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Surface-sterilization of *Glomus mosseae* sporocarps for studying endomycorrhization in vitro

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Abstract The effects of sterilization time, sterilizing agents (ethanol, Chloramine T, calcium hypochlorite) and antibiotics (streptomycin and gentamycin) on *Glomus mosseae* (BEG 12) sporocarp germination and contamination were evaluated. Incubation for 10 s in 96 % ethanol, followed by 10 min in a solution of 2% Chloramine T, 0.02% streptomycin, 0.01% gentamycin and Tween 20, and then 6 min in 6% calcium hypochlorite greatly reduced fungal and bacterial contamination from sporocarps and caused little change in germination rate in water agar medium.

Key words *Glomus mosseae* · Sporocarps · Surface sterilization · Germination

Introduction

Arbuscular mycorrhizal (AM) fungi, which are ubiquitous in soils throughout the world (Smith and Read 1997) and well known for their impact on the growth and health of economically important plants (Lovato et al. 1995), cannot be cultured alone axenically. Thus the production of high-quality inoculum under controlled conditions is a problem and a subject of interest for certain commercial firms. Viable, surface-sterilized spores are a prerequisite not only for gnotobiotic or in vitro experiments but also for producing starter inoculum, free of contaminants, for commercial purposes.

Chloramine T (2%), with 200 μg m/l streptomycin and a trace of wetting agent, is most commonly used for sterilizing spores (Mosse 1959). Variations in the sterilization time, the composition of the sterilizing solution, and inclusion of ultrasonic and ultraviolet radiation have also been proposed (Boudarga et al. 1990; Fracchia et al. 1998; Tommerup and Kidby 1980; Walley and Germida 1996).

Glomales species like *Glomus mosseae* form hypogeal sporocarps during their life cycle, in which spores are surrounded by a weft of mycelium. Sporocarps could be used as propagules to initiate AM but they harbor many saprophytic microorganisms that can influence both spore germination and AM formation and thus require sterilization (Fracchia et al. 1998). We present here the first report of a procedure for obtaining surface-sterilized sporocarps of *G. mosseae*.

Materials and methods

Production of *G. mosseae* sporocarps

Sporocarps of *G. mosseae* (Nicolson & Gerdemann) (Gerdemann & Trappe) (BEG 12) were produced using sorghum (*Sorghum bicolor* L. var. Esquirol) as a host plant in pots containing 400 ml of autoclaved calcined clay (Oil Dry type III, Laporte Absorbents Europe). Plants were grown in a constant-environment room (22/24 °C, 16-h photoperiod, 300 μmol photon/m^2/s, 70% relative humidity) for 90 days. Sporocarps were recovered from 3-month-old pot cultures by wet sieving and decanting (Gerdemann and Nicholson 1963). Homogeneous sporocarps were collected manually under a dissecting microscope using a fine spatula, washed in tap water and maintained in tap water at 5 °C until use.

Surface sterilization

Sporocarps (500–1000) were washed in sterile water by slow vortexing to remove loosely adhering particles, transferred to fresh sterile water, vortexed again and rinsed with sterile water until this remained clear. They were then transferred using a sterile Pasteur pipette to a sterile Millipore filtering apparatus (Millipore SA) with a 0.8-μm filter. After a rinse with 50 ml sterile water, the sporocarps where washed successively with three sterilizing solutions: (1) 96% ethanol, (2) a mixture of 2% Chloramine T (w/v), 0.02% streptomycin (w/v), 0.01% gentamycin (w/v) and two drops of Tween 20, (3) 6% calcium (Ca) hypochlorite (w/v), pumped through the Millipore column at a pressure of 5 hg/cm.

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Table 1 Sterilizing solutions and treatment times (I 96% ethanol, II 2% Chloramine-T, 0.02% streptomycin, 0.01% gentamycin and Tween 20, III 6% calcium hypochlorite)

Solution	Treatment times												
	Experiment 1					Experiment 2				Experiment 3			
	1	2	3	4	5	6	7	8	9	10	11	12	13
I	10 s	10 s	10 s	10 s	10 s	10 s	10 s	10 s	10 s	10 s	60 s	90 s	180
II	10 min	10 min	10 min	10 min	10 min	10 min	12 min	14 min	16 min	10 min	10 min	10 min	10 min
III	0 min	2 min	4 min	6 min	8 min	6 min	6 min	6 min	6 min	6 min	6 min	6 min	6 min

Three experiments with 13 different incubation times were performed (Table 1). The treatment in experiment 1 giving the highest number of uncontaminated, germinated sporocarps was used as a control in experiment 2. Similarily, the best results from experiment 2 were used in experiment 3, and so on. Thus, during the first experiment, incubation time was varied for solution 3, in experiment 2 for solution 2, and in experiment 3 for solution 1. After each treatment, sporocarps were rinsed with sterile water, and transferred to a sterile Whatman filter paper moistened with sterile water in a sterile Petri dish. All operations were performed under a sterile laminar-flow hood.

