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Failure to decontaminate *Glomus clarum* NT4 spores is due to spore wall-associated bacteria

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Abstract Exposure of spores of *Glomus clarum* NT4 to solutions of chloramine-T (2.5–10% w/v) for 10–120 min failed to fully decontaminate all spores. Scanning electron microscopy did not show the presence of contaminants on treated spores, but transmission electron microscopy revealed bacterial cells embedded within the outer spore wall layer. Bacteria that remained protected within the spore walls were detected only when the spores were placed on appropriate media. Nutrient agar and tryptic soy agar supported relatively high levels of contaminant growth and were regarded as good media for assessing contamination, whereas the detection of contaminant growth on water agar required prolonged incubation. Contamination and germination of *G. clarum* NT4 spores following decontamination treatments were dependent on spore age. Generally, lower concentrations of chloramine-T and shorter incubation periods were required to reduce contamination of freshly harvested spores than of mature spores. Exposure to 10% chloramine-T for 120 min was required to reduce the levels of contamination of mature spores to $\leq 10\%$. Unfortunately, spore germination was compromised by rigorous decontamination treatments, thus the success of any decontamination procedure should be evaluated prior to its routine use. Moreover, if the interpretation of experimental results rests on the assumption of true surface sterility of VAMF spores, we suggest that the axenic condition of spores be confirmed prior to experimentation on a medium that encourages contaminant growth.

Key words VAMF · Mycorrhizae · Chloramine-T · Sterilization · Germination

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Introduction

Surface-sterilized VAMF spores are often a desirable or necessary requisite for gnotobiotic or in vitro experiments. Surface decontamination may be required both to avoid proliferation of contaminants and to ensure that contaminating organisms associated with VAMF spores do not bias experimental results.

A number of methods are routinely cited in the literature as providing surface-sterile VAMF spores. Mosse (1962) used a solution of 2% chloramine-T, 200 $\mu g\ ml^{-1}$ streptomycin, and a trace of detergent as a decontaminant. Many variations of this method have been reported, but procedures typically involve exposure of spores to a decontaminant solution, followed by rinsing in sterile water (Mertz et al. 1979; Tommerup and Kidby 1980; Mayo et al. 1986; Tilak et al. 1989; Boudarga et al. 1990; Safir et al. 1990; Paula and Siqueira 1990; Tsai and Phillips 1991).

Unfortunately, although procedures to surface decontaminate VAMF spores are routinely used, the level of decontamination achieved is seldom reported. Moreover, much of the limited information available is contradictory. For example, although Mosse (1962) achieved 90% spore decontamination, Mertz et al. (1979) reported that in their hands Mosse's method produced only 32–68% axenic spores. As a further complication, in vitro germination of surface-decontaminated VAMF spores has frequently been evaluated either on water agar (Azcon-Aguilar et al.1986; Safir et al. 1990; Vierheilig and Ocampo 1990) or minimal media with limited or no carbohydrate source (Hepper 1979; Tsai and Phillips 1991; Bécard et al. 1992). In these studies, the presumption of surface decontamination may have rested largely on an inadequate provision of nutrients for contaminant growth.

During studies in our laboratory in which we examined interactions between VAMF and plant growth-promoting rhizobacteria, we used a modification of the method proposed by Tommerup and Kidby (1980) to routinely surface decontaminate VAMF spores. It be-

came apparent, however, that surface decontamination of *Glomus clarum* NT4 spores was not always successful. Here we report that the failure of the surface decontamination procedure was due to the presence of bacteria intimately associated with VAMF spore walls.

Materials and methods

Fungal spores

Spores of *G. clarum* NT4 (INVAM no. SA101) (Talukdar and Germida 1993) were produced in pot cultures using corn (*Zea mays* L. var. Golden Bantam) grown for 90 days in a 1:1 (v/v) soil:sand mixture as the host crop. Following multiplication, pot cultures were stored at 5 °C and spores were recovered from the soil either immediately (i.e., freshly harvested) or at 16-, 24- or 52 weeks, hereafter referred to as spore age. Prior to each experiment, spores were held at –20 °C for 14 days to improve and synchronize spore germination (Safir et al. 1990).

Spores were recovered from pot cultures by wet sieving and sucrose density gradient centrifugation [20:60% sucrose (w/v)] (Daniels and Skipper 1982). Spores were collected from the sucrose interface using a Pasteur pipette and thoroughly washed in running tap water. Debris and damaged or discoloured spores were removed manually. Spores were isolated within 24 h of initiating an experiment and were maintained in tap water at 5 °C during experimental preparation.

