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

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ABSTRACT

At present, over 300 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 m 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 °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 (+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

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life cycle with the production of several hundreds to thousands of spores after 2 m. This method opens the door for the long-term maintenance at ultra-low temperature of AMF isolates within international repositories.

Introduction

Arbuscular mycorrhizal fungi (AMF) are root symbionts forming associations with an estimate of 80 % of land plants (Smith & 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 & Scheublin 2007).

In the recent years, the taxonomy and phylogeny of AMF have received an increasing attention. The Glomeromycota 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 & Walker 2010; Oehl *et al.* 2011). Over 300 species are identified at present (Schüßler & 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 (BEG) 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 & Schenck 1990; Plenchette *et al.* 1996; Declerck & 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; Cárdenas-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 & 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 (Dalpe 1987). Tommerup (1988) also successfully preserved a number of AMF species by L-drying. *Rhizophagus fasciculatus* (synonym *Glomus fasciculatum*) was preserved for 4 y at −4 °C in a dried soil (Douds & Schenck 1990). More recently, Declerck & 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 °C min^{-1}) from +20 °C to −35 °C and rapid (18 °C min^{-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 & Engelmann 2007) 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 & 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.

Materials and methods

Biological material

Twelve AMF isolates originating from different biotopes were considered (Table 1). 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* L. clone DC2) roots. The isolates were maintained on the modified Strullu-Romand (MSR) medium (Declerck *et al.* 1998), solidified with 3 g l^{-1} phytagel (Sigma–Aldrich, USA). The Petri plates were incubated in an inverted position in the dark at 27 °C. After 4–5 m, several hundred to thousand of spores were obtained in each Petri plate.

Table 1 – AMF isolates tested for the encapsulation–drying cryopreservation method.

AMF isolates	Authorities	Synonymy	Local code	Other code	Origin	Biotope
Rhizophagus sp.	C. Walker & Schuessler, 2010	*Glomus sp.*	MUCL 41833	DAOM 233750	Canaries Islands	Tropical
Rhizophagus sp.	C. Walker & Schuessler, 2010	*Glomus sp.*	MUCL 41835	DAOM 233751	Unknown (contaminant of a Danish isolate)	Unknown
Rhizophagus sp.	C. Walker & Schuessler, 2010	*Glomus sp.*	MUCL 43195	DAOM 212349	Wasaga beach, Ontario, Canada	Temperate
Rhizophagus sp.	C. Walker & Schuessler, 2010	*Glomus sp.*	MUCL 43196	DAOM 229456	Unknown (contaminant of a New Zealand isolate)	Unknown
Rhizophagus sp.	C. Walker & Schuessler, 2010	*Glomus sp.*	MUCL 43204	DAOM 229457	Clarence-Creek Ontario	Temperate
Rhizophagus sp.	C. Walker & Schuessler, 2010	*Glomus sp.*	MUCL 46239	DAOM 234181	Cap-aux-Meules, iles-de-la Madeleine, Québec, Canada	Temperate
Rhizophagus sp.	C. Walker & Schuessler, 2010	*Glomus sp.*	MUCL 49424	FTSR203	Martinique	Tropical
Rhizophagus irregularis	(Błaszk, Wubet, Renker & Buscot) C. Walker & Schuessler, 2010 [as '*irregulare*']	*Glomus irregularis*	MUCL 43194	DAOM 181602 DAOM 197198	Pont-Rouge, Québec	Temperate
Rhizophagus fasciculatus	(Thaxt) C. Walker & Schuessler, 2010	*Glomus fasciculatum*	MUCL 46100	NA	Unknown	Tropical
Glomus aggregatum[a]	N.C. Schenck & G.S. Sm., 1982	NA	MUCL 49408	105 73	Brittany, France	Temperate
Rhizophagus diaphanus	(J.B. Morton & C. Walker) C. Walker & Schuessler, 2010	*Glomus diaphanum*	MUCL 49416	STR05-130A	Eschikon-lindau, Switzerland	Temperate
Rhizophagus intraradices	(N.C. Schenck & G.S. Sm.) C. Walker & Schuessler (2010)	*Glomus intraradices*	MUCL 49410	DAOM 197198	Florida, USA	Temperate

NA = not applicable.
a Species of uncertain position [C. Walker & Schuessler, (2010)].

