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ORIGINAL PAPER

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Breaking dormancy in spores of the arbuscular mycorrhizal fungus *Glomus intraradices*: a critical cold-storage period

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Abstract To elucidate the effect of cold storage on spore dormancy in the arbuscular mycorrhizal (AM) fungus *Glomus intraradices*, spores were cold stratified at 4°C, for either 0, 3, 7, 14, 90 or 120 days, prior to germination tests at 25°C. The results showed that cold stratification longer than 14 days significantly increased spore germination. Moreover, the longer cold storage periods clearly reduced spore mortality from 90% to 50% and considerably altered the hyphal growth pattern. Long polarized hyphae were only observed after cold stratification periods longer than 14 days, involving consequences for root infectivity. The results clearly show that environmental factors, e.g., coldness, can affect the physiology of AM fungal spores.

Keywords Germination · Dormancy · Growth pattern · Mycorrhiza · Glomales

Introduction

Spore dormancy is a common phenomenon in the fungal kingdom and the term "dormancy" has been used to describe a large range of physiologically inactive stages (Van Etten et al. 1983). It is therefore necessary to first define precisely the definition of dormancy used in this study. A dormant spore will be considered as "one that fails to germinate although it is exposed to physical and chemical conditions that support germination and hyphal growth of apparently identical, but non-dormant, spores of the same species" (Tommerup 1983). Therefore, dormancy is apparently due to an internal physiological state, which is broken by a critical activation step, resulting in spore germination.

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It has been observed that germinability is dependant on the period of the year at which arbuscular mycorrhizal (AM) spores are formed or collected (Mosse 1959; Gemma and Koske 1988). Gemma and Koske (1988) showed that although the majority of spores of *Gigaspora gigantea* are formed in late summer and autumn, the period of germination is between December and July. In temperate biomes, spore dormancy of AM fungi is of prime importance for survival of the fungi in soil by synchronizing spore germination with periods of rapid root growth and favorable conditions for root colonization (Tommerup 1985). Under controlled conditions, Tommerup (1983) observed that storage for several weeks was necessary to induce spore germination in *Glomus caledonium*, *Glomus monosporum*, *Gigaspora calospora* and *Acaulospora laevis*. Optimal temperatures of spore storage were different for each species. Other studies showed that incubation at low temperature (ca. 5°C) for several weeks often induced spore germination (Hepper and Smith 1976; Gemma and Koske 1988). From these observations, it seems likely that spores are innately dormant when first formed, and may require exposure to cold before being able to germinate.

It is a common laboratory practice to store freshly isolated AM fungal spores at 4°C prior to use (Waltrud et al. 1978; Graham 1982; Elias and Safir 1987; Chabot et al. 1992a). This storage may inadvertently serve to break spore dormancy. In many studies assessing the influence of chemical and physical factors on AM fungus spore germination (see review articles: Siqueira et al. 1985; Vierheilig et al. 1998), the effect of spore dormancy has often been ignored. It therefore remains unclear whether these factors can break spore dormancy, or whether the results have been influenced by spore dormancy. To obtain comparable data in AM spore germination experiments, it is therefore essential to clarify the fundamental bases of dormancy.

Therefore, in the present work, the effects of cold storage on germination and hyphal growth of spores of *Glomus intraradices* were tested. Spore germination and hyphal growth patterns were observed in situ.

Materials and methods

Biological material

Spores of the AM fungus *Glomus intraradices* Schenck & Smith (DAOM 197198; Department of Agriculture, Ottawa, Canada) were obtained from monoxenic cultures with Ri T-DNA-transformed carrot (*Daucus carota* L.) roots (Chabot et al. 1992a; St-Arnaud et al. 1996) maintained on M medium in Petri dishes (Bécard and Fortin 1988) [0.6% (w/v) gellan gum from ICN Biochemical, Aurora, Ohio] at 25°C in an incubator.