Surface sterilization of VAMF spores

A modification of the surface-sterilization procedure proposed by Tommerup and Kidby (1980) was used. Spores were washed to remove loosely adhering contaminants by vortexing 400–600 spores for 3 min in 5 ml sterile 0.1 mg ml^{-1} sodium dodecyl sulfate (SDS) (BDH, Toronto, Ont.) solution. Spores were then transferred using a sterile Pasteur pipette to a sterile filter apparatus made by cutting the end off a 10-ml autoclavable syringe and heat annealing a 45-μm pore size Swiss monofilament fabric screen (B. and S.H. Thompson, Scarborough, Ont.) to the open end. Spores held in the filter apparatus were rinsed with 500 ml sterile 0.1 mg ml^{-1} SDS solution, transferred to a second sterile filter apparatus and immersed in the filter-sterilized (0.2 μm) decontaminant solution. The filter apparatus was conveniently sized to fit snugly within a 50-ml centrifuge tube that could be capped tightly and vortexed with the filter apparatus in place. Sterile centrifuge tubes contained 20 ml filter-sterilized (0.2 μm) decontaminant solution.

Spores were exposed to fresh decontaminant solution at 20- to 30-min intervals by removing the filter apparatus from one centrifuge tube and transferring it to a second tube containing fresh solution. Following spore transfer, tubes were capped, vortexed for 30 s, and subsequently maintained at 30 °C in a water bath. The total exposure time ranged from 20 to 120 min. After exposure to the decontaminant solution was completed, spores were rinsed with 500 ml 0.1 mg ml^{-1} SDS, followed by 1 l sterile reverse osmosis water. Except for warming the decontaminant solutions in the water bath, all manipulations were performed in a laminar flow hood.

Decontaminant solutions contained 2.5–10% (w/v) chloramine-T (Sigma, St. Louis, Mo.) and 0.1 mg ml^{-1} SDS in reverse osmosis water. The concentration of available chlorine in a 5.0% (w/v) solution of chloramine-T was determined by titration with sodium thiosulfate (Vogel 1951).

Contamination and germination of VAMF spores

Treated and rinsed spores were suspended in a small volume of sterile water (ca.3 ml) and subsequently transferred in 7-μl droplets onto the media (100 × 15-mm Petri dishes) using an automated microvolume multidispense pipette fitted with a sterile 100 μl Eppendorf pipette tip. Plates were inverted and incubated in the dark for 3–21 days at 27 °C. Although non-surface-sterile control treatments were included in all studies, extensive contaminant growth typically obscured the spores and these plates were discarded.

Four media were evaluated for supporting VAMF spore germination and contaminant growth: water agar (WA) [1% (w/v) agar in deionized water], yeast extract agar (YEA) [0.02% (w/v) yeast extract and 1% (w/v) agar (Tommerup and Kidby 1980)], nutrient agar (NA) [0.8% (w/v) dehydrated nutrient broth and 1% (w/v) agar], and 1/10 strength tryptic soy agar (1/10-TSA) [0.3% (w/v) dehydrated tryptic soy broth and 1% (w/v) agar]. All complex media components and agar were Difco products (Difco, Detroit, Mich.). Contamination and spore germination were evaluated at 3–21 days by examining spores at 25–50 × magnification. Spores were considered germinated when a germ tube (> 100 μm in length) was clearly visible. Treatments were replicated three to five times, and experiments were conducted twice with similar results.

Microscopy studies

Spores examined using a scanning electron microscope (SEM) were fixed in 2.5% (v/v) gluteraldehyde buffered in 0.2 M S-collidine (pH 7.39) for 15 min, washed in three changes of S-collidine buffer and postfixed in 1% (w/v) osmium in S-collidine buffer for 1 h on ice. Fixed spores were transferred to nucleopore filters, mounted on aluminium studs, dried over P_2O_5, coated with gold, and examined with a Phillips 505 electron microscope.

