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Storage Conditions for the Long-Term Survival of AM Fungal Propagules in Wet Sieved Soil Fractions

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Abstract

The survival of 20 glomalean isolates belonging to 16 species in four genera (*Glomus*, *Acaulospora*, *Gigaspora* and *Scutellospora*) has been evaluated under different storage conditions for the conservation of their germplasm, in order to guarantee a back-up reserve and an alternative supply source for start cultures. Soil from pot cultures of the different fungal isolates was wet sieved and fractions of soil debris containing mycelium and spores were collected. These fractions were stored at room temperature (18–24°C), +4°C, –18°C, –80°C, in liquid nitrogen, or were lyophilised. Six of the fungi were also stored in water at either room temperature or +4°C. Samples were removed after different times of storage and inoculated onto a test plant in soil. After 6, 7 or 8 months, the entire soil was wet sieved from the inoculated pots and fungal viability assessed by the occurrence of newly formed spores and/or sporocarps. Sporulation was observed for all isolates stored at all temperatures. Storage in water did not affect survival of the six tested fungi. These results indicate the resilient nature of AM fungi, and demonstrate the feasibility of long-term storage of their germplasm using a simple protocol which can be applied to a wide range of isolates.

Keywords: Glomales, mycelium, spores, storage, survival

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1. Introduction

Arbuscular mycorrhizal (AM) fungi are mycosymbionts essential for the growth and survival of many plants (Smith, 1992). They form root associations with more than 80% of higher plant families and are represented by more than 130 species in the order Glomales (Smith and Read, 1997; Walker, 1992). AM fungi are ecologically obligate biotrophs that have not been cultured on defined synthetic media in the absence of living roots (Siqueira et al., 1985). Reliable maintenance of AM fungal cultures is important for both research and for widespread use of the fungi at the commercial level but culturing success depends very much on the host plant, the fungal isolate, and the physical and chemical properties of the soil or substrate (Smith and Read, 1997). Appropriate storage conditions are essential for large culture collections in order to ensure a back-up reserve of germplasm.

AM fungal propagules exist either as free mycelium or spores in soil, or as mycelial colonies within roots, and each of these forms have the potential to initiate an infection. Spores of some AM fungi have a dormancy period which can extend from a few weeks to several months depending on the species or isolate (Tommerup, 1983). Various techniques have been developed for long-term preservation of AM fungi *ex-planta* such as dry storage, freeze-drying, cryopreservation with or without cryoprotectant, and lyophilization, but investigations have involved different species and conditions so that it is difficult to standardise a procedure. For example, Young (1982) reported that some propagules of AM fungi are capable of germinating and colonising roots of bioassay plants after 2 to >28 years storage in air dried soils kept at room temperature. The range of spore types in this study included representatives of *Glomus*, *Acaulospora*, *Gigaspora* and *Sclerocystis* species. Douds and Schenck (1991) showed for a few species (*Gigaspora margarita*, *Glomus mosseae*, *Glomus intraradices*, *Acaulospora longula*) that spores have different responses to storage duration and moisture availability in soil, reflecting the complexity of problems for storing AM fungi in soil.

Ferguson and Woodhead (1982) advised storing whole pot cultures in dry soil at 3–5°C. However, Siqueira et al. (1985) suggested that the simplest and most successful method of storage of AM fungi was to leave spores in partially dried, native soil and to store the entire sample in a sealed plastic container at 4–6°C. Louis and Lim (1988) reported that pre-germination treatment of the mycorrhizal inoculum, by prolonged storage in dry soil at 25–30°C followed by a brief cold treatment at 4°C, enhanced germination of *G. clarum* spores on water agar. Spores of *G. clarum* have also been reported to survive storage better in wet than dry sand or soil (Daft et al., 1987). Mugnier and Mosse (1987) stored sporocarps of *G. mosseae* for 4 years at 4°C in a moist atmosphere, whilst Afek et al. (1994) found that dried inoculum (mixture of roots, soil and spores) of *G.*