Encapsulation procedure

For each experiment described below, the AMF isolates were encapsulated in alginate beads following the procedure described by Declerck & Van Coppenolle (2000). Briefly, spores were isolated from the Petri plates by solubilisation of the MSR medium (Doner & Bécard 1991) and further separated from roots with forceps, filtered on a sterilized (121 °C for 15 min) nylon mesh (40 μm) and suspended in a 2 % (w/v) solution of sodium alginate (acid sodium salt from brown algae, Sigma–Aldrich, UK) sterilized at 121 °C for 15 min. Groups of 50 ± 5 spores were recovered with a micropipette and dropped into a sterilized (121 °C for 15 min) solution of 0.1 M $CaCl_2$ 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 m old cultures of *Rhizophagus sp.* MUCL 43204 and encapsulated in alginate beads as described above. The beads were subsequently stored in a Petri plate overnight at 15 °C. The beads were then immersed for 1 d in trehalose (0.5 M) 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 °C min^{-1}) from room temperature (+20 °C) to −35 °C followed by a fast decrease in temperature (18 °C min^{-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.* 1996). 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 m old cultures of *Rhizophagus sp.* MUCL 43204 were encapsulated in alginate beads, incubated at 4 °C overnight in trehalose (0.5 M) and dried for 24 h 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 °C min^{-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 µl) 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 J g^{-1}).

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

Spores of 5 m 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 °C min^{-1}) from room temperature (+20 °C) to −35 °C followed by a fast decrease in temperature (18 °C min^{-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 °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 (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 °C min^{-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 m 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 d. 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 d 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 crushed AMF cultures on the %PIB of 12 AMF isolates

For each isolate (Table 1), the gelling medium, containing spores and roots of 5 m 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 crushed two times for 30 s at the 8th speed 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 µm) 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, dried at 27 °C for 2 d and cryopreserved in the freezer at −130 °C). The isolates were cryopreserved for 1 d, 1 m, 3 m, and 6 m. 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 m 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 m. Spore production was evaluated under a binocular microscope at 10–40× 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 & Hayman (1970). Colonization was estimated under a bright-field light microscope at 50–250× 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 %. 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 m old culture was 50 and 25 % respectively. Interestingly the highest %PIB, was observed with spores isolated from 5 m old cultures. The %PIB was 80 % and significantly differed as compared to the %PIB of encapsulated spores isolated from 4.5 and 4 m old cultures.

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 1). 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.

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 3 h. Whatever the assay, the drying of beads before cryopreservation had no detrimental effect on the %PIB. For each assay, the %PIB was 100 % (data not shown). To the contrary, after cryopreservation at −100 °C for 3 h, the %PIB of the non-dried beads was zero for all the assays (data not shown). 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 % (data not shown).

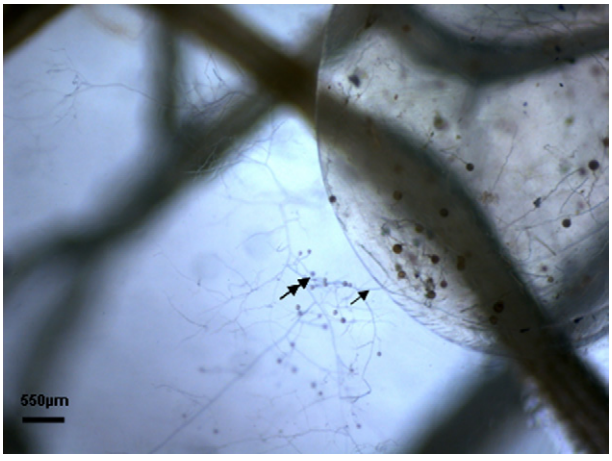

Fig 1 – Encapsulated spores of *Rhizophagus sp*. MUCL 43204 incubated in trehalose (0.5 M) and cryopreserved for 3 h at −100 °C, associated to a transformed carrot root. Details showing hyphal re-growth through the calcium chloride coating (arrow) and spore production (double arrow).

The DSC data for beads osmohydrated with trehalose and dried or not are presented in Fig 2. 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 J g^{-1} (Fig 2) 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 2).

