula lacrymans* DSM3106* *Serpula lacrymans* DSMIHD061* *Serpula lacrymans* IHD061*	Fungal isolates grown on charcoal filter paper strips (CFS) on the surface on culture medium for 3-5 weeks and transferred in cryovials	Glycerol (10%)	Cryopreservation	Placed 24 h in the gas phase of a LN tank (i.e at a cooling rate of approx 1-10 °C/min until -120 to -140 °C), then transferred into LN	24hours	In water bath at 25-30°C	Stielow et al. (2011)

Serpula himantioides DSM5043* *Melanogaster broomeianus* DSM 23845* *Melanogaster broomeianus* DSM 28281* *Rhizopogon luteolus* Strain RI* *Boletus edulis* DSM 4409* *Clitocybe gibba* DSM 8517*							
Cortinarius sp. 52207 (Cosp51) *Laccaria bicolor* 52211 (LbicLs2) *Laccaria bicolor* 52212 (LbicLs5sp) *Laccaria bicolor* 52213 (LbicLs6sp) *Lactarius rufus* 52214 (Lr30) *Paxillus involutus* 52218 (Pi30) *Paxillus involutus* 52220 (Pi51) *Suillus bovinus* 52149 (N1Sbo) *Suillus bovinus* 52150 (N2Sbo)	Fungal cultures were grown directly in cryovials on MFM medium for 7-9 weeks.	Glycerol (10%)	Cryopreservation	8°C/min from +20°C to +4°C; 1°C/min from +4°C to -50°C; 10°C/min from -50°C to - 100°C. The isolates were then directly transferred into a freezer	1month	In water bath at 38°C for 2min	Crahay et al. (2013a)

Suillus bovinus 52151 (N3Sbo)	at -130°C.
Suillus bovinus 52152 (N5Sbo)	
Suillus bovinus 52153 (N7Sbo)	
Suillus bovinus 52154 (E1Sbo)	
Suillus bovinus 52155 (E2Sbo)	
Suillus bovinus 52156 (E3Sbo)	
Suillus bovinus 52157 (E4Sbo)	
Suillus bovinus 52158 (MG3Sbo)	
Suillus bovinus 52159 (MG4Sbo)	
Suillus bovinus 52160 (MG5Sbo)	
Suillus bovinus 52161 (MG6Sbo)	
Suillus bovinus 52162 (Ls1Sbo)	
Suillus bovinus 52163 (LS2Sbo)	
Suillus bovinus 52164 (Ls3Sbo)	
Suillus bovinus 52165 (Ls4Sbo)	
Suillus bovinus 52166 (Lst1Sbo)	
Suillus bovinus 52167 (Lst4Sbo)	
Suillus bovinus 52168 (Lst6Sbo)	

Suillus bovinus 52169 (Lst8Sbo)

Suillus bovinus 52170 (HR1Sbo)

Suillus bovinus 52171 (HH1Sbo)

Suillus bovinus 52172 (P1Sbo)

Suillus bovinus 52174 (P3Sbo)

Suillus bovinus 52175 (P4Sbo)

Suillus bovinus 52177 (OF1Sbo)

Suillus bovinus 52178 (OF2Sbo)

Suillus bovinus 52179 (OF3Sbo)

Suillus bovinus 52180 (OF4Sbo)

Suillus bovinus 52181 (Z1Sbo)

Suillus bovinus 52182 (Z2Sbo)

Suillus bovinus 52183 (Z3Sbo)

Suillus bovinus 52184 (Z4Sbo)

Suillus bovinus 52185 (DS1Sbo)

Suillus bovinus 52186 (DS2Sbo)

Suillus bovinus 52188 (DS4Sbo)

Suillus luteus 52100 (E1Slu)

