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# Influence of surface sterilization and cold treatment on germination of AM spores

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Vesicular arbuscular mycorrhizal(VAM) fungi are a unique group of ubiquitous soil microorganism known to form symbiotic association with roots of economically important crop plants. AM propagules such as isolated spores, sheared mycorrhizal roots are virtually able to initiate AM symbiosis and establish the pre-symbiotic phase with the transformed root. The spores were sterilized with 96 % ethyl alcohol and treated for 30, 60 and 90 seconds. Surface sterilization of AM spore *viz*., *Glomus mosseae*, *G. intrardices* and *G. caledonium* with 96 per cent ethyl alcohol for 60 sec exhibited higher germination per cent without affecting the viability of spores. Further cold treatment of spores at $4^0$C for a period of 30 days improved the spore germination to 90 % and 56.5 % in *Glomus intraradices* and *Glomus caledonium*, respectively, whereas *Glomus mosseae*, recorded maximum with 20 days of cold treatment. Though germination was observed higher with 30 days and cold treatment, stratification at $4^0$C for a perod of 16-20 days resulted better germination of spores.

Key words : AM fungi, Sterilization, Spore germination, *Glomus*

## INTRODUCTION

The first culture of hairy roots colonized by the AM fungus was achieved by Mugnier and Mosse (1987). AM propagules such as isolated spores, vesicles and sheared mycorrhizal roots are used to initiate AM symbiosis and establish the pre-symbiotic phase with the transformed root. The first *in vitro* sporulation of AM fungus was obtained by Becard and Fortin (1988) using carrot hairy roots colonized by *Glomus intraradices*

AM propagules such as isolated spores, vesicles and sheared mycorrhizal roots are virtually able to initiate AM symbiosis and establish the pre-symbiotic phase with the transformed root. Chlamydospores of *Glomus* sp (Mosse and Hepper, 1975; Mugnier and Mosse, 1987) and non-sporocarpic azygospore of *Gigaspora margarita* (Becard and Fortin, 1988; Diop *et al*., 1992) are also preferred as starter inoculum. Recently, sporocarps of *Glomus mosseae* have also been used in an attempt to establish *in vitro* cultures (Budi *et al*., 1999).

Spores are usually collected from the field, or from pot cultures, by wet sieving (Gerdemann and Nicolson, 1963). Mycorrhizal roots used to initiate monoxenic cultures come from plants grown in pot cultures, with field collected soil or AM propagules. Leek (*Allium porrum* L.) plants are widely used because of their susceptibility to colonization.

Proper selection and efficiency of sterilization process are keys for the success of axenic or monoxenic AM fungal cultures. Variations in the sterilization time, the composition and concentration of the sterilizing agent may have significant impact on the process of sterilization.

Viable, surface sterilized spores are a prerequisite not only for *in vitro* experiments but also for producing starter inoculum, free of contaminants, for commercial purposes. Germination of AM spores may also get affected by the use of surface sterilants. Hence, inorder to study the effect of sterilization and cold treatment on germination of AM fungal spores following studies were conducted.

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. Dormancy can be broken by critical activation step, which results in spore germination (Tommerup, 1985). Studies showed that incubation at low temperature ($5^0$C) for several weeks often induced spore germination (Hepper and Smith, 1976). Hence, to study the influence of surface sterilants as well as cold treatment on germination of AM spores lab experiments were conducted under controlled condition.

## MATERIALS AND METHODS

### *AM cultures used :*

The arbuscular mycorrhizal cultures *viz*., *Glomus mosseae*, *Glomus intraradices* and *Glomus caledonium* were used in this study. The cultures were obtained from the culture collection centre at TERI, New Delhi. The cultures were maintained in 1:1 sterile sand: soil mixture either with maize or onion as a host.

***Isolation of AM spores from pot culture inoculum :***

AM spores were isolated using the method of wet sieving and decantation as described by Gerdemann and Nicolson (1963) from the pot inoculum.