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

Beads, containing spores of *Rhizophagus sp*. MUCL 43204 sampled from 5 m 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. 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 (~12 °C min^{-1}) from room temperature (+20 °C) to −110 °C and subsequent slow decrease in temperature (~1 °C min^{-1}) from −110 °C to −130 °C (i.e. $\%PIB = 75 \pm 13.5$). 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 °C min^{-1}) from room temperature (+20 °C) to −35 °C followed by a fast decrease in temperature (18 °C min^{-1}) from −35 °C to −100 °C) (i.e. $\%PIB = 37.5 \pm 25$) and to the %PIB of beads cryopreserved with direct freezing in liquid nitrogen until −196 °C (i.e. $\%PIB = 27.5 \pm 25.6$) (very fast temperature decrease of 200 °C min^{-1}) ($P < 0.0001$ and 0.0001 respectively).

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

Beads containing spores of *Rhizophagus sp*. MUCL 43204 isolated from 5-m old cultures were dried at 27 °C for 1, 2, 3, and 5 d. 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 d drying (data not shown). The % PIB measured after 10 d of incubation on the MSR medium (at 27 °C in the dark) was 100 % for the beads dried 1 and 2 d (water content = 20.6 ± 2.1 and 8.1 ± 4.6 %, respectively). After 3 d drying, the beads water content were significantly decreased and the %PIB decreased to 80 % (data not shown). After 5 d drying, no spore germination was observed in the beads. The spores appeared empty (Fig 3).

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

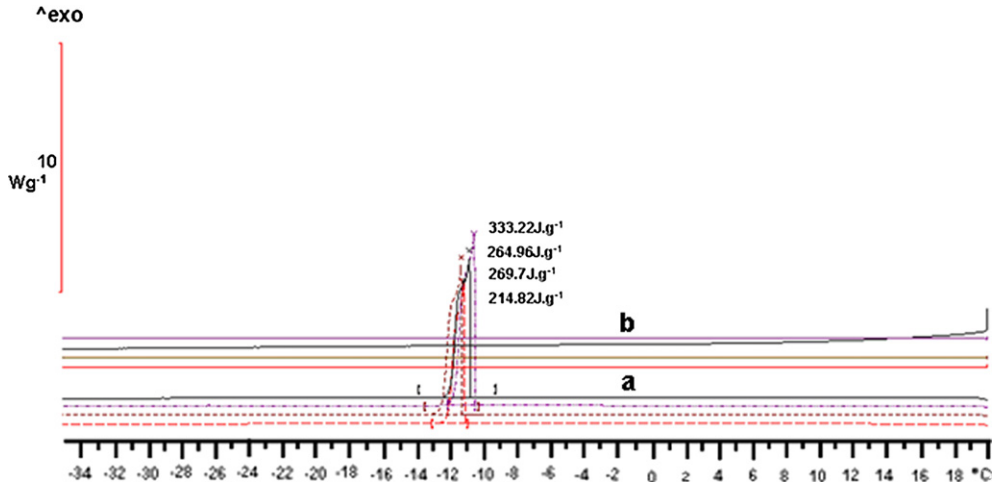

Fig 2 – Thermograms resulting from thermal analysis (using DSC) of alginate beads containing spores of *Rhizophagus sp.* MUCL 43204. (a) Beads osmodehydrated with trehalose and not dried, (b) beads osmodehydrated with trehalose and dried for 24 h in an incubator set at 27 °C. Each curve represents one bead ($n = 4$).

(Table 1) isolated from 5m old cultures, dried for 2 d before cryopreservation at −130 °C for 1 d, 1 m, 3 m, and 6 m is presented in Table 2.

Irrespective of the AMF isolates, the drying had no effect on the %PIB before cryopreservation. For each treatment, the %PIB was 100 % (Table 2). With the exception of encapsulated spores of *Rhizophagus fasciculatus* MUCL 46100 cryopreserved for 1, 3, and 6 m, 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 m later than the spores of the non-cryopreserved encapsulated treatment. The ability of the encapsulated 6 m cryopreserved germinated spores to re-initiate the fungal life cycle was evaluated after 2 m of association (Table 3). 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*