Suillus luteus 52101 (E2Slu)
Suillus luteus 52102 (Ew2Slu)
Suillus luteus 52103 (Ew3Slu)
Suillus luteus 52106 (MG4Slu)
Suillus luteus 52107 (MG5Slu)
Suillus luteus 52108 (N1Slu)
Suillus luteus 52111 (Na2Slu)
Suillus luteus 52112 (Na3Slu)
Suillus luteus 52113 (Na4Slu)
Suillus luteus 52114 (A9Slu)
Suillus luteus 52116 (MM1Slu)
Suillus luteus 52117 (P1Slu)
Suillus luteus 52118 (P2Slu)
Suillus luteus 52119 (P4Slu)
Suillus luteus 52120 (P8Slu)
Suillus luteus 52121 (LM2Slu)
Suillus luteus 52122 (LM3Slu)
Suillus luteus 52123 (LM4Slu)

Suillus luteus 52124 (Ls1Slu)
Suillus luteus 52125 (Ls2Slu)
Suillus luteus 52126 (Ls3Slu)
Suillus luteus 52127 (Lst1Slu)
Suillus luteus 52128 (Lst2Slu)
Suillus luteus 52129 (Lss4Slu)
Suillus luteus 52130 (Lss5Slu)
Suillus luteus 52131 (Lss6Slu)
Suillus luteus 52132 (Lss44Slu)
Suillus luteus 52133 (HH1Slu)
Suillus luteus 52134 (HH3Slu)
Suillus luteus 52136 (HH19Slu)
Suillus luteus 52139 (HR7Slu)
Suillus luteus 52140 (HR8Slu)
Suillus luteus 52142 (OF2Slu)
Suillus luteus 52144 (OF5Slu)
Suillus luteus 52146 (DS3Slu)
Suillus luteus 52147 (DS4Slu)

	Suillus luteus 52148 (DS5Slu) *Suillus variegatus* 52189 (P1Sva) *Suillus variegatus* 52190 (P2Sva) *Suillus variegatus* 52191 (Z1Sva) *Suillus variegatus* 52192 (Z3Sva) *Suillus variegatus* 52193 (T3.1Sva) *Suillus variegatus* 52194 (T4.1Sva)							
Arbuscular mycorrhizal fungi	*Glomus caledonius***	Spores in soil from plant trap culture of 6 months old air dried 5 months at 22°C and then dried over silica gel for 21 days to 0.2 to 0.4% water content	NA	Liquid-drying under vaccum	NA	ND	at 80°C for up to 40 min	Tommerup and Kidby (1979)

	*Acaulospora laevis***	Spores in soil from plant trap culture of 6 months air dried 5 months at 22°C	NA	Liquid-drying under vaccum	NA	ND	at 80°C for up to 40 min	Tommerup and Kidby (1979)
	*Glomus monosporus***	Spores in soil from plant trap culture of 6 months old air dried 8 months at 22°C and then dried over silica gel for 21 days to 0.2 to 0.4% water content	NA	Liquid-drying under vaccum	NA	ND	at 80°C for up to 40 min	Tommerup and Kidby (1979)
	*Glomus caledonius*** *Gigaspora sp***.	Spores in soil from plant trap culture of 6 months old air dried 5 months at 22°C and then dried over silica gel for 21 days to 0.2 to 0.4% water content	NA	Liquid-drying under vaccum	NA	ND	at 80°C for up to 40 min	Tommerup and Kidby (1979)

	*Glomus claroideum***	Spores in soil of trap plant culture stored in plastic bag	NA	At 4°C	NA	5yaers	NA	Wagner et al. (2001)
	Glomus geosporum BEG 11	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old	NA	In osmosed water at room temperature and at 4°C	NA	20months	At room temperature by rehydration with osmosed water	Kuszala et al. (2001)
	Glomus claroideum BEG 31	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old	NA	In osmosed water at room temperature and at 4°C	NA	14months	At room temperature by rehydration with osmosed water	Kuszala et al. (2001)
	Glomus fasciculatum BEG 53							