Spore production and stratification

Cultures 2–3 months old, containing spores, were used for the experiments. To measure the effects of different cold periods on germination, Petri dishes were stored at 4°C for either 0, 3, 7, 14, 90 or 120 days. Following cold stratification of intact monoxenic cultures, single viable spores (see assessment below) were transferred individually with a blade from cultures to germination dishes.

Assessment of spore viability or mortality

After cold storage and at the end of the experiment, spore viability was assessed under the microscope. Spores were considered viable when they displayed a clear, whitish to creamish color, and a dense, thinly granulated content. Spores were considered dead when either the content was partially or totally transparent, or when membranes and wall remnants had turned dark brown.

Experimental design

The experiment was carried out during summer, and laid out in a randomized complete block design with three blocks and six treatments. For each treatment in each block, 20 spores were divided between two Petri dishes (standard, 90 mm diameter) and arranged in two rows of five spores, on M medium (Bécard and Fortin 1988). The dishes were sealed with parafilm and incubated in the dark at 25°C. Spore germination was quantified after 3, 7, 9, 11, 14, 16, 18, 21, 23, 25, 28, and 60 days. Germination was considered to have occurred if one or more germ tubes were clearly visible. After 60 days, images of the hyphal growth patterns were taken and spore mortality was determined.

Imaging tools

Germination growth patterns were observed in situ without staining of hyphae, using a Leitz diavert microscope (inverted microscope). Images were recorded using a Hitachi VK-C150 camera. The final photo plate (Fig. 3A, B, D) was assembled using Adobe Photoshop 3.0.

Statistical analysis

Statistical analysis was done using the GLM procedures in SAS (SAS Institute 1996). ANOVA was used to show differences in spore germination for each observation time. Multiple comparisons were performed using the Waller-Duncan test.

Results

Influence of cold storage on spore germination

Time course of *G. intraradices* spores germination was recorded after the different cold stratification periods (Fig. 1). No significant differences in germination occurred between the various cold treatments at the beginning of the experiment (day 3). More than 30% of the spores had germinated by day 3 in all treatments. However, from day 7 until the end of the experiment, the control (0 days of cold storage) showed significantly lower germination percentage than the longest cold treatment (120 days). This difference between treatments increased with time. From day 16, significantly more spores germinated in the 90-day and 120-day cold treatments, compared to the control. From day 25, there was a significant difference between the control and the 14-, 90- and 120-day stratification treatments. On day 60, two statistically distinct germinability groups were observed: (1) the 0-day and 3-day cold treatments, with a germination of approximately 50%, and (2) the 14-day, 90-day, and 120-day cold treatments, with a germination of approximately 90%. The germination percentage was intermediate (70%) for the 7-day cold treatment.

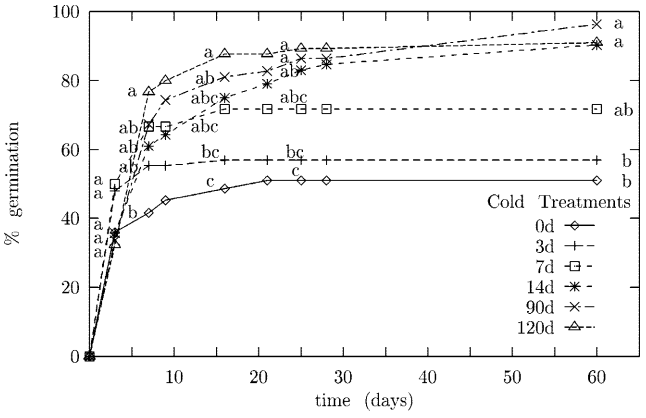

Fig. 1 Time-course of germination of *Glomus intraradices* spores which had been cold stratified for 0–120 days (*d*). Sample size is of 10 spores×2 replicates×3 blocks. ANOVA was used to show differences in spore germination for each observation time. *Different letters* indicate significant differences for a given time (Waller-Duncan multiple comparisons test). For clarity, *letters* are only included for time points where changes occurred, on days 3, 7, 16 and 25