Spores were processed and prepared for transmission electron microscopy (TEM) by the Department of Pathology, Royal University Hospital, University of Saskatchewan, Saskatoon. Spores were fixed in 2% (v/v) gluteraldehyde buffered in 3% (v/v) 0.1 M cacodylate (pH 7.35) for 1 h, washed in three changes of cacodylate buffer and postfixed in 2% (w/v) osmium in cacodylate buffer for 1 h on ice. The spores were subsequently dehydrated through a graded ethanol series, cleared in propylene oxide and embedded in Epon. Ultrathin sections of 40–300 nm were cut with diamond knives in a Reicher ultracut microtome. Sections were mounted on Cu grids, stained in uranyl acetate and lead hydroxide and examined with a Zeiss EM 10 electron microscope.

Results

Contamination and germination of VAMF spores

Both NA and 1/10-TSA supported relatively high levels of detectable contaminants within 72 h (Fig. 1). Levels of contaminants associated with treated spores on YEA were typically less than 50% of those observed on NA and 1/10-TSA. Although WA supported growth of microorganisms associated with untreated spores, few contaminants associated with treated spores were detected.

Incubation of spores for longer than 72 h on either NA or 1/10-TSA resulted in extensive contaminant growth; as many as 50% of the plates were completely covered by spreading fungi and bacteria and were discarded. Of the remaining plates, contaminants originating from single VAMF spores frequently spread to engulf neighboring spores, thus hindering contamination estimates. Although contaminant growth was not as extensive on WA and YEA, detection of contaminants

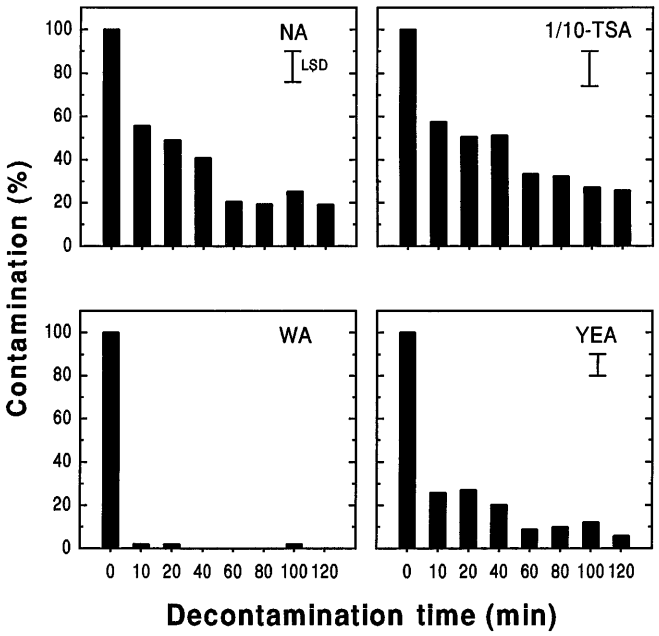

Fig. 1 Apparent contamination of 16-week-old *Glomus clarum* NT4 spores incubated for 72 h following decontamination in 5% chloramine-T. Values are means of five replicate determinations

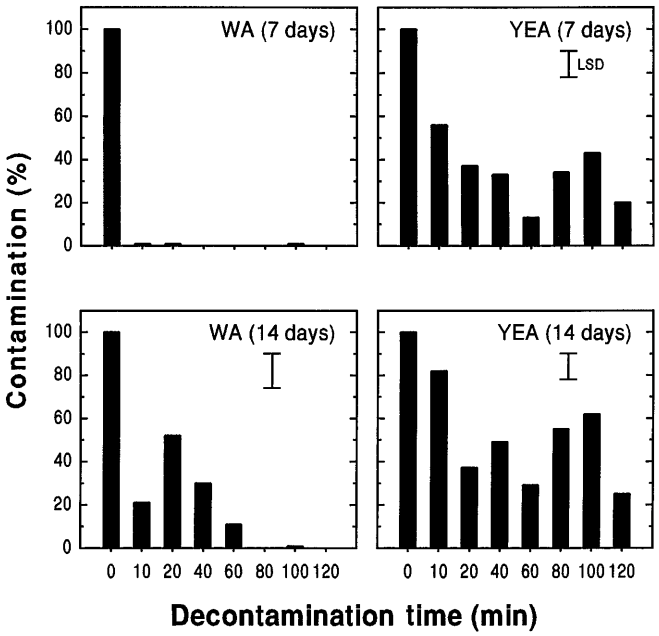

Fig. 2 Apparent contamination of 16-week-old *G. clarum* NT4 spores incubated on water agar or yeast extract agar for 7 and 14 days following decontamination in 5% chloramine-T. Values are means of four replicate determinations