mosseae was effective when stored for 4 or 8 weeks at 24°C, or below 10°C when moist. Tommerup (1988) has successfully preserved spores of *A. laevis*, *A. trappei*, *G. monosporum*, *G. caledonium*, *S. calospora*, dry hyphae of *G. tenue*, *G. fasciculatum*, *S. calospora*, and mother-cells of *A. laevis* by L-drying and storage under vacuum. Lyophilization has only proved effective for species with thick-walled spores like *G. clarum*, *G. constrictum*, *G. macrocarpum* and *G. pustulatum* (Dalpé, 1987).

Not all AM fungi can be cryopreserved by standard techniques. The best method of cryoprotection and cryopreservation was found to be slow drying of pot culture soil and freezing (3 months) of spores in situ, that is in the soil in which they were produced (Douds and Schenck, 1990). This procedure was satisfactory for some representative species of five genera of AM fungi. Safir et al. (1990) suggested storage of soil inoculum at –10°C for 28 to 58 days, in order to improve and synchronize spore germination of *G. mosseae* and *G. fasciculatum*. Addy et al. (1998) demonstrated that when in vitro cultures of *G. intraradices* were slowly cooled prior to freezing the majority contained active hyphae, whereas hyphal activity was almost completely eliminated by freezing in non-precooled cultures. Morton et al. (1993) have reported to store all germplasm from the International Collection (INVAM) in dry growth substrate at 4°C (cold room) or at –196°C (liquid nitrogen). Successful storage of pot cultures in liquid nitrogen for six years or longer has been reported for a certain number of fungi from the International INVAM Collection but the species are not indicated (INVAM, 1997).

There are major difficulties in comparing this fragmentary information in order to draw general conclusions about the effect of storage conditions on the infectivity of different AM fungal isolates and forms of inoculum. However, the response of AM fungal propagules to factors limiting *ex-planta* viability is essential to their long-term storage. The aim of the present work was to develop a simple protocol for long-term *ex-planta* conservation of viable glomalean germplasm which can be generalised to different AM fungal species, in order to guarantee a back-up reserve and an alternative supply source for start cultures. For this, the survival of 20 glomalean isolates belonging to 16 species in four genera (*Glomus*, *Acaulospora*, *Gigaspora* and *Scutellospora*) was tested under different storage temperatures using soil sievings of spores and mycelium.

2. Material and Methods

Source and maintenance of fungi

All cultures originated from the BEG (Banque Européenne des Glomales) and

Table 1. Details of glomalean fungi, host plant, culture medium and sieved fractions.

Fungal species	ID[1] code	Origin	Plant[2]	Culture medium[3]	µm	FC[4]	µm
Glomus							
geosporum (Nicol. & Gerd.) Walker	BEG 11	UK	Leek	Alkaline soil +Terragreen	160	<FC	< 250
mosseae (Nicol. & Gerd.) Gerdemann & Trappe	BEG 12	UK	Leek	Alkaline soil	100	<FC	< 160
claroideum Schenck & Smith emend. Walker & Vestberg	BEG 14	Denmark	Leek	Alkaline soil	100	<FC	< 160
caledonium (Nicol. & Gerd.,) Trappe & Gerdemann	BEG 20	UK	Leek	Alkaline soil	160	<FC	< 250
coronatum Giovannetti	BEG 22	Australia	Leek	Alkaline soil	250	<FC	< 350
claroideum Schenck & Smith emend. Walker & Vestberg	BEG 23	Czech Rep.	Leek	Alkaline soil	100	<FC	< 160
claroideum Schenck & Smith emend. Walker & Vestberg[5]	BEG 31	Finland	Clover	Acid soil+gravel	100	<FC	< 160
versiforme (Karsten) Berch	BEG 47	USA	Leek	Alkaline soil	100	<FC	< 160
fasciculatum (Thaxter) Gerd. & Trappe emend.	BEG 53	Canada	Leek	Alkaline soil +Terragreen	60	<FC	< 100
fasciculatum (Thaxter) Walker & Koske	BEG 53	Canada	Leek	Alkaline soil +Terragreen	100	<FC	< 160
clarum Nicolson & Schenck	BEG 142	Brazil	*Tephrosia* sp.	Acid soil+perlite	100	<FC	< 160
clarum Nicolson & Schenck	BEG 142	Brazil	*Tephrosia* sp.	Acid soil+perlite	160	<FC	< 250
intraradices Schenck & Smith	LPA 54	Denmark	Leek	Alkaline soil	160	<FC	< 250
clarum Nicolson & Schenck	LPA 64	Brazil	*Tephrosia* sp.	Acid soil+gravel	100	<FC	< 160
Gigaspora							
rosea Nicolson & Schenck	BEG 9	USA	Leek	Terragreen	160	<FC	< 250
candida Bhattacharjee, Mukerji, Tewarii & Skoropad	BEG 17	Taiwan	Leek	Alkaline soil +Terregreen	160	<FC	< 250