	Glomus clarum LPA 64*	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old	NA	In osmosed water at room temperature and at 4°C	NA	13months	At room temperature by rehydration with osmosed water	Kuszala et al. (2001)
	Acualospora longula BEG 8	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old	NA	In osmosed water at room temperature and at 4°C	NA	7months	At room temperature by rehydration with osmosed water	Kuszala et al. (2001)
	Acualospora scrobiculata BEG 33	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old	NA	In osmosed water at room temperature and at 4°C	NA	15months	At room temperature by rehydration with osmosed water	Kuszala et al. (2001)

	Glomus geosporum BEG 11	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in lyophilization bottles	NA	Lyophilization and then at room temperature	NA	26months	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)
	Glomus mosseae BEG 12	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in lyophilization bottles	NA	Lyophilization and then at room temperature	NA	12months	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)
	Acualospora longula BEG 8 *Glomus claroideum* BEG 14	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in lyophilization bottles	NA NA	Lyophilization and then at room temperature	NA	15months	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)

	Glomus claroideum BEG 23	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in lyophilization bottles	NA	Lyophilization and then at room temperature	NA	2months	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)
	Glomus claroideum BEG 31	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in lyophilization bottles	NA	Lyophilization and then at room temperature	NA	16months	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)
	Glomus coronatum BEG 22	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in lyophilization bottles	NA	Lyophilization and then at room temperature	NA	9months	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)

	Acualospora levis BEG 13	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in lyophilization bottles	NA	Lyophilization and then at room temperature	NA	17months	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)
	Acualospora levis BEG 26	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in lyophilization bottles	NA	Lyophilization and then at room temperature	NA	10months	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)
	Acualospora scrobiculata BEG 33	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in lyophilization bottles	NA	Lyophilization and then at room temperature	NA	28months	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)

	*Glomus mosseae***	S pores from 6-8months pot culture old excised from porocarps	NA	At 4°C in saturated salt solutions	NA	4years		Mugnier and Mosse (1987)
	*Acaulospora leavis*** *Acaulospora trappei*** *Scutellispora calopsora*** *Glomus caledonium*** *Glomus tenue*** *Glomus fasciculatum***	Spores and mycelium in soil of plant trap cultures	NA	Liquid-drying under vaccum and storage at 4°C	NA	8years	at 80°C for up to 40 min	Tommerup (1988)
	*Glomus occultum*** *Glomus mosseae* 322* *Glomus mosseae* 336* *Glomus versiforme* 231* *Glomus macrocarpum* 925* *Glomus claroides* 884* *Glomus intraradix* 208*	Spores dried in soil pot culture for 3-4 days and put in cryovials before freezing	NA	Cryopreservation	Directly in the freezer at ~-60-70°C	3-24h	In water bath at 35-40°C	Douds and Schenck (1990)

*Glomus occultum***							
Glomus sp. 878B*							
Entrophospora sp. 283*							
Gigaspora margarita 680*							
Gigaspora margarita 185*							
Scutellispora dipapillosa 400*							
Acaulospora longula 316* *Gigaspora margarita* 105* *Glomus etunicatum* 329*	Spores dried in soil pot culture for 3-4 days and put in cryovials before freezing	NA	Cryopreservation	Directly in the freezer at ~-60-70°C	3 months	In water bath at 35-40°C	Douds and Schenck (1990)
Glomus geosporum BEG 11	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in plastic eppendorf tubes or in cryovials	NA	Cryopreservation	Directly at: -18°C, -80°C or in LN	26 months for all the preservation temperatures	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)

	Glomus mosseae BEG 12	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in plastic eppendorf tubes or in cryovials	NA	Cryopreservation	Directly at: -18°C, -80°C or in LN	21, 21 and 14months respectively	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)
	Glomus claroideum BEG 14	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in plastic eppendorf tubes or in cryovials	NA	Cryopreservation	Directly at: -18°C, -80°C or in LN	15 months for all the preservation temperatures	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)
	Glomus claroideum BEG 20	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in plastic eppendorf tubes or in cryovials	NA	Cryopreservation	Directly at: -18°C, -80°C or in LN	20 months for all the preservation temperatures	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)