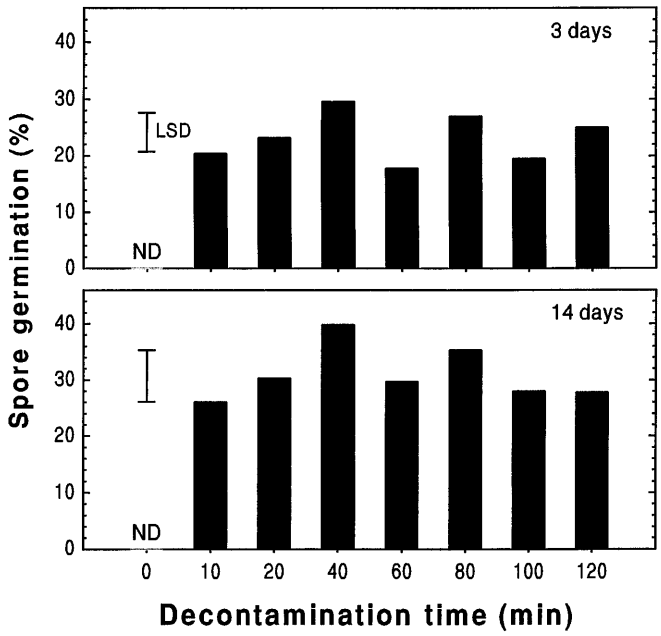

Fig. 3 Influence of decontamination time on the germination of 16-week-old *G. clarum* NT4 spores incubated for 3 and 14 days. Values are means of five replicate determinations (*ND* not determined)

continued to increase with time (Fig. 2). An incubation period of 14 days was required for detectable levels of contamination on YEA to approach those observed within 72 h on either 1/10-TSA or NA. Although contaminants were observed on WA after 14 days, levels remained relatively low and did not approach those obtained on 1/10-TSA, NA or YEA (Figs. 1, 2).

Contaminant growth surrounding VAMF spores obscured germ tube emergence and growth; thus, assessment of germination was limited to spores incubated on WA. The germ tube emerged through remnants of old subtending hyphae. Germination of VAMF spores was limited, achieving a maximum of only 40% (Fig. 3). Although a high proportion of these spores had germinated within 72 h, spore germination continued for up to 14 days. Germination was initially enhanced by treatment in 5% chloramine-T, and maximum levels were obtained following a 40-min exposure to the decontaminant (Fig. 3). Although prolonged exposure to chloramine-T may have inhibited germination of some spores, germination levels as high as 28% were obtained after exposure to 5% chloramine-T for 120 min.

Surface contamination of VAMF spores, evaluated on 1/10 TSA, was reduced both by increasing the chloramine-T concentration and by prolonging the exposure time (Table 1). However, even the most rigorous treatments failed to completely decontaminate the VAMF spores. Moreover, spore germination was compromised by such rigorous decontamination treatments.

Freshly harvested spores were more sensitive to the decontamination procedure than were mature spores (Tables 1 and 2). Whereas mature spores required and withstood fairly rigorous decontamination treatments (Table 1), contamination of freshly harvested spores was readily reduced by mild treatments consisting of

Table 1 Contamination and germination of 24-week-old *Glomus clarum* NT4 spores after exposure to solutions of 5.0, 7.5, and 10.0% (w/v) chloramine-T for up to 120 min. Contamination values are means with standard errors of five replicate plates with 20–39 spores per plate; germination values are means with standard errors of five replicate plates with 26–42 spores per plate

Chloramine-T concentration (%)	Sterilization time (min)	Contamination (%)	Germination (%)
5	30	50±3	27±5
	60	43±7	23±4
	90	29±6	24±4
	120	26±9	22±5
7.5	30	25±6	23±4
	60	15±4	18±6
	90	13±5	18±6
	120	12±4	17±3
10	30	19±6	27±5
	60	14±7	16±3
	90	13±8	9±3
	120	8±4	9±4

Table 2 Contamination and germination of freshly harvested *G. clarum* NT4 spores after exposure to solutions of 2.5, 5.0, and 7.5% (w/v) chloramine-T for up to 120 min. Contamination values are means with standard errors of five replicate plates with 21–42 spores per plate; germination values are means with standard errors of three replicate plates with 78–108 spores per plate

Chloramine-T concentration (%)	Sterilization time (min)	Contamination (%)	Germination (%)
2.5	30	29±2	40±6
	60	23±9	25±6
	90	16±4	15±5
	120	14±5	2±1
5.0	30	18±2	16±2
	60	14±2	13±1
	90	11±3	8±2
	120	10±1	3±3
7.5	30	17±3	14±3
	60	11±3	5±2
	90	11±3	2±1
	120	8±2	1±1

low concentrations of chloramine-T and short exposure times (Table 2). However, even mild decontamination treatments significantly reduced the germination of freshly harvested spores.