Table 1. Continued.

Fungal species	ID[1] code	Origin	Plant[2]	Culture medium[3]	µm	FC[4]	µm
Acaulospora							
longula Spain & Schenck	BEG 8	UK	Clover	Acid soil+gravel	60	< FC	< 100
laevis Gerdemann & Trappe	BEG 13	UK	Clover	Acid soil+gravel	160	< FC	< 250
laevis Gerdemann & Trappe	BEG 26	China	Clover	Acid soil+perlite	160	< FC	< 250
scrobiculata Trappe	BEG 33	UK	Clover	Acid soil+gravel	100	< FC	< 160
Scutellospora							
nodosa Blaszkowski	BEG 4	UK	Clover	Acid soil+gravel	160	< FC	< 250

[1]Details of BEG isolates can be found at http://www.bio.ukc.ac.uk/BEG.
[2]Leek: *Allium porrum* L. cv. Electra; onion†: *A. cepa* L. hybrid Topaze; clover : *Trifolium repens* L. cv. Diper.
[3]Alkaline soil: clay loam, pH (H_2O) 8, 24 ppm Olsen P; acid soil: silty clay, pH (H_2O) 4.9, 11.6 ppm Olsen P.
[4]Size of sieved soil fractions collected.
[5]Walker and Vestberg, 1998.

LPA (Laboratoire de Phytoparasitologie) collections held at UMR INRA / Université de Bourgogne BBCE-IPM, Dijon, France. Cultures used were from individual leek, onion, clover or *Tephrosia* sp. pot cultures. The fungi, host plant and growth medium used within the study are listed in Table 1.

Preparation of fungal material

Soil was wet sieved from fresh 6 to 18 month old pot cultures of the different fungal isolates. According to the fungus, different sieved fractions (Table 1) of soil debris containing mycelium and spores were collected, recuperated on a 50 µm Filtys nylon filter (nylon tissue, group SEFAR, Germany), mixed together and divided into sub-samples of approximately 0.8 ml. Each sub-sample was heterogeneous in spore maturity and contained between 100 and 200 spores plus mycelium, vesicles or auxiliary cells. Sub-samples were placed in 1.8 ml cryopreservation vials, 1.5 ml plastic Eppendorf tubes or lyophilization bottles, depending on storage conditions to be used.

Ex-planta storage of inoculum

Sub-samples were partly dried by exposing to air for 3 to 4 days at room temperature (18–24°C), and then stored either at room temperature (18–24°C), +4°C, –18°C, –80°C or in liquid nitrogen (sub-samples were immersed in a cryotank), or were lyophilised for 48 hours and then maintained at room temperature. Six of the fungi (*G. geosporum* BEG 11, *G. claroideum* BEG 31, *G. fasciculatum* BEG 53, *G. clarum* LPA 64, *A. longula* BEG 8, *A. scrobiculata* BEG 33) were also stored in osmosed water after sieving, at either room temperature or +4°C.