Test of viability and sterility

Twenty surface-sterilized sporocarps from each treatment were transferred with a sterile spatula to a Petri dish (100×15 mm) containing 0.6% water agar. The dishes were sealed with parafilm, inverted and incubated in the dark at 25 C for 2–21 days. Germination and contamination were evaluated under a dissecting microscope at ×16–40 magnification. There were 10 replicate dishes per treatment.

Three media were used to detect contaminants not developing on water agar: (1) 2% (w/v) Luria Broth (LB) (Sigma) in 0.6% (w/v) water agar, (2) 2% (w/v) malt extract (Difco) in 0.6% water agar and (3) 2% (w/v) Soyton (Difco) in 0.6% water agar. Germination and contamination were evaluated as above. Tests were replicated five times.

Statistical analysis

Data was analyzed using the Stat-ITCF program. Significant differences were tested by the Newman-Keuls test at $P<0.05$. Percentages were arcsin transformed prior to analysis.

Results

No loss of viability (sporocarp germination) was observed for *G. mosseae* sporocarps when treated with Ca hypochlorite for up to 6 min (Table 2). The percentage germination (Fig. 1) remained high even when exposed to Ca hypochlorite for 8 min, where contamination by bacteria and fungi was similar to the 6-min treatment. Sporocarps disinfected for 2 or 4 min in Ca hypochlorite, showed higher contamination than after 6 or 8 min, but percentage germination did not differ.

When sterilization times with ethanol and Ca hypochlorite were kept constant, a decrease in sporocarp germination was observed with increase in sterilization time from 10 to 16 min with Chloramine-T, streptomycin and gentamycin, but contamination was not significantly ($P=0.05$) affected (Table 2).

Table 2 Germination and contamination of *Glomus mosseae* sporocarps after 21 days incubation on water agar media. The sterilization treatments were 10 s 96% ethanol (solution I), 10 min 2% chloramine T, 0.02% streptomycin, 0.01% gentamycin and Tween 20 (solution II), and 6 min 6% Ca hypochlorite (solution III) with the variation in times shown in the table. Values are means (±SE) for 10 replicates. Values for each treatment followed by the same letter in each column do not differ significantly ($P=0.05$)

Sterilization time	% Germination	% Contamination
Solution III (min)		
0	92.0±7.15 a	30.0±8.16 a
2	92.5±5.40 a	20.0±3.00 a
4	90.5±4.38 a	16.0±8.50 a
6	90.0±4.70 a	5.0±4.08 b
8	84.0±6.10 a	5.5±7.97 b
Solution II (min)		
10	88.0±9.40 a	3.5±6.68 a
12	76.5±5.79 b	2.2±3.63 a
14	63.0±8.88 c	2.5±4.25 a
16	50.5±7.25 d	2.0±3.49
Solution I (s)		
10	90.0±9.42 a	1.5±3.37 a
60	85.5±8.83 a	2.0±8.83 a
90	67.5±10.0 b	1.0±2.10 a
180	3.2±4.60 c	1.0±2.00 a

Decrease in sporocarp germination was also observed when a constant sterilization time with Ca hypochlorite on Chloramine-T, streptomycin and gentamycin was preceded by increasing times of sterilization with ethanol (Table 2). Sporocarp treatment with ethanol for 3 min significantly ($P=0.05$) decreased germination.

The best treatment combination for sporocarp surface sterilization allowing good germination was 10 s 96% ethanol, 10 min Chloramine-T, streptomycin, gentamycin and Tween 20, and 6 min Ca hypochlorite. This combination was used to study sporocarp germination and contamination on nutrient-rich media.