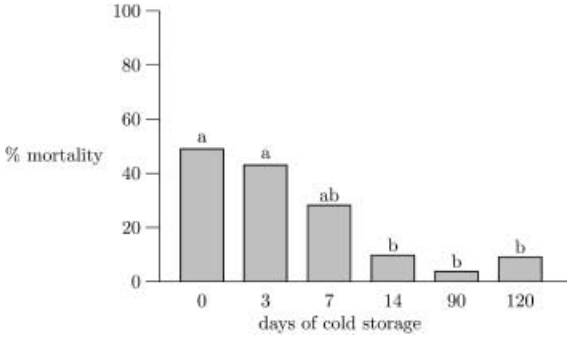

Fig. 2 Influence of the duration of cold stratification on spore mortality on day 60. Sample size is of 10 spores×2 replicates×3 blocks. ANOVA was performed on mortality percentages. *Different letters* indicate significant differences between storage times (Waller-Duncan multiple comparisons test)

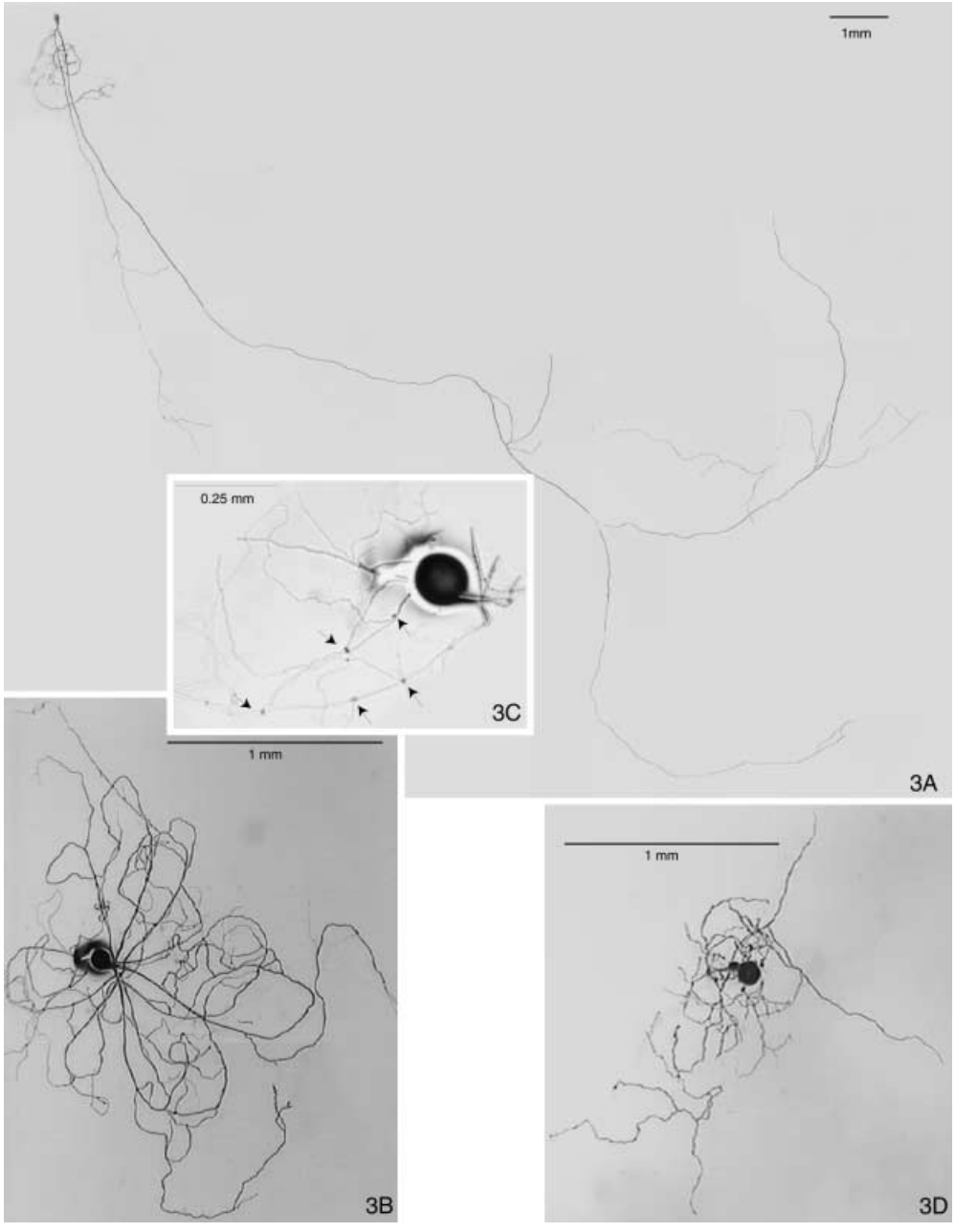

Fig. 3A–D Characteristic germination patterns of spores of *Glomus intraradices*. **A** G-type pattern, characterized by a long polarized hypha extending over several centimeters. **B D** g-type germination pattern, with curling hyphae limited to a few millimeters around the spore. **C** Detail of **B** showing numerous anastomosis points (*arrows*); **C** was imaged using a Leitz Laborlux 12 microscope, equipped with a Wild photoautomat MPS 55 (Kodak Ektachrome 160 Tungsten slide film)

Influence of cold storage on spore mortality

Spores which had not germinated by the end of the experiment (60 days) and which showed the described characteristics (see Materials and methods) were considered dead. Spore mortality was considerably decreased (<10%) following cold stratification for 14 days, 90 days or 120 days, compared to the control (0 days) and 3-day cold treatments, where spore mortality reached approximately 50% (Fig. 2).

Hyphal growth pattern