	Glomus claroideum BEG 23	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in plastic eppendorf tubes or in cryovials	NA	Cryopreservation	Directly at: -18°C or-80°C	10 and 7 months respectively	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)
	Glomus claroideum BEG 31	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in plastic eppendorf tubes or in cryovials	NA	Cryopreservation	Directly at: -18°C, -80°C or in LN	18, 25 and 25months respectively	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)
	Glomus coronatum BEG 22	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in plastic eppendorf tubes or in cryovials	NA	Cryopreservation	Directly at: -18°C or-80°C	9 and 9 months respectively	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)

	Glomus versiforme BEG 47	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in plastic eppendorf tubes or in cryovials	NA	Cryopreservation	Directly at: -18°C, -80°C or in LN	8, 8 and 7 months respectively	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)
	Glomus fasciculatum BEG 53	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in plastic eppendorf tubes or in cryovials	NA	Cryopreservation	Directly at: -18°C, -80°C or in LN	14, 14 and 12 months respectively	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)
	Glomus clarum BEG142	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in plastic eppendorf tubes or in cryovials	NA	Cryopreservation	Directly at: -18°C, -80°C or in LN	17 months for all the preservation temperatures	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)

	Glomus clarum LPA 64*	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in plastic eppendorf tubes or in cryovials	NA	Cryopreservation	Directly at: -18°C, -80°C or in LN	8, 13 and 13 months respectively	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)
	Glomus spp LPA 17*	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in plastic eppendorf tubes or in cryovials	NA	Cryopreservation	Directly at: -18°C, -80°C or in LN	8, 8 and 6 months respectively	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)
	Glomus intraradicess LPA 54*	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in plastic eppendorf tubes or in cryovials	NA	Cryopreservation	Directly at: -18°C or in LN	9 and 9 months respectively	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)

	Gigasora rosea BEG 9	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in plastic eppendorf tubes or in cryovials	NA	Cryopreservation	Directly at: -18°C or-80°C	17 and 17 months respectively	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)
	Gigasora candida BEG 17	Spores, vesiculs and or mycelium seived from 6-8months pot culture old, dried for 3-4days and puted in plastic eppendorf tubes or in cryovials	NA	Cryopreservation	Directly at -18°C	10 months	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)
	Acualospora longula BEG 8	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in plastic eppendorf tubes or in cryovials	NA	Cryopreservation	Directly at: -18°C, -80°C or in LN	15, 27and 15months respectively	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)

	Acualospora levis BEG 13	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in plastic eppendorf tubes or in cryovials	NA	Cryopreservation	Directly at: -18°C, -80°C or in LN	17, 26 and 26 months respectively	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)
	Acualospora levis BEG 26	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in plastic eppendorf tubes or in cryovials	NA	Cryopreservation	Directly at: -18°C, -80°C or in LN	14, 13 and 8 months respectively	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)
	Acualospora scrobiculata BEG 33	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in plastic eppendorf tubes or in cryovials	NA	Cryopreservation	Directly at: -18°C, -80°C or in LN	15, 28 and 15 months respectivly	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)

	Scutellispora nodosa BEG 47	Spores, vesiculs and or mycelium sieved from 6-8months pot culture old, dried for 3-4days and putted in plastic eppendorf tubes or in cryovials	NA	Cryopreservation	Directly at -18°C	13months	At room temperature by rehydration with osmotic water	Kuszala et al. (2001)
	Claroideoglomus claroideum PER 9.1 *Claroideoglomus claroideum* PER 8.5 *Septoglomus constrictus* PER 7.2 *Paraglomus brasilianum* Ecu 18.VF	*In vivo* produced spores and vesicules incapsulated in alginate beads and dried for 48h	Trehalose (0,5M)	Cryopreservation	12°C/min from room temperature (+20°C) to -110°C then 1°C/min- from -110°C until -130	1month	In water bath at 35°C for 15min	Lalaymia et al. (2013b)