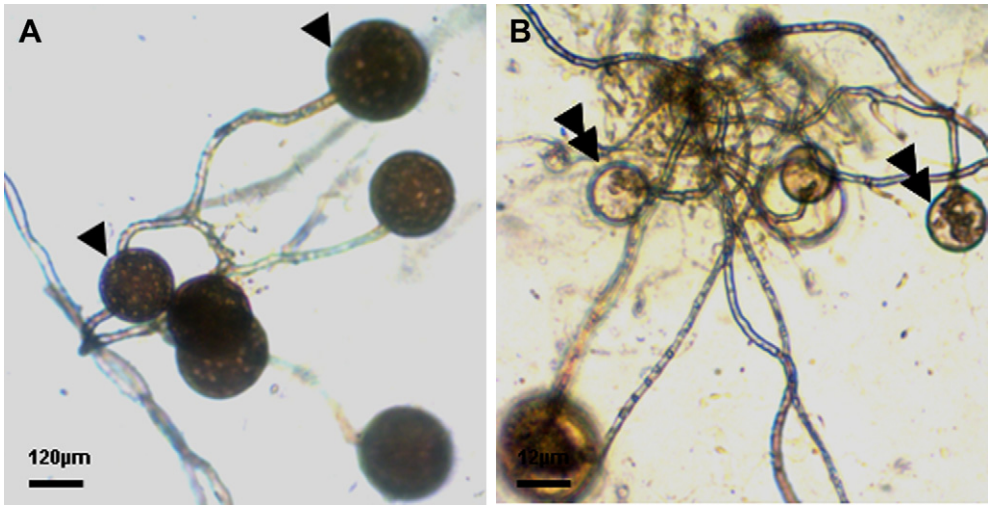

Fig 3 – Spores of *Rhizophagus sp.* MUCL 43204 isolated from 5 m old cultures, encapsulated in beads and dried for 2 d (A) and 5 d (B) at 27 °C. Details showing healthy spores (arrows) and damaged (empty) spores (double arrows).

Table 2 – %PIB after drying (2 d) and cryopreservation at −130 °C for 1 d, 1 m, and 3 m of beads containing AMF propagules (i.e. spores and mycorrhizal/non-mycorrhizal roots) sampled from 5 m 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.

AMF isolates	%PIB				
	Before cryopreservation	After cryopreservation			
		1 d	1 m	3 m	6 m
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]
Rhizophagus 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 column 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 m of cryopreservation.

MUCL 49416 and *Rhizophagus sp.* MUCL 43204 cultures respectively.

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 & 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 & Van Coppenolle 2000). Here we achieved the cryopreservation for several months at −130 °C of 12 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 m 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 m 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 & Van Coppenolle 2000), conidia of several filamentous fungi (Chandler 1994) and ectomycorrhizal fungi (Mauperin *et al.* 1987; Paloschi de Oliveiral *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-

Table 3 – Estimation of spores production and root colonization of 12 AMF cultures from 6 m cryopreserved isolates after 2 m 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 MUCL 46100	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

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 m old cultures. This findings corroborated the earlier study conducted by Declerck & 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). The hydroxyl groups of this cryoprotectant substitute the cellular water molecules and interact with the polar residues of membrane and proteins. This substitution 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 m 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 m 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, polysaccharides, and heat shock proteins, for membrane and proteins protection (Fuller *et al.* 2004). 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 & 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 5m old cultures are higher than 4.5 and 4m 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 thermograms 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 (Bart & Maurizio 2005; Engelmann *et al.* 2008). However, this value may vary depending on the type of sample (Block 2003). In our study, after 1 d of beads drying at 27 °C, encapsulated spores were still viable and germinated (data not shown). 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 d (8.1 ± 4.6 % of beads water content). Beads treated with trehalose and dried for 2 d at 27 °C had the highest and most homogenous %PIB.

The %PIB was the highest for encapsulated spores treated with trehalose, dried for 2 d and cryopreserved at −130 °C in the freezer (by fast cooling (~12 °C min^{-1}) until −110 °C followed by a slow cooling (~1 °C min^{-1}) until −130 °C) as compared with the cryopreservation at −100 °C with the controlled cooling (a slow decrease (1 °C min^{-1}) from room temperature (+20 °C) to −35 °C followed by a fast decrease in temperature (18 °C min^{-1}) from −35 °C to −100 °C) and with the cryopreservation in liquid nitrogen with the very fast cooling rate (i.e. 200 °C min^{-1}). In common practices for fungal cryopreservation, fungi are cooled slowly at 1 °C min^{-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 °C min^{-1} (Mazur 1977), the bulk of extracellular water is slowly crystallized, leaving gradually a high extracellular concentrated solution (Mazur 1984; Fuller *et al.* 2004). 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 °C min^{-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.

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 m 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 m 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 m old cultures, (2) the incubation overnight in trehalose (0.5 M), (3) the drying at 27 °C for 2 d, (4) the cryopreservation in the freezer at −130 °C with two steps decrease in temperature: (1) 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. This protocol allowed the successful cryopreservation at −130 °C for several months of 12 isolates belonging to six 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.

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