Infectivity tests of stored inoculum

Viability and mycorrhizal capacities of inoculum sub-samples were verified after different times of storage by inoculation onto host plants. Samples were put at room temperature and rehydrated with osmosed water, then were immediately inoculated into the same sterilised soil or soil mixes and onto the same host plant as the culture from which they originated. Soil (see Table 1 for characteristics) was γ-irradiated and sterilised two hours at 180°C before using to fill plastic containers of 400 ml. Gravel and Terragreen (OIL DRI® Typ III R US - Special, CONEX GmbH) used in mixes were also sterilised at 180°C. Soil or soil mixes were watered to field capacity. The inoculum was placed on the root of sterile pre-germinated seedlings at transplanting in order to optimise the opportunity for mycorrhiza establishment, and the root was covered with the sterile soil or soil mix.

All experiments were carried out in a growth room. Day/night temperatures

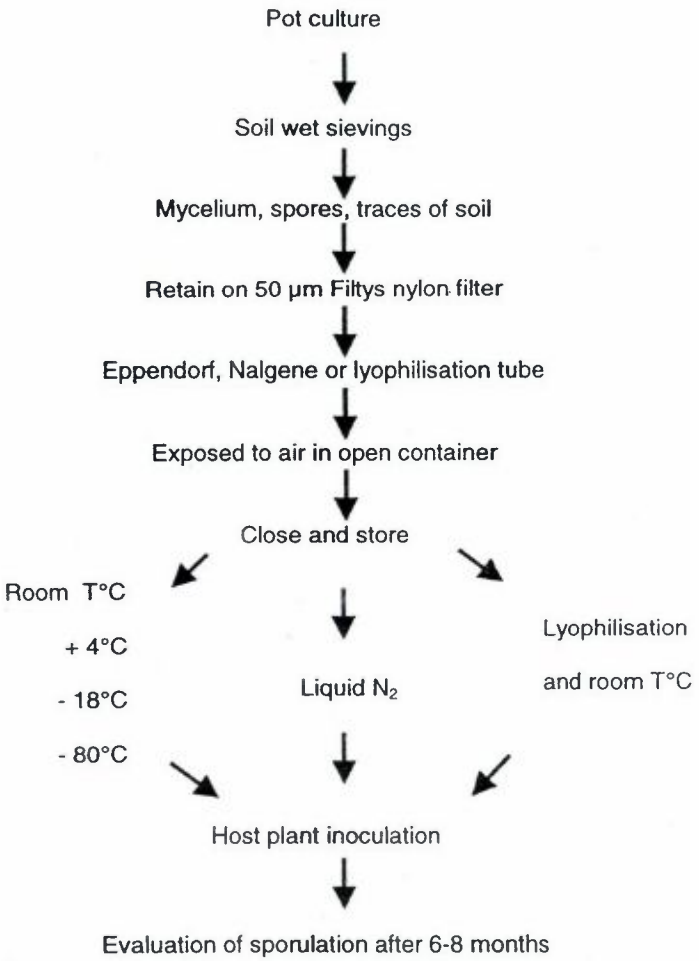

Figure 1. Protocol for long-term conservation of glomalean germplasm *ex-planta*.

were 27°C/25°C for *G. clarum* (LPA 16, LPA 64) and 22°C/19°C for all other fungi. Other growth conditions were: 16 hour photoperiod, 60–70% relative humidity, 300 µE/m^2/sec illumination. Pots were watered daily with osmosed water and fertilised once a week with a modified (P/10) Long Ashton nutrient solution (Hewitt, 1966).