We observed two distinct types of hyphal growth: (1) straight hyphae with few branches, and (2) recurved, undulate and heavily branched hyphae. At the end of the experiment, in general, growth patterns could be classified into one of these two growth-type groups. In the first group, the growth pattern was characterized by a clearly distinguishable main hypha, which was several centimeters long (Fig. 3A). The longest hypha in this group reached 80 mm. In the second group, the emerging hyphae continuously curled on itself and branched (Fig. 3B, D), with growth restricted to a few millimeters around the spore, and without any visible dominant hypha. This growth pattern gave rise to numerous anastomoses (Fig. 3C).

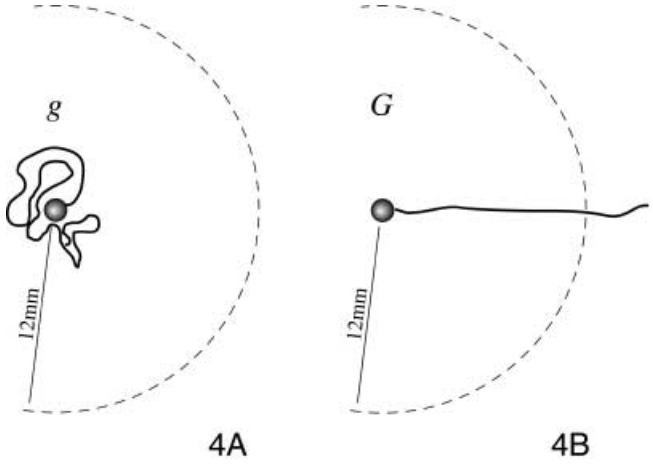

Fig. 4A, B Schematic illustrations of the two germination growth patterns. **A** g restricted-type germination, with the longest hypha within a 12-mm radius around the spore, and **B** G extensive-type with the longest hypha growing beyond a radius of 12 mm from the spore

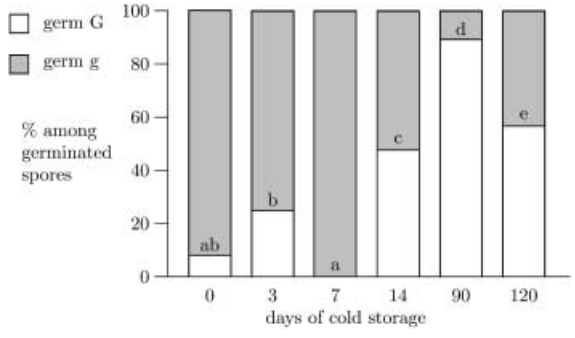

Fig. 5 Influence of cold storage duration on the percentage of germination types G and g. Only germinated spores on day 60 have been considered here, which is why the total of proportions of G+g, for each cold treatment, is always equal to 100%. Sample size is of 10 spores×2 replicates×3 blocks. ANOVA was performed on G percentages. *Different letters* indicate significant differences between storage times (Waller-Duncan multiple comparisons test; we have verified that the null value obtained for the young spores that stayed at 4°C for 7 days did not modify the grouping)

