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Ultrastructure of spore development in *Scutellospora heterogama*

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Abstract The ultrastructural detail of spore development in *Scutellospora heterogama* is described. Although the main ontogenetic events are similar to those described from light microscopy, the complexity of wall layering is greater when examined at an ultrastructural level. The basic concept of a rigid spore wall enclosing two inner, flexible walls still holds true, but there are additional zones within these three walls distinguishable using electron microscopy, including an inner layer that is involved in the formation of the germination shield. The spore wall has three layers rather than the two reported previously. An outer, thin ornamented layer and an inner, thicker layer are both derived from the hyphal wall and present at all stages of development. These layers differentiate into the outer spore layer visible at the light microscope level. A third inner layer unique to the spore develops during spore swelling and rapidly expands before contracting back to form the second wall layer visible by light microscopy. The two inner flexible walls also are more complex than light microscopy suggests. The close association with the inner flexible walls with germination shield formation consolidates the preferred use of the term 'germinal walls' for these structures. A thin electron-dense layer separates the two germinal walls and is the region in which the germination shield forms. The inner germinal wall develops at least two sub-layers, one of which has an appearance similar to that of the expanding layer of the outer spore wall. An electron-dense layer is formed on the inner surface of the inner germinal wall as the germination shield develops, and this forms the wall surrounding the germination shield as well as the germination tube. At maturity, the outer germinal wall develops a thin, striate layer within its substructure.

Keywords *Scutellospora* · Spore ultrastructure · Arbuscular mycorrhizal fungi · Germination shield · Germinal walls · Glomeromycota

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Introduction

Spore development in *Scutellospora* species is one of the most intricate in the Kingdom Fungi. Not only are two distinct and complex sets of walls formed (spore wall and inner flexible walls), but the synthesis of a germination shield within the inner wall complex is unique, a feature shared only with some other glomeromycotan fungi. Wall structure within spores has played a significant role in the classification of the Glomeromycota, and a range of terminology has been used to describe the various layers present within glomeromycotan spores (Walker 1983; Franke and Morton 1994). These descriptions were based on light microscope observations of spore wall layers and included staining characteristics, laminations, degree of rigidity and ease of separation. These distinctions warranted the separation of the genus *Scutellospora* from *Gigaspora*, in which the former formed spores with one or more inner 'flexible' walls and which germinated from a specialised structure, the germination shield, positioned on or between these inner walls (Walker and Sanders 1986). The process of development of the germination shield was not fully elucidated by the authors, but a likely mode of formation was inferred from

the micrographs of Gibson (1985). The inner, flexible walls are continuous around the spore contents and, unlike the spore walls, are not contiguous with walls of the subtending hypha. As these walls are intimately involved in the development of the germination shield, they have since been termed 'germinal walls' (INVAM 2006), and we will use and justify this descriptor. Franke and Morton (1994) later described the light microscopy of spore development in *Scutellospora heterogama* and *S. pellucida* in detail. They recognised five or six stages respectively. Stage 1 involved an initial stage of spore growth and expansion during which the two-layered spore wall developed. Stage 2 involved the differentiation of the spore wall into a thin, homogenous, outer layer and a thicker, laminate, inner layer. Stages 3 and 4 of spore development involved the successive formation of two germinal walls, each also with two layers at maturity. At stage 5 in *S. heterogama*, the germination shield formed with completed differentiation of the germinal walls. Five additional species of *Scutellospora* differentiated spore wall layers in a similar pattern, but formed only a single, bilayered germinal wall with the subsequent development of the germination shield on this wall (Morton 1995). More recently, De Souza et al. (2005) revealed that spores of *S. reticulata* had a more complex spore wall, with three distinct layers of increasing thickness towards the spore centre. The developmental stages of the germinal walls and germination shield, however, were considered to be identical to previously reported patterns.

No complementary studies of the ultrastructural development of *Scutellospora* spores have been published, possibly because of the reported problems of fixation (Maia et al. 1993). The ultrastructure of mature spores of *S. nigra* (Old et al. 1973), *S. heterogama* and *S. pellucida* (Gibson 1985) has been described briefly, and both studies showed that wall complexity was greater than predicted from light microscopy. In this paper, we provide further details of spore and germinal wall organisation in *S. heterogama* and reveal that the formation of germinal walls is integral to germination shield formation.

Materials and methods

Fungi, host plants and culture methods

Two isolates of *S. heterogama* (Nicol. & Gerd.) Walker and Sanders (BEG35 and BEG40) from two geographical sources were pot-cultured in 2.5-l pots on *Pueraria phaseoloides* or *Desmodium ovalifolium* in an attapulgite clay (Agsorb 8/16, Oil-Dri, Wisbech, UK) substrate. Plants were maintained in a controlled environment tropical greenhouse with supplementary lighting (Rodriguez et al. 2005). Spores were wet-sieved from pot cultures (IBG 2006) and divided into groups on the basis of colour (maturity): mature spores (150–250 μm diameter) were red-brown with clear contents, spores of an intermediate developmental stage (150–250 μm diameter) were orange with dense opaque contents and immature spores (50–150 μm diameter) were white with dense opaque contents.

Light microscopy

Spores were mounted in water, polyvinyl-lactoglycerol (PVLG) or 1:1 PVLG-Melzer's reagent using standard protocols (IBG 2006). Some spores were bleached with 5.25% (*wt/v*) sodium hypochlorite until they lost most of their pigmentation to improve visualisation of the germination shield. Spores were then washed in distilled water and mounted as described above. Fully differentiated germination shields could be seen in most of the dark orange-coloured spores, as germination shields retained more colour than the spore walls after bleaching. They were also faintly visible in mature spores when viewed directly under a low-power dissecting microscope, but more clearly in spores that had been bleached and crushed. Intermediate stages in the development of germination shields could not be detected, possibly because differentiation occurred rapidly.

Scanning electron microscopy

Intact spores were fixed for 3 h in 2.5% (*wt/v*) glutaraldehyde in phosphate-buffered saline at pH 7.2 (PBS) and dehydrated through a graded acetone series. They then were critical-point dried and sputter coated with gold using standard protocols. Spores were examined using a Philips 525 M scanning electron micrograph with an accelerating voltage of 80 kV.

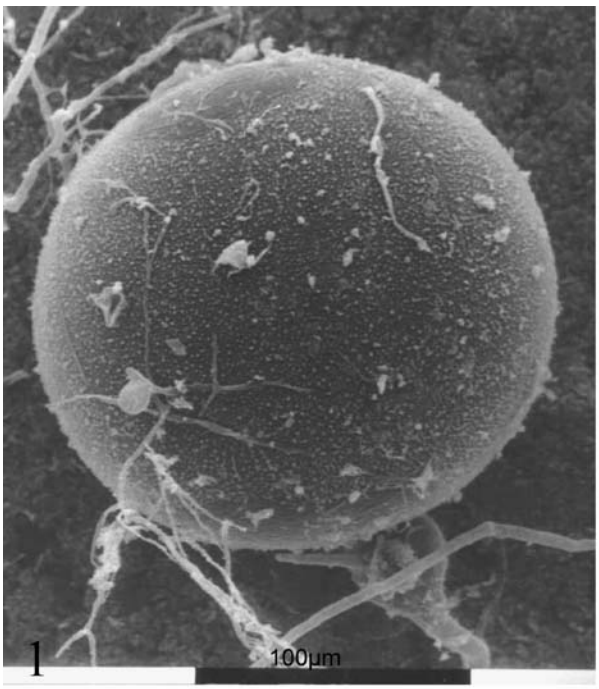
Transmission electron microscopy

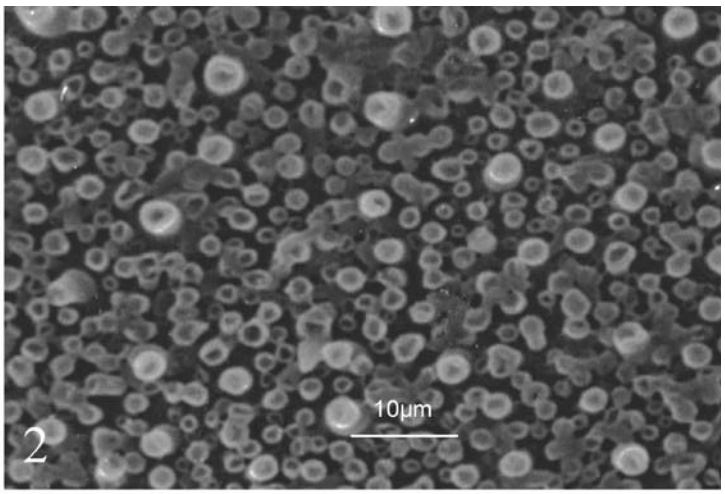
Spores of *S. heterogama* from each colour group were placed in 2.5% (*wt/v*) glutaraldehyde in PBS as above and observed under a dissecting microscope while being gently broken with a fine needle to allow fixative to enter (Miller and Jeffries 1994). This stage was usually necessary to overcome previously reported fixation difficulties. After a further fixation in 1% (*wt/v*) OsO_4 in 0.1 M cacodylate buffer for 3 h, spores were embedded, sectioned and stained using conventional methods (Miller and Jeffries 1994). Micrographs were obtained using either a Philips EM410 or a JEOL 1230 electron microscope.

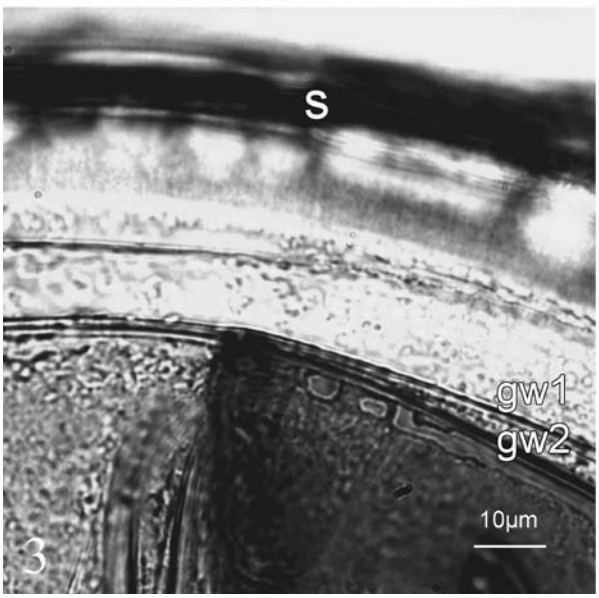
Results

Both isolates of *S. heterogama* shared most features; therefore, the observations here are reported for isolate

BEG35; BEG40 is only singled out where differences were noted. Spores from each developmental state were mainly spherical (Fig. 1), but some were ovoid. Scanning electron microscopy of whole spores accentuated detail of their papillate surface ornamentation (Figs. 1 and 2). Individual papillae were cupulate, 0.2–2.0 μm in diameter and varied in height from 0.5 to 0.8 μm. Thin, short hyphae often protruded from the swollen area of the unornamented hyphal attachment (Fig. 1). By light microscopy, gently broken mature and intermediate spores revealed a spore wall composed of an outermost, ornamented wall approximately 1.5 μm thick covering a thicker wall, approximately 5–8 μm thick. Electron microscopy (see below) showed an inner wall differentiated into two zones. Beneath these walls were two germinal walls, each approximately 1–2 μm thick, that often separated on crushing (Fig. 3). In Meltzer's







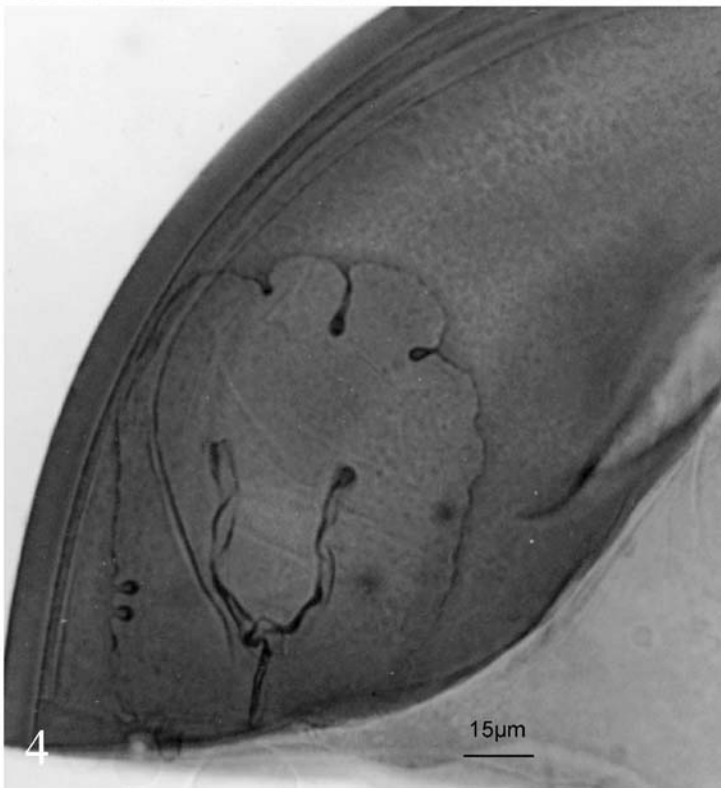


Fig. 1 Scanning electron micrograph (SEM) of mature spore of *Scutellospora heterogama* BEG35 with attachment. ×430

Fig. 2 Scanning electron micrograph (SEM) of mature spore of *Scutellospora heterogama* BEG35 surface showing cupulate ornamentation. ×2,200

Fig. 3 Light micrograph (LM) of crushed orange spore of *Scutellospora heterogama* BEG35 showing spore wall (*s*) and germinal walls (*gw1*, *gw2*) separating where a germination shield (*dark area within spore*) is formed. ×1,000

Fig. 4 Light micrograph (LM) of crushed red-brown spore of *Scutellospora heterogama* BEG35 showing germination shield in surface view. ×660

reagent, the inner germinal wall became a pale-purple-to-pink colour, whereas the spore wall stained more slowly, becoming a reddish-brown colour. Spores of *S. heterogama* BEG40 showed a similar range of colour to those of BEG35, although mature spores were paler and slightly larger (200–260 μm). The spore wall did not stain dark red in Meltzer's reagent. The germination shield (Fig. 4) formed between the two germinal walls, and in isolate BEG35, appeared as a simple, uni-lobed shield, without significant invagination of the margins (Fig. 4). In contrast, the germination shield of BEG40 had a highly invaginated margin, and frequently, was multi-lobed.

Ultrastructure of spore development

Immature spores (white) White spores with condensed contents are formed as a swelling from the apex of the hyphal attachment and initially possess a two-layered wall structure, although a third inner layer soon develops within the expanding spore (Fig. 5; layer 3). The wall of the hyphal attachment is continuous with the wall of the subtending hypha with an outer, thin, electron-grey layer and a thicker, inner fibrillar layer. During spore development, the hyphal attachment is full of cytoplasm contiguous with that within the developing spore (Fig. 6). Once the spore wall has differentiated and the spore turns orange, a septum appears in the region separating the developing spore from the attachment (Fig. 7). During this period, the cytoplasm within the attachment becomes vacuolated. Cytoplasm was rarely observed in mature spore hyphal attachments, and in pot culture, this region usually became colonised by bacteria. The outer electron-grey layer (layer 1; Fig. 5) around the swelling spore becomes ornamented with cupulate papillae (Figs. 2, 5 and 9). The ornamentation is strictly confined to the spore and does not extend beyond the narrow neck delimiting the hyphal attachment. The second layer of the developing spore wall (layer 2; Fig. 5) is homogeneous and more electronlucent. As the spore swells, it develops an additional inner layer that is fibrillar, electron-dense and extends into the hyphal attachment where it tapers away (Fig. 6). Synthesis of this new layer is associated with the presence of small, membrane-bound vesicles with electron-dense contents in the cytoplasm adjacent to the developing wall and within the developing wall itself (Fig. 5). This layer thickens rapidly within the developing spore (Figs. 5 and 8) and then decreases in thickness as it rigidifies. Despite this reduction in width, it remains the widest layer of the entire mature spore wall. As it thickens, the outer part of the layer appears electron-dense (Fig. 8), whereas the developing inner part appears less electron-dense. This pattern suggests that the increase in width of the layer is generated by expansion of existing material rather than synthesis of new material. This layer often had a striated appearance as a result of microchatter of the diamond knife during sectioning, also suggesting that this layer is less solid than the other layers of the spore wall. Once spores have reached full size, the three layers of the spore wall are clearly delineated (Figs. 8, 9 and 10), and in older spores, layer 2 often becomes degraded and invaded by bacteria.

Intermediate spores (orange) Development of the germinal walls begins after the spore wall is fully differentiated. The spore contents are avacuolate, multi-nucleate and lipid-rich. Initially, the developing germinal wall is homogenous but soon develops an inner electron-denser zone (Figs. 9 and 10). The initial stages of germination shield formation are associated with an accumulation of small, dense, membrane-bound bodies in the spore cytoplasm next to the wall in the region where the germination shield is developing (Figs. 11 and 12). The next stages of germination shield occur very rapidly as cytoplasm, including the dense, membrane-bound bodies, is extruded into the electronlucent region of the developing germinal walls (Figs. 11 and 12), presumably via a pore through the electron-dense region of the developing germinal wall (Fig. 13). As the cytoplasm is extruded, a thin, electron-dense layer is also formed in the centre of the outer electronlucent zone of the developing

Fig. 5 Thin section (TS) of white spore of *Scutellospora heterogama* BEG35 showing thin, outer ornamented layer (*1*), inner, electronlucent layer (*2*) and the initiation of the development of an inner, spore wall layer (*3*) associated with cytoplasmic and intramural vesicular activity. ×17,000

Fig. 6 Thin section (TS) of attachment region of white spore of *Scutellospora heterogama* BEG35 showing orientation of spore walls 2 and 3 (*arrows*) to the walls of the subtending hypha (*h*). ×2,700

Fig. 7 Tangential section through attachment region of mature spore of *Scutellospora heterogama* BEG35 showing septum (*s*) in neck. The germinal walls (*arrow*) do not extend into this region. This tangential section does not have scale bar, as the wall thickness is not representative of correct width. ×1,500

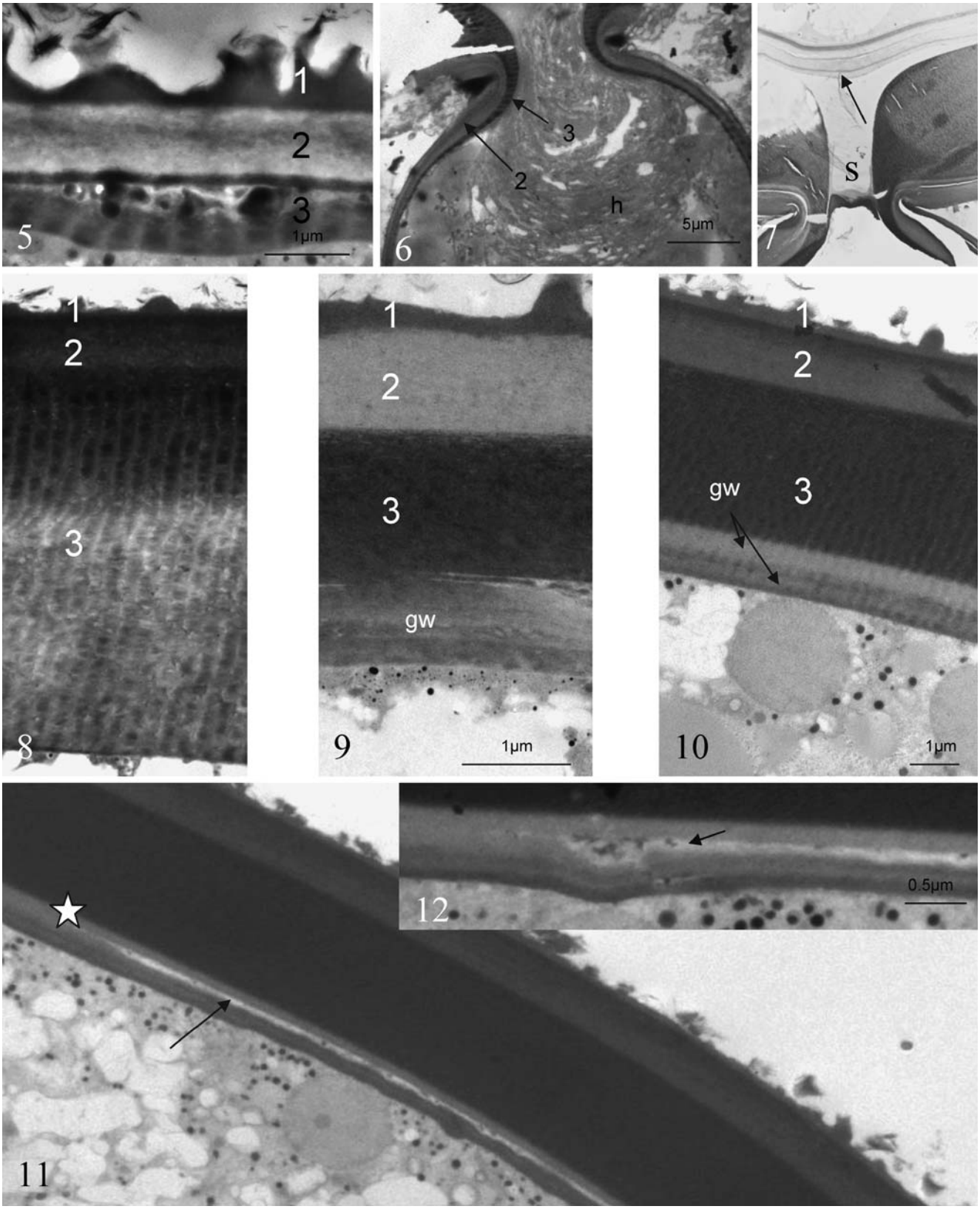
Fig. 8 Tangential section of white spore of *Scutellospora heterogama* BEG35 showing expanded inner spore wall layer (*3*), which has vertical striations caused by knife chatter. This tangential section does not have scale bar, as the wall thickness is not representative of correct width. ×11,000

Fig. 9 Thin section (TS) of orange spore of *Scutellospora heterogama* BEG35 with spore wall (*layers 1–3*) and thin electronlucent initial layer (*arrow*) of developing germinal wall region. ×20,000

Fig. 10 Thin section (TS) of dark orange spore of *Scutellospora heterogama* BEG35 with fully differentiated spore wall (*layers 1–3*) and germinal wall region now differentiated into two regions of different electron density (*gw*; *two arrows*). ×7,000

Fig. 11 Thin section (TS) of dark orange spore of *Scutellospora heterogama* BEG35 showing initiation of germination shield where cytoplasm (*arrow*) has moved into the outer electronlucent layer of the developing germinal wall. Note presence of many small, dense vesicles in cytoplasm adjacent to this region. ×3,600

Fig. 12 Higher magnification of *starred* region shown in Fig. 11 showing edge of developing germination shield (*arrow*) and associated dense, membrane-bound vesicles. ×26,000



germinal wall, and this zone marks the region where the two germinal walls will split (Fig. 14). As avacuolate, lipid-rich cytoplasm and nuclei continue to flow through the pore, a new thin, homogenous, electron-dense, fibrillar wall layer, approximately 0.05 μm thick, is laid down around the expanding germination shield and extends around the edges

of the pore and onto the inner surface of the germinal wall (Fig. 13). This same material is also involved in the formation of septa that isolate the chambers of the germination shield as it matures in the developing red-brown spores (Fig. 15). The septa appear to be formed from two layers of this electron-dense material, which are either side of an electron-grey material that appears to be continuous with the inner germinal wall (Figs. 13 and 15).

Mature spores (red-brown) In the mature spore, the germinal wall complex becomes separated from the spore wall, leaving a gap (zone a; Fig. 16) that often contains electron-dense deposits. The fully formed germinal wall complex comprises a series of at least six differentially staining zones (Figs. 17 and 18) along with the inner wall layer 10, which is contiguous with that of the germination shield. Germinal wall 1 comprises an electron-dense zone (layer 5) sandwiched between two lighter-staining zones (layers 4 and 6) of similar appearance (Figs. 16 and 17). The electron-dense layer within this outer germinal wall exhibits short, fine electron-dense striations running towards the spore periphery (Fig. 18). Layer 7 is the region where the germinal walls separate and is where the germination shield is formed. Germinal wall 2 comprises layer 8, which is identical in appearance to layer 6 of germinal wall 1 and is of common origin, below which is layer 9, which has similar electron density to that of the thick, inner layer of the spore wall. Layer 9 sometimes bears similar knife microchatter, suggesting it is of a similar material (Fig. 17). Layer 10 is the new wall layer that is formed on the inner surface of germinal wall 2 (Figs. 14 and 16) during germination shield formation. The new inner wall (layer 10) within the spores becomes thicker where the germination shield is present and is continuous with the wall around the germination shield (Fig. 15). In sections of mature spores, which have developed a germ tube (Fig. 19), the electron-dense wall of the germination shield envelopes the developing germ tube, which breaks through germinal wall 1 and then penetrates the spore wall layers. Stages in the development of the spore wall are illustrated diagrammatically in Fig. 20 up to the stage where the germinal walls begin to differentiate. Figure 21 illustrates the processes involved in germination shield development.

Discussion

This study reveals that the spore and germinal walls in *Scutellospora* are more complex in their development than revealed by light microscopy. The basic concept of a rigid spore wall enclosing two inner germinal walls (Franke and Morton 1994) still holds true, but additional zones within these three walls are distinguishable at the ultrastructural level, and an additional wall is formed during germination shield development. It appears that the spore wall has three layers rather than the two reported from light microscopy. The outer spore wall observable by light microscopy actually consists of an outer, thin, ornamented layer and an inner, slightly thicker layer both derived from the hyphal wall and present at all stages of development (Fig. 20a,b). A third inner layer develops during spore swelling as a true new spore wall layer because it is synthesized separately from any connection to wall layers of the hyphal attachment. This layer is thin initially but expands rapidly (Fig. 20c) and then contracts into a thick, laminate layer (Fig. 20d) visible as the second spore wall layer by light microscopy. The small vesicles with electron-dense contents that are associated with the synthesis of this new layer are similar to those that are associated with development of the germinal walls and the synthesis of the germination shield. Their presence is an indicator of wall development, as they are not present in the cytoplasm of mature spores or the associated germination shield. Mosse (1970) also associated the presence of small, dense, membrane-bound vesicles with sites of wall synthesis in *Acaulospora laevis* and suggested they may contain wall pigment.

No obvious laminations were observed in the spore wall of *S. heterogama* at the ultrastructural level, but Gibson (1985) observed arcuate microfibrils in the thick layer of the outer layer of the spore wall of *S. pellucida* (and in

Fig. 13 Thin section (TS) of pore region of developing germination shield showing germinal wall 1 (*gw1*) and continuity of inner wall layer 10 (*arrow*) around germinal wall 2 to the wall of the germination shield. Septa (*s*) are present in this part of the germination shield. ×4,400

Fig. 14 Thin section (TS) of region around edge of germination shield (*gs*) in mature red-brown spore of *Scutellospora heterogama* BEG35 showing thin, electron-dense layer 7 split and separating the two germinal walls. Germinal wall 1 comprises zones *4–6*, germinal wall 2 comprises zones *8–9*. Zone *10* is the wall around the germination shield. ×7,200

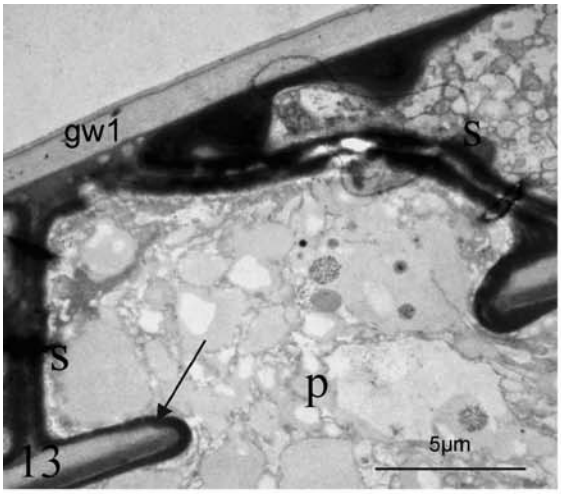
Fig. 15 Thin section (TS) through germinal walls of mature spore of *Scutellospora heterogama* BEG35 with germination shield. Note the presence of septa (*s*) compartmentalising the germination shield. The septum on the *right* is forming. ×2,000

Fig. 16 Tangential section of complete mature spore of *Scutellospora heterogama* BEG35 showing spore wall separated from germinal walls 1 and 2 (*gw1*, *gw2*) by zone (*a*). The germinal walls have separated along the electron-dense layer 7. This tangential section does not have scale bar, as the wall thickness is not representative of correct width. ×6,500

Fig. 17 Thin section (TS) inner wall group showing sub-layers 4–10. ×50,000

Fig. 18 Higher magnification of wall sub-layer 5 showing striations above an electron-dense zone. ×80,000

Fig. 19 Thin section (TS) of germinated compartment of germination shield (*gs*) showing germ tube (*gt*) emerging into region between the spore wall (*sw*) and germinal wall 1 (*gw1*). The peg wall is synthesised from the wall of the germination shield. ×2,400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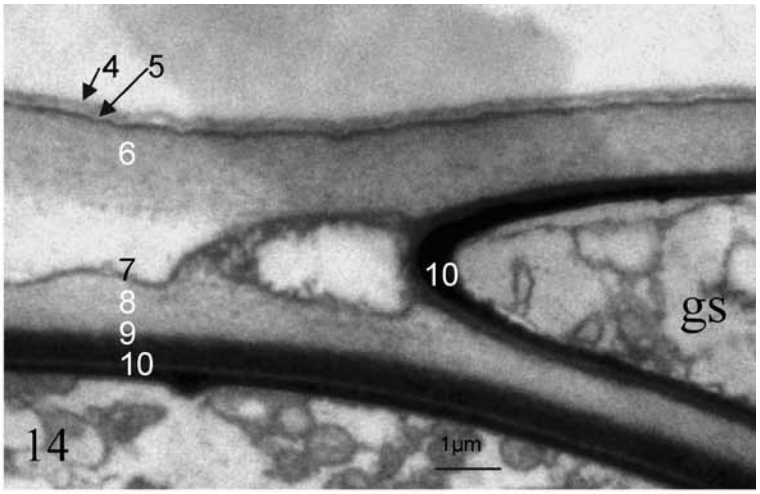
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