Entire soil from pots was wet-sieved after 6, 7 or 8 months and fungal viability assessed by the occurrence of newly formed spores and/or sporocarps. Root colonisation was also checked in random samples from plants inoculated with *G. claroideum* BEG 14, *G. rosea*, BEG 9, *A. laevis* BEG 26 and *S. nodosa*

Table 2. Number of storage months up to which AM fungal viability was detected under different conditions. Results from 2 or 3 replicate experiments, except where marked with an asterisk.

Fungal species	ID code[1]	Room T(°C)[2]		+4°C[2]		Storage conditions			
		H_2O		H_2O		−18°C	−80°C	Liquid N_2	Lyophilisation
Glomus									
geosporum	BEG 11	20	26	20	26	26	26	26	26
mosseae	BEG 12		21		?	21	21	14	12*
claroideum	BEG 14		15		15	15	15	15	15*
caledonium	BEG 20		20		20	20	20	20	nd
coronatum	BEG 22		9		9	9	9	nd	9
claroideum	BEG 23		10		2*	10	7	nd	2*
claroideum	BEG 31	14	14	14	14	16	25	25	16*
versiforme	BEG 47		8		8	8	8	7*	nd
fasciculatum	BEG 53	14	7*	14	12	14	14	12	nd
clarum	BEG 142		17		17	17	17	17	nd
spp	LPA 17		6*		6*	8	8	6*	nd
intraradices	LPA 54		2*		2*	9*	nd	9*	nd
clarum	LPA 64	13	13	13*	13	8*	13	13	nd
Gigaspora									
rosea	BEG 9		8		8	17	17	nd	nd
candida	BEG 17		5		5*	10	nd	nd	nd

Table 2. Continued.

Fungal species	ID code[1]	Room T(°C)[2]		+4°C[2]		Storage conditions			
		H_2O		H_2O		−18°C	−80°C	Liquid N_2	Lyophilisation
Acaulospora									
longula	BEG 8	7*	27	7*	27	15	27	15	15
laevis	BEG 13		26		26	17	26	26	17
laevis	BEG 26		13		13	14	13	8*	10
scrobiculata	BEG 33	15*	15	15*	15	15	28	15*	28
Scutellospora									
nodosa	BEG 4		6		13	13*	nd	nd	nd

[1]See Table 1.
[2]Sieved soil fractions are either suspended in osmosed water (H_2O) or partially air dried.
nd: Not determined.
?: Viability not detected at 21 months.

BEG 4 after storage at –18°C. Root systems were washed, cleared in 10% potassium hydroxyde at 120°C for 1 hour 30, then stained with 0.5% trypan blue in lactoglycerol for 15 minutes at 120°C. Roots were examined microscopically for fungal colonisation.

3. Results and Discussion

The protocol developed in the present work for long-term storage of glomalean fungi is summarised in Fig. 1. Fungi were considered to have survived a storage condition if sporulation reached a minimal number of 400 spores in pots 6 to 8 months after inoculation, or if sporocarps were produced in the case of *G. mosseae*. Results for the viability of the 20 different fungi are summarised in Table 2. Inoculum was tested after 2 months storage and onwards, and the large majority of the fungi were tested after a minimum of 8 months storage.

All the fungal isolates, except one (*G. mosseae* BEG 12), survived the conditions under which and for the time they were maintained, even at low temperatures without cryoprotectant. Sporulation was observed for all isolates stored below 0°C, including the tropical isolates of *G. clarum* (BEG 142 and LPA 64). *G. caledonium* BEG 20, *G. geosporum* BEG 11 and *A. laevis* BEG 13 were still viable after storing for 17, 20 or 26 months whatever the tested conditions. Other fungi like *A. longula* BEG 8, *G. claroideum* BEG 31 and *A. scrobiculata* BEG 33 were found to survive up to 25 to 28 months under different storage conditions, including –80°C, liquid N_2 and lyophilisation (Table 2). Species belonging to the genera *Acaulospora* and *Glomus* sporulated abundantly, and *G. claroideum* BEG 14 and BEG 31, and *A. laevis* BEG 13, were the most prolific after storing. Sporulation was, on the contrary, less in pots inoculated with the genera *Scutellospora* or *Gigaspora*. Roots of plants inoculated with *G. claroideum* BEG 14, *G. rosea*, BEG 9, *A. laevis* BEG 26 and *S. nodosa* BEG 4 stored at –18°C were all mycorrhizal.