Measurements of growth patterns

We defined a measurable criterion to classify all germination patterns, including those which exhibited mixed hyphal growth patterns. The longest hyphal elongation of each germinated spore was measured at the end of the experiment (day 60). Two categories were defined (Fig. 4): g (for restrictive germination), with the longest hypha within a 12-mm radius around the spore, and G (for extensive germination), with the longest hypha beyond a radius of 12 mm around the spore.

Cold storage influence on G and g growth patterns

Influence of cold storage duration on the percentages of germination types G and g were expressed (Fig. 5). When spores were subjected to 4°C for 0–7 days, the g growth pattern could be observed most frequently. By contrast, >50% of germinated spores showed the G hyphal growth pattern after the 14-day and 120-day cold treatments. The highest proportion of G-type germination (89%) occurred after 90 days at 4°C.

Discussion

Dormancy is due to an internal physiological state, which is broken by an activation step (Van Etten et al. 1983; Tommerup 1983). In the light of our in vitro experiments, one such activation step for *Glomus intraradices* spores is cold storage at 4°C for at least 2 weeks.

We had anticipated from common observations that the absence of cold storage would delay the germination time: this was not the case, as germination usually occurred immediately, irrespective of the cold stratification period. However, when spores were submitted to cold stratification for <2 weeks, subsequent exposure to favorable conditions for germination resulted in increased spore mortality. Spores which did not germinate during the germination test were no longer dormant at the end of the test, but dead.

The role of cold, "winter-like" conditions in breaking spore dormancy needs to be further investigated as results are equivocal on this point (Douds and Schenck 1991; Louis and Lim 1988). Spore dormancy in *G. intraradices* and likewise in *Gigaspora margarita* and *Acaulospora longula* has been broken by storage at 23°C at varying soil matric potentials (Douds and Schenck 1991). Louis and Lim (1988) showed that germinability of a tropical isolate of *Glomus clarum* was enhanced after 3–6 months of dry storage at 25–30°C. However, in their study, the highest germination was obtained when a 6-month dry storage period at 30°C was followed by cold stratification at 4°C for 2–4 weeks. Some other reports suggest that certain species do not show inherent dormancy. Indeed, Douds and Schenck (1991) concluded that *Glomus mosseae* spores showed no dormancy, as identical germination rates were obtained with or without a storage period. However, Hepper and Smith (1976) increased germinability of the same species by stratification at 6°C for several weeks. Interestingly, Safir et al. (1990) obtained increased and synchronized spore germination of *G. mosseae* and *Glomus fasciculatum* after 28 days at –10°C, whereas spore storage at 4°C did not improve germination rates over storage at room temperature.

From these studies and the results obtained by Douds and Schenk (1991) with *Gigaspora margarita*, *Glomus mosseae*, *Glomus intraradices* and *Acaulospora longula*, it can be concluded that dormancy varies considerably from one species to another. In the case of *G. mosseae*, contradictory results may be linked to its geographical distribution. This species occurs in a wide range of biomes and therefore it is possible that different isolates from different geographical regions exhibit different

strategies adapted to their particular environment. It is likely that spore dormancy, even under laboratory conditions, is controlled by a basic internal biological clock and that cold stratification is not absolutely necessary to break dormancy. However, it may trigger or accelerate the physiological modifications involved. Sward (1981) refers to an "endogenous inhibitor of germination contained in the spore wall" that alteration of the spore wall by a surface-sterilization procedure may release, "allowing subsequent activation of germination processes". According to Louis and Lim (1988), a germination promoter or inhibitor may be respectively activated or deactivated during cold stratification, which would then trigger a germination response induced during dry storage. This signal must involve molecular and genetic events.