The possibility that incomplete decontamination of the spores was due to the failure of chloramine-T to supply adequate levels of chlorine was investigated (data not shown). The concentration of chlorine in a freshly prepared solution of 5.0% chloramine-T was 0.51%. Incubation at 5 °C for 100 min reduced chlorine levels to 0.47%. Similarly, warming the solutions had no marked effect on the chlorine concentrations, i.e., 94–100% of the chlorine initially present in the chloramine-T solutions remained in solution following incubation at 30 °C for 20 min.

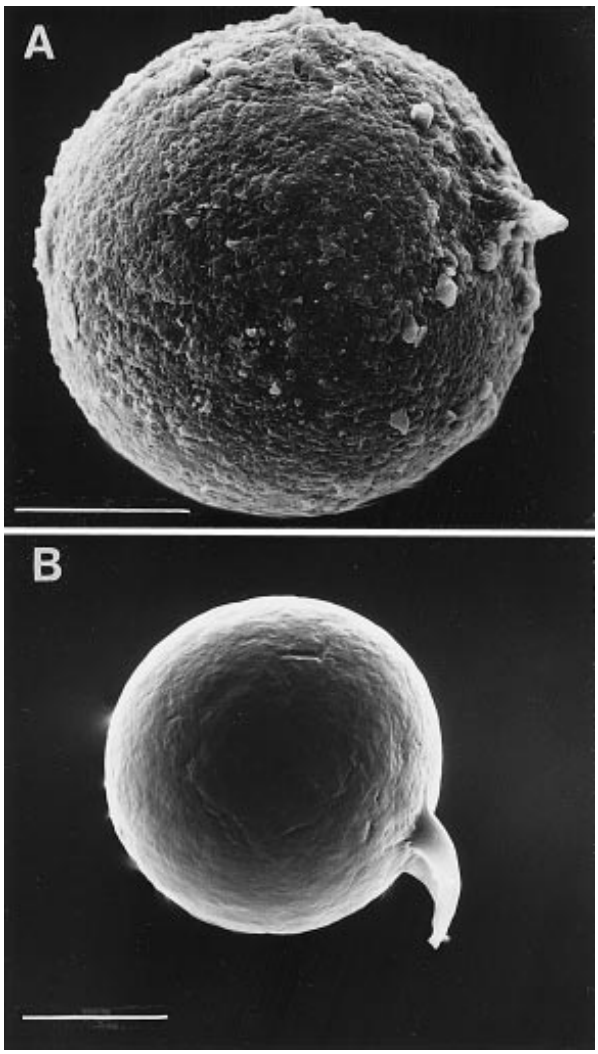

Fig. 4 Scanning electron micrographs of intact 52-week-old *G. clarum* NT4 spores: **A** before decontamination; **B** after decontamination in 5% chloramine-T for 30 min. *Bars* 50 μm

Ultrastructure study of VAMF spore walls

Scanning electron micrographs revealed that even mild decontamination treatments (e.g., 5% chloramine-T for 30 min) effectively removed both debris and contaminants from the surface of *G. clarum* NT4 spores (Fig. 4). However, transmission electron micrographs revealed that a variety of irregularly shaped bacterial cells, ranging in size from 0.9 to 1.6 μm, and apparently cocci-shaped cells, ranging in size from 0.1 to 0.2 μm in diameter, were embedded within the walls of *G. clarum* NT4 spores (Fig. 5). Bacteria and evidence of microbial activity were generally limited to the outer spore wall surface, and penetration into the spore wall further than 3.7 μm was not observed. Erosion of spore walls by the bacteria was more pronounced in older spores. Typically, *G. clarum* NT4 spore walls consisted of three visually distinct layers: the outer wall of largely fragmented material, 2–3 μm thick; the middle wall,