	*Glomus intraradices***	Fungi 6weeks old in vitro cultured on root organ culture on Minimum medium (MM)	NA	Cryopreservation	2°C/min from 23°C to 3°C, and incubation for 10 days at 3°C, transfert in refrigator at 1°C for 2 days and the directly in the freezer at -12°C	2days	At 1°C over night	Addy et al. (1998)
	Rhizophagus sp, MUCL 41385	*In vitro* produced spores encapsulated in alginate beads	Trehalose (0,5M)	Cryopreservation	1°C/min from +20°C to -35°C then 18 °C/min from -35°C until -100°C	3hours	In water bath at 35°C for 15min	Declerck and Van Coppenolle (2000)
	Rhizophagus sp. MUCL 41833 *Rhizophagus sp.* MUCL 41835	*In vitro* produced spores and vesicules	Trehalose (0,5M)	Cryopreservation	12°C/min from room	6months	In water bath at 35°C for	Lalaymia et al. (2012)

	Rhizophagus sp. MUCL 43195 *Rhizophagus sp.* MUCL 43196 *Rhizophagus sp.* MUCL 43204 *Rhizophagus sp.* MUCL 46239 *Rhizophagus sp.* MUCL 49424 *Rhizophagus irregularis* MUCL 43194 *Rhizophagus fasciculatus* MUCL46100 *Glomus aggregatum* MUCL 49408 *Rhizophagus diaphanus* MUCL 49416	incapsulated in alginate beads and dried for 48h			temperature (+20°C) to -110°C then 1°C/min- from -110°C until -130°C		15min	
	Rhizophagus sp. MUCL 49422 *Rhizophagus proliferus* MUCL 41827 *Rhizophagus clarus* MUCL 46238 *Rhizophagus sp.* MUCL 45686 *Glomus sp.* MUCL 43208	*In vitro* produced spores and vesicules incapsulated in alginate beads and dried for 48h	Trehalose (0,5M)	Cryopreservation	12°C/min from room temperature (+20°C) to -110°C then 1°C/min- from -110°C	1month	In water bath at 35°C for 15min	Lalaymia et al. (2013b)

	Gigaspora sp. MUCL 52331	until -130°C
	Claroideoglomus claroideum MUCL 54351	

MMN: Modified Melin Norkrans
PGM modified Pridham-Gottlieb medium
MFM: modified Fries Medium
LN: Liquid Nitrogen
NA: Not Applicable in this mode of preservation

Isolates code:

- DR: Culture collection of Dana L. Richter; ATCC, American Type Culture Collection, Manassas, Virginia. USA
- S238 N: Culture collection of USDA Forest Service. Forestry Sciences Laboratory, Corvallis. OR. USA
- KTP: Culture collection of CSIRO Division of Forestry and Forest Products Private Bag PO, Wembley 60014 Australia
- SIV : Culture collection of Equipe de microbiologie forestière Nancy-France)
- ATTC: American Type Culture Collection, Manassas, Virginia. USA
- CCBAS: Culture Collection of Basidiomycetes, Institute of Microbiology, Academy of Sciences of the Czech Republic
- MUCL: Mycothèque de l'Université catholique de Louvain
- BEG: Banque Européenne des Glomales
- Code isolates in Kuek et al. (1992): E or H letters followed by numbers are isolates from the culture collection of the Commonwealth Scientific and Industrial Research Organization's (CSIRO)

Annex II

- Code isolates in Crahay et al. (2013a): The first number refers to the code number assigned to the isolate in the Mycothèque de l'Université catholique de Louvain (MUCL), Louvain-la-Neuve, Belgium and the number between brackets refers to the origin code of the isolate
- Code isolates in Lalaymia et al. (2013b): PER and VF are isolates in the MUCL *in vivo* collection.
- *: The culture collection from which the isolate was purchased is not determined
- ** No culture code was found in the publication from which the information was purchased