Mosse (1988) gave the following description of germinating spores of *G. intraradices* in monoxenic culture conditions: "main germ tubes arising from spores radiated out in straight lines, showing the usual strong apical dominance and sparse branching". This corresponds to our G-type germination pattern. Our data show that this pattern was only consistently observed after a cold treatment of 14 days or more. By contrast, spores exposed to <14 days at 4°C showed principally g-type germination patterns. This is, to the best of our knowledge, the first description of this germination pattern in *G. intraradices*.

The growth of AM fungal germ-tubes is influenced by various exogenous factors, which affect overall length and/or branching. Powell (1976) first reported "fan-like" structures of predominantly septate hyphae, which were formed in the proximity of host roots. Further studies have found enhanced branching of germ-tubes (Graham 1982), elicited hyphae (Giovannetti et al. 1993), or hyphal elongation (Elias and Safir 1987; Bécard and Piché 1989; Bécard et al. 1992; Chabot et al. 1992b; Morandi et al. 1992; Poulin et al. 1993), following the application of root exudates, flavonoids and/or CO_2 to germinated spores (Vierheilig et al. 1998). Nevertheless, how such hyphal branching should be interpreted remained unclear. More recently, several authors have described some specific branching aspects of germinating hyphae, responding to application of root exudates (Nagahashi and Douds 1999) or light (Nagahashi et al. 2000), and the existence of a "branching factor" has been proposed (Buee et al. 2000). However, hyphal branching can also be associated with environmental stress or other abnormal situations. Hepper (1979) found that applying various inhibitors to germinating spores of *Glomus cale-donius* resulted in different degrees of hyphal branching and growth.

In the fungal kingdom, "proliferation of hyphal branches, often forming characteristic knots and with abnormal morphology, typically occurs in the appressed/inhibited regions" (Rayner and Coates 1987). Hyphal growth in fungi is controlled by three factors: (1) hyphal polarity, (2) branching initiation, and (3) spatial distribution of hyphae (Moore 1984). In our experiment, profuse branching of the g-type germination pattern was often correlated with curling of the hyphae. Such "spiral growth", either clockwise or anticlockwise, is characteristic of many fungi grown on media with a high agar content, or at the margin of mature colonies (Trinci 1984). Therefore, "curling" hyphae are known to occur under stressful situations. Furthermore, a recent study in our laboratory has observed that application of root exudates to germinating spores of *Glomus intraradices* led to the exclusive development of long runner hyphae, whereas absence of root exudates led to spiral growth in the proximity of the spore (Pinior et al. 1999). Thus, at least two factors seem to have an influence on growth patterns following germination in *G. intraradices*: cold storage of the spores and application of root exudates. In the present study, the g germination type was associated with numerous anastomoses, referred to as "self fusions" (Rayner 1991), often observed in AM fungi (Mosse 1988; Tommerup 1988).

Ecologically, germinating hyphae forming a g-type pattern have a reduced probability of meeting host roots, compared to those from a G germination type. Several studies have shown that successful fungal germination is not necessarily followed by successful root colonization, especially if spores have been allowed to germinate by favorable moisture and temperature conditions irrespective of their normal period of dormancy (Tommerup 1984; Nemec 1987; Douds and Schenck 1991). Such interruptions of spore dormancy did not always prevent germination from occurring, but may increase the occurrence of spiral g growth patterns and thus prevent successful root colonizations.

Spore age is known to influence germinability (Godfrey 1957; Koske 1981; Siqueira et al. 1985). In our study, spores from cultures >6 months old showed no significantly different germination and hyphal growth patterns (data not shown) from spores in the present experiment, excluding a simple age effect on germinability (slight delay in initial germination).

Further studies are needed in order to better understand the physiological and genetic regulators that permit a spore to germinate after a completed dormant period, and also to investigate which are the exact factors responsible for spore mortality.

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