***Effect of sterilization on germination of AM spores :***

Viable, surface sterilized spores are pre-requisite for the *in vitro* experiments. Efficiency of sterilization process is the key factor for the success of axenic or monoxenic AM fungal cultures. The spores were sterilized by the method described by Budi *et al*. (1999). Ethyl alcohol (96 %) was used here with varying exposure timings.

***Treatment details :***

Treatments subjected to the spores of AM fungi *viz.*, *G. mossseae, G intraradices* and *G. caledonium*.

$T_1$ – 96 % Ethyl alcohol for 30sec

$T_2$ –96 % Ethyl alcohol for 60 sec

$T_3$ – 96 % Ethyl alcohol for 90 sec

The spore of three species of AMF were surface sterilized as above and washed with sterile water with the use of bacteriological filters. Spores present in the membrane filter were washed several times with sterilized distilled water to remove the traces of sterilizing agent. There after aseptically the spores were transferred from the membrane filter to the sterile Petriplates containing water agar and incubated for germination of spores at 24 ± $1^0$C. Development of germ tube and mycelium from the spores indicate the germination of AM spores. The plates were observed after 8 – 10 days of incubation under the stereo microscope for spore germination.

The per cent germination was determined as below:

$$\text{AM spore germination (\%)} = \frac{\text{Number of spores germinated}}{\text{Total number of spores kept for germination}} \times 100$$

***Effect of cold treatment on germination of AM spores:***

This study was conducted to assess the influence of cold treatment on germination of AM spores. Surface sterilized spores of *G. mossseae, G intraradices* and *G. caledonium* were used. Each spore types were collected separately in a vial with sterile water. Seven treatments were followed with three replication. The collected spores were kept at $4^0$C for period ranging from 4 - 30 days for cold stratification as below.

***Treatments :***

- Cold storage of spores for a period of 0, 4, 8, 12, 16, 20 and 30 days
- Incubation temperature: $4^0$C

After cold treatment the spores were brought to room temperature and placed in water agar plates and incubated at 24±$1^0$C for germination. For each treatment 10 spores with three replications were used.

## RESULTS AND DISCUSSION

The results obtained from the present investigation are presented below :

***Effect of sterilization on in vitro germination of AM spores on water agar medium (after 8-10 days of incubation):***

Spores before being used for *in vitro* establishment in hairy roots, it needs to be surface sterilized. Based on earlier results, ethanol 96 % was used as sterilizing agent. In order to find out the optimum exposure time for the germination of AM spores under *in vitro* conditions, spores were exposed to ethanol 96 % for three different time periods (30 sec, 60 sec and 90 sec) and incubated at 24±$1^0$C for 8-10 days. The germination of AM spores in different treatments were observed.

The germination per cent was significantly higher in all the three AM cultures *viz*., *Glomus mosseae* (55.2%), *Glomus intraradices* (65.7%) and *Glomus caledonium* (50.2%) when they were exposed to 96 per cent ethyl alcohol for a period of 60 seconds. Significant reduction in germination was observed when exposed to 30 sec in *Glomus mosseae* (40.5%), *Glomus intraradices* (50.3 %) and *Glomus caledonium* (35.7%) compared to 60 sec exposure. When exposed to 90 sec also germination of AM spores *viz*., *Glomus mosseae* (35.5%), *Glomus intraradices* (45.2 %) and *Glomus caledinuim* (30.4%) was significantly reduced (Table 1). Significant interaction was observed between AM cultures as well as exposure timings on germination of AM spores.

By observing the overall germination percentage, it was found that ethanol 96 % treatment for 60 sec produced significantly higher germination (57.03 %). While comparing the cultures used, *Glomus intraradices* recorded maximum germination (53.73 %) than other cultures used. All the cultures respond similarly with the varying exposure to ethyl alcohol (96 %).