Although contaminants developed from some sporocarps on the nutrient-rich media, about 90% were found to be sterile (Table 3). Sporocarps did not germinate on Soyton and LB agar. Water agar allowed the

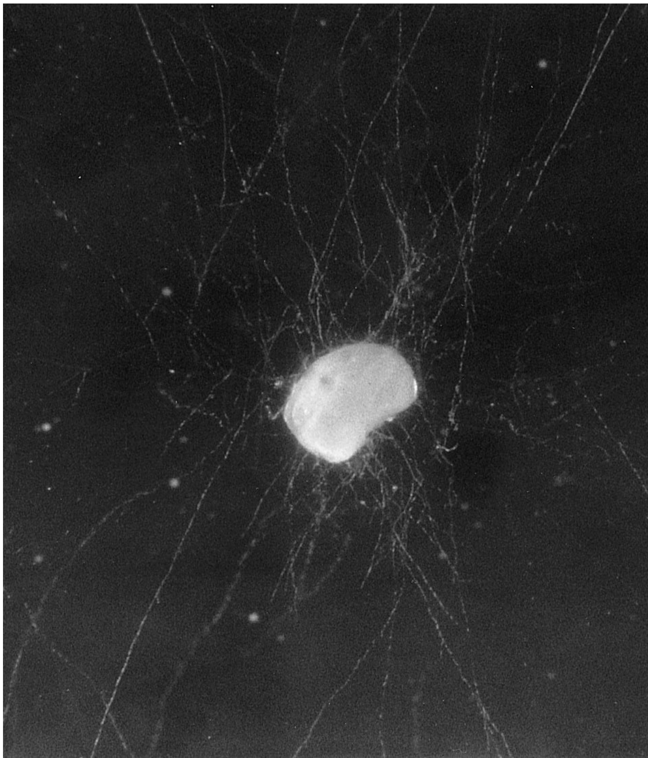

Fig. 1 Germinating hyphae of *Glomus mosseae* (BEG 12) arising from surface-sterilized sporocarps after incubation on water agar (0.6%) medium for 21 days at 25 °C in the dark

Table 3 Germination and contamination of *G. mosseae* sporocarps incubated on four media for 21 days following surface sterilization in 96 % ethanol for 10 s, 2% Chloramine T, 0.02% streptomycin, 0.01% gentamycin and Tween 20 for 10 min, and 6% Ca hypochlorite for 6 min. Values are means (±SE) for five replicates. Values followed by the same letter in each column do not differ significantly ($P=0.05$)

Medium	% Germination	% Contamination
Water agar	88.84 ± 5.49 a	3.66 ± 4.15 a
Malt extract agar	18.66 ± 2.95 a	13.34 ± 6.20 a
Soyton agar	0.00 ± 0.00 b	9.22 ± 7.11 a
Luria Broth agar	0.00 ± 0.00 c	12.00 ± 9.08 a

highest sporocarp germination, while malt extract agar was more suitable for detecting contaminated sporocarps. In general, sporocarp contamination by bacteria was detected after 4 days incubation on rich media and 7 days on water agar medium, whereas fungal contamination was detected after 2 days incubation on all media.

Increasing time of incubation did not eliminate contamination by bacteria and fungi completely, except that ethanol applied for 3 min completely eliminated fungal contamination.

Discussion

The surface-sterilization procedure using a combination of ethanol, Chloramine-T, streptomycin, gentamycin and Tween 20, and Ca hypochlorite produced satisfactory amounts (about 90%) of *G. mosseae* sporocarps with a high level of germination and no visible bacterial or fungal contamination after 3 weeks incubation. It would be interesting to test many commercial antibiotics and other disinfectants used for surface sterilization of AM spores (Colozzi-Filho et al. 1994; Tommerup and Kidby 1980) to improve these procedures further, althought sterilization time appears to be crucial for sporocarp germination.

The first successful use of 96% ethanol, combined with other agents, was reported by Strulu and Romand (1986) to obtain sterile AM roots. The same treatment with 96% ethanol (4 min) followed by 6 min in Ca hypochlorite or 10 min in Chloramine-T, streptomycin, gentamycin and Tween 20 effectively destroyed unwanted microorganisms but killed all sporocarps (data not shown). Contaminants were not completely eliminated using a shorter time in ethanol, 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, sporocarps remained alive and could be successfully treated with the other two sterilizing solutions, indicating a high sensitivity to ethanol as sterilizing agent.

Sporocarps of *G. mosseae* also harbor many saprophytic fungi (Fracchia et al. 1998), which were apparently destroyed by the chlorine in 2% Chloramine T and 6% Ca hypochlorite. However, the effectiveness of this treatment will depend greatly on the quality of the sporocarps.

Because it appears difficult to surface sterilize all sporocarps, to optimize yield of sporocarps it is advisable to test for residual contaminants on nutrient-rich media after surface disinfection. In this study, Malt extract agar medium allowed contaminant growth with a short incubation, and contaminated sporocarps could be identified and eliminated from the sample. Sporocarps are efficient propagules for initiating AM formation and the development of the described procedure for surface sterilization greatly assists production of high quality starter inoculum free of unwanted microorganisms for in vitro and molecular studies.

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