Differences in survival were observed with *G. mosseae* BEG 12. This fungus survived room temperature, –18°C, –80°C and liquid nitrogen during 14 months. In contrast, 4°C during 14 and 21 months were lethal. Mugnier and Mosse (1987) stored sporocarps at 4°C in a moist salt-satured atmosphere and spores were excised as required. Under these conditions spores retained the ability to germinate after four years storage at 4°C. However, in our study no sporocarps were present in the stored inoculum but only free spores and mycelium. Further experiments are necessary in order to determine why storage at 4°C of propagules other than sporocarps affects the survival of *G. mosseae*.

Storage in water, either at room temperature or at 4°C, did not affect survival of the six fungi tested. To our knowledge, this is the first report of long-term storage of AM fungal propagules in water. There are reports of AM in

aquatic plants and of AM fungal spores in associated sediments, suggesting that these fungi should survive periods of high water conditions. For example, Khan (1993) isolated spores and sporocarps of *G. fasciculatum*, *G. mosseae*, *Sclerocystis rubiformis*, *Gigaspora margarita* and an unidentified *Scutellospora* sp. from aquatic sediments and soils.

The protocol developed here has proved successful for the long-term storage of 20 different isolates of glomalean fungi, under different environmental conditions including at low temperatures without cryoprotectant. In a previous attempt to store glomalean fungi *ex-planta* at low temperature, Douds and Schenck (1990) concluded it was necessary to prepare inoculum for cryoprotection and cryopreservation by slowly drying pot cultures and freezing the fungal propagules in situ (in the soil or substrate in which they were produced). The protocol we describe differs in that wet-sieved fractions of fresh pot cultures containing spores and external hyphae with some soil debris were first exposed to room temperature in an open storage container before freezing. It appears that preliminary slow drying somehow conditions the different fungi so that they are able to resist low temperature and freezing. It is known that injury to cells can occur during freezing and thawing. The formation of intracellular ice crystals and the effects of the concentration of solutes during the process are the most important factors responsible for freezing injury. The fact that enough propagules survive for mycorrhizal establishment after low temperature or freezing, without preliminary treatment, underlines the need for more research to elucidate the mechanisms by which AM fungi are able to survive a wide range of hostile conditions.

Glomalean fungi are obligate biotrophs but they have a wide host range (Allen, 1996). In nature, the propagules must remain viable from one period of root growth to the next. According to Walker (1992), some glomalean fungi such as *A. scrobiculata* and *G. mosseae* have a very wide geographical distribution, whilst species of *Gigaspora* and *Scutellospora* may be more frequent in tropical than in temperate conditions. However, the present observations showed similarities in storage survival between tropical (*G. clarum* BEG 142, LPA 64) and temperate isolates, suggesting that their distribution is not simply due to a different ability to survive extreme temperatures and that intact they can resist difficult conditions. The successful storage in water, at room temperature and at 4°C, of both tropical and temperate glomalean fungi could have an ecological significance for their persistence in flooded habitats, and their ability to survive extreme temperatures could contribute more to their world-wide distribution.

In conclusion, our results suggest that the extraction of glomalean fungi by soil wet sieving and their subsequent exposure to air drying conditions are determining factors for their successful long term storage. The protocol described in this paper provides an original and simple procedure for storing different

species of glomalean fungi over long periods under different conditions. The results also give further insight into the biology of this group of biotrophic organisms and into their ability to survive in hostile environmental conditions.

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