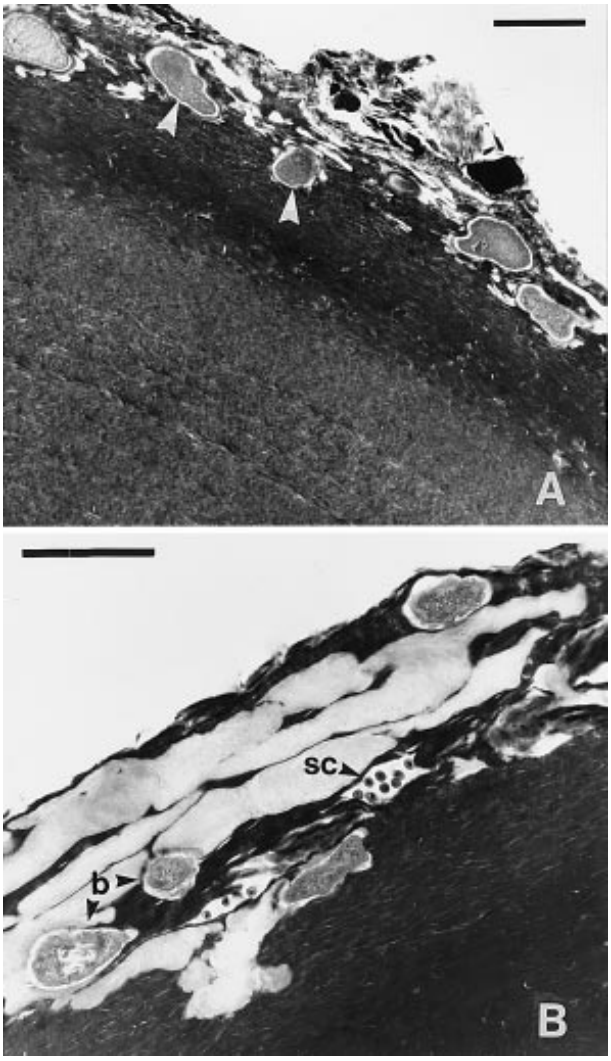

Fig. 5 Transmission electron micrograph of sectioned *G. clarum* NT4 spores showing presence of structures consistent with the general appearance and size of bacteria embedded in the spore walls. **A** Spores stored for 8 weeks. Bacterial cells are limited to the spore wall surface (*arrows*). **B** Spores stored for 52 weeks. Bacterial cells (*b*) and small cells (*sc*) are embedded within the spore wall. *Bars* 2 μm

9.5–10.5 μm thick; and the inner wall of two to three thin bands delineating the inner surface of the wall (Fig. 6).

Discussion

Satisfactory evaluation of the surface sterility of VAMF spores rested largely on the selection of media that encouraged contaminant growth. Although it is not surprising that the media relatively rich in nutrients supported more contaminant growth than WA, it is significant that contaminants otherwise undetectable on WA flourished in the presence of nutrients within 72 h. This observation demonstrated that contaminants presented

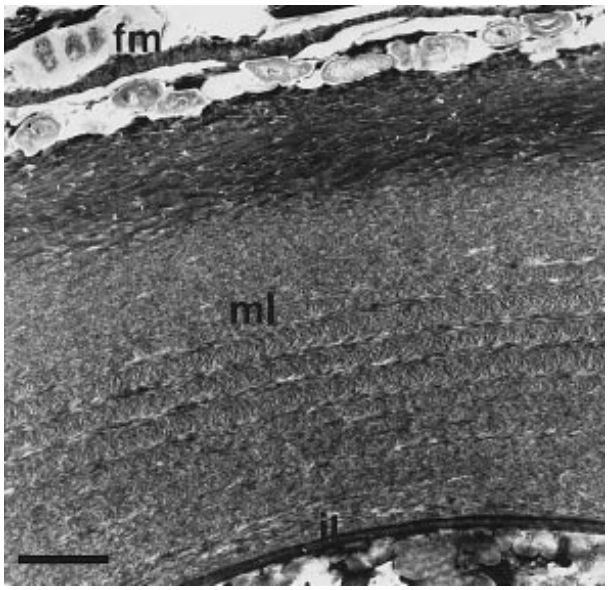

Fig. 6 Transmission electron micrograph of sectioned 52-week-old *G. clarum* NT4 spore wall showing three visually distinct layers: outer heterogeneous band of fragmented material (*fm*), middle wall layer (*ml*) comprised of a series of bands having a curved fibrillar structure, and an inner dense layer (*il*) delineating the inner surface of the spore wall. *Bars* 2 μm

with an appropriate nutrient supply made a rapid transition to an active growth stage. Actively growing bacteria in intimate association with VAMF spores could function as a supply of metabolic products to the host VAMF spore (Mosse 1970).

The potential for metabolite-mediated interactions between VAMF spores and associated organisms was suggested by Mosse (1970) who hypothesized that bacteria-like organelles in the cytoplasm of *Endogone* spores would directly supply metabolic products to the host spore during periods of rapid multiplication. Others have demonstrated that microorganisms can both stimulate (Mosse 1959; Azcon-Aguilar et al. 1986; Mayo et al. 1986; Mugnier and Mosse 1987; Will and Sylvia 1990) and inhibit or delay (Daniels and Trappe 1980; Paulitz and Linderman 1989) VAMF spore germination. Thus, undetected contaminants in intimate contact with a spore could exert a considerable influence on events leading to spore germination and early saprophytic growth. Indeed, the presence of undetected contaminants associated with VAMF spores may account, in part, for the wide variation in spore germination reported in the literature.

When assessed on NA, contamination of mature *G. clarum* NT4 spores exposed to 5.0% chloramine-T for 120 min remained as high as 26%. Using a similar decontamination method followed by incubation of spores for 14 days on YEA, Tommerup and Kidby (1980) reported that complete decontamination of *Glomus caledonius* and *G. monosporus* spores was achieved after 20 min, but that *Acaulospora laevis* spores required 40 min to be completely decontami-

nated. It is likely that species-dependent spore characteristics, including spore wall thickness and surface features account, in part, for differences in the degree to which successful decontamination can be achieved.

SEM failed to reveal any residual contaminants on the surface of VAMF spores following any decontamination procedure. However, as demonstrated using TEM, failure to achieve complete decontamination of *G. clarum* NT4 spores was related to the presence of bacteria embedded within the spore walls. Cocci-shaped cells (0.1–0.2 μm in diameter) embedded within the spore walls were consistent with observations of small or dwarf cells in soil (Bae and Casida 1973; Casida 1977) and could represent a bacterial resting state. Casida (1977) reported that some soil microorganisms decrease cell size to less than 0.21 μm in response to reduced nutrient availability, particularly when associated with a surface.

The observation that lower concentrations of chloramine-T and shorter incubation periods were required to reduce contaminant levels of freshly harvested spores as compared to more mature spores is consistent with the observation that bacteria eventually became embedded in the walls of *G. clarum* NT4 spores, i.e., contaminants of freshly harvested spores may have been restricted largely to the surface of the spores and were thus subject to chloramine-T contact, whereas bacteria embedded within the walls of older spores remained protected.

Others have noted the presence of bacteria embedded in VAMF walls of *G. caledonius* spores (MacDonald 1981; MacDonald and Chandler 1981). MacDonald and Chandler (1981) suggested that bacteria deeply embedded in VAMF spore walls might be physically protected from sterilant solutions and posited that eventual growth of embedded bacteria could account for the spontaneous contamination of apparently axenic VAMF spores. Our results confirm that bacteria also can be embedded in *G. clarum* NT4 spore walls. Moreover, although surface decontamination procedures removed the bacteria from the spore surface, as revealed by SEM, some bacteria remained protected within the spore walls and were detected only when the spores were placed on appropriate media.

The pursuit of complete decontamination can be costly in terms of spore germination. MacDonald (1981) recognized the difficulty of achieving complete surface sterility of VAMF spores and regularly screened for contamination on nutrient media after surface decontamination. Thus, as an alternative to achieving complete decontamination of a large number of spores, it may be advisable to use a decontamination procedure that provides only a portion of decontaminated spores but has a limited impact on germination. Treated spores could be plated on a medium, such as 1/10-TSA, that encourages contaminant growth and, following a short incubation period, contaminated spores could be identified and culled from the sample. Unfortunately, because spore germination may proceed during screening procedures, this practice may limit the design of subsequent experiments.

Our results underscore the importance of evaluating the success of a decontamination procedure using conditions conducive to contaminant growth. Moreover, the relative success of a method, both in terms of decontamination and subsequent germination levels achieved, may be dependent both on VAMF species and spore age. Consequently, it is advisable that the success of any decontamination procedure be evaluated prior to its routine use, particularly if the interpretation of experimental results rests on the assumption of true surface sterility of the VAMF spores.

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