Further, no contaminants were noticed with 60 sec and 90 sec exposure to ethanol apart from recording higher germination. However, with 30 sec exposure, germination along with other microbial contaminants were noticed. Due to higher germination as well as no contaminants, 96 per cent ethyl alcohol treatment for 60 sec was considered to be optimum for surface sterilization of AM spores

**Table 1 : Effect of sterilization on *in vitro* germination of AM spores on water agar medium (after 8-10 days of incubation)**

| AM cultures | Germination of AM spores (%) Exposure to 96 % ethanol | | | |
|---|---|---|---|---|
| | 30 sec | 60 sec | 90sec | Mean |
| *Glomus mosseae* | $40.50^{d}$ | $55.20^{b}$ | $35.50^{e}$ | $43.73^{b}$ |
| *Glomus intraradices* | $50.30^{c}$ | $65.70^{a}$ | $45.20^{d}$ | $53.73^{a}$ |
| *Glomus caledonium* | $35.70^{e}$ | $50.20^{b}$ | $30.40^{f}$ | $38.70^{c}$ |
| Mean | $42.16^{b}$ | $57.03^{a}$ | $37.03^{c}$ | |
| | S.E. ± | | C.D.(P=0.05) | |
| Culture (C) | 2.13 | | 4.48 | |
| Time (T) | 2.13 | | 4.48 | |
| C*T | 3.69 | | 7.77 | |

subjected to *in vitro* condition.

***Effect of cold treatment on spore germination of AM spores :***

Dormancy of AM spores is a major problem in using the spore for *in vitro* studies. Hence to break the dormancy, different periods of cold treatments have been employed with three different species of AM fungus and the recorded results are presented in Table 2.

The results showed the influence of cold treatment on germination at varying levels. No significant impact on spore germination was observed upto 8 days of cold treatment in all the three AM species tested. With an increase in cold treatment period, beyond 8 days increased the spore germination of all the three AM species. Higher percentage of germination was observed under 30 days of cold treatment as 90 % and 56.5 % in *Glomus intraradices* and *Glomus caledonium*, respectively, whereas *Glomus mosseae*, recorded maximum with 20 days of cold treatment(75 %). Though germination percentage was recorded higher with 30 days of cold treatment, no significant difference was observed between 20 and 30 days of cold treatment. Among the spores tested, *Glomus intraradices* (59.04 %) recorded higher germination and the least was recorded with *Glomus caledonium* (43.08) irrespective of the spore types.

With reference to overall results of cold treatment of spores on germination, significantly higher germination percentage was shown when kept for 30 days. There was no significant difference till 8 days of cold treatment. But germination was significantly increased further. As the number of days of cold treatment increases, significantly higher germination percentage was found. Significant variation between cultures were observed after 16 days of cold treatment. A period of 16-20 days cold treatment at $4^{0}$C resulted better germination of AM spores.

***Sterilization of AM spores :***

Glomales species form hypogeal sporocarps, in which spores are surrounded by a weft of mycelium. This may have saprophytic microorganisms that can influence both germination and AM formation and thus, require sterilization (Fracchia and Mujica, 1998).

Proper selection and efficiency of sterilization process are keys of the success of axenic AM fungal cultures. The spores may carry bacteria between wall layers, making disinfection difficult or even impossible (Walley and Germida, 1996). A solution containing the strong oxidizing agent, chloramine T and a surfactant (eg. Tween 20) is widely used to sterilize AM fungal spores. The first successful use of 96% ethanol, combined with other agents, was reported by Strulu and Romand (1986) to obtain sterile AM roots.

In the present study, surface sterilization with 96 per cent ethyl alcohol for 60 sec effectively destroyed the unwanted microorganisms without affecting the viability of spores and also increased the germination percentage from 50.2 to 65.70 % in different species of AM fungi. The use of ethanol 96 % for 90 sec recorded lesser germination percentage as it effectively destroyed

**Table 2 : Effect of cold treatment on germination of AM spores (after 8-10 days of incubation)**

| AM cultures | Germination of AM spores (%) Period of cold treatment of AM spores | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 days | 4 days | 8 days | 12 days | 16 days | 20 days | 30 days | Mean |
| *Glomus mosesae* | $31.20^{k}$ | $32.00^{jk}$ | $33.10^{jk}$ | $41.50^{hi}$ | $60.00^{e}$ | $75.00^{d}$ | $70.50^{d}$ | $49.04^{b}$ |
| *Glomus intraradices* | $30.00^{jk}$ | $33.50^{ij}$ | $38.70^{ij}$ | $55.00^{efg}$ | $82.00^{bc}$ | $84.10^{ab}$ | $90.00^{a}$ | $59.04^{a}$ |
| *Glomus caledonium* | $30.10^{k}$ | $31.20^{k}$ | $33.50^{k}$ | $44.00^{hi}$ | $48.20^{gh}$ | $49.10^{fgh}$ | $56.50^{ef}$ | $43.08^{c}$ |
| Mean | $30.4^{e}$ | $32.20^{d}$ | $35.10^{d}$ | $46.83^{c}$ | $63.40^{b}$ | $69.40^{a}$ | $72.33^{a}$ | |
| | | | S.E. (d) | | C.D. (P=0.05)_) | | | |
| Strain (S) | | | 1.59 | | 3.22 | | | |
| Duration (D) | | | 2.44 | | 4.92 | | | |
| S*D | | | 4.22 | | 8.53 | | | |

unwanted microorganisms but killed all sporocarps also and hence the germination percentage was reduced. When shorter period of ethanol treatment (30 sec) was given, contaminants were not completely eliminated, probably due to the presence of bacteria embedded in the hyphal surface and in the spore walls of *G. mosseae* (Filippi *et al*., 1998). However, the effectiveness of this treatment will depend greatly on the type of spores. According to Sward (1981) presence of endogenous inhibitor of germination contained in the spore wall has been released and activated by the alteration of spore wall. The surface sterilization procedure may alter the spore walls leads to increase in germination of AM spores.

***Effect of cold treatment on spore germination :***

Spore dormancy is common phenomenon in the fungal kingdom and the term dormancy has been used to describe a large range of physiologically inactive stages. A dormant spore will be considered as one that fails to germinate although if it is exposed to physical and chemical conditions that support germination and hyphal growth (Tommerup, 1985). Therefore, dormancy is due to an internal physiological state which is broken by critical activation step resulting in spore germination.

Cold storage may have the potential to break the dormancy of spores. Therefore, in the present work, the effect of cold storage on germination and hyphal growth of AM spores were tested. No significant difference in spore germination was observed upto 8 days of cold treatment. Further increase in cold storage resulted increase in germination of all the AM species tested. *G. intraradices* and *G. caledonium* recorded higher level of germination at 30 days, whereas *G. mosseae* recorded higher level with 20 days of cold treatment at $4^0$C. But beyond 20 days of cold treatment, no significant difference in germination of AM spores was noticed.

Spore dormancy in *G. intraradices*, and likewise in *Gigaspora margarita* and *Acaulospora longula* has been broken by storage at $23^0$C at varying soil matric potentials (Douds and Schenck, 1991). Hepper and Smith (1976) increased germinability of *G. mosseae* species by stratification at $6^0$C for several weeks. 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.

Cold treatment of *G. intraradices* spores at $4^0$C for different time period showed the enhanced germination at 14 days of cold treatment and this clearly shows that environmental factors e.g. coldness can affect the physiology of AM fungal spores which helps for the increased germinability.

The present results suggest the use of 96 per cent ethanol for a period of 60 seconds resulted in better sterilization as well as did not cause any inhibition on germinability of AM spores. Similarly stratification of spores at $4^0$C for a period of 14 – 20 days resulted maximum germination of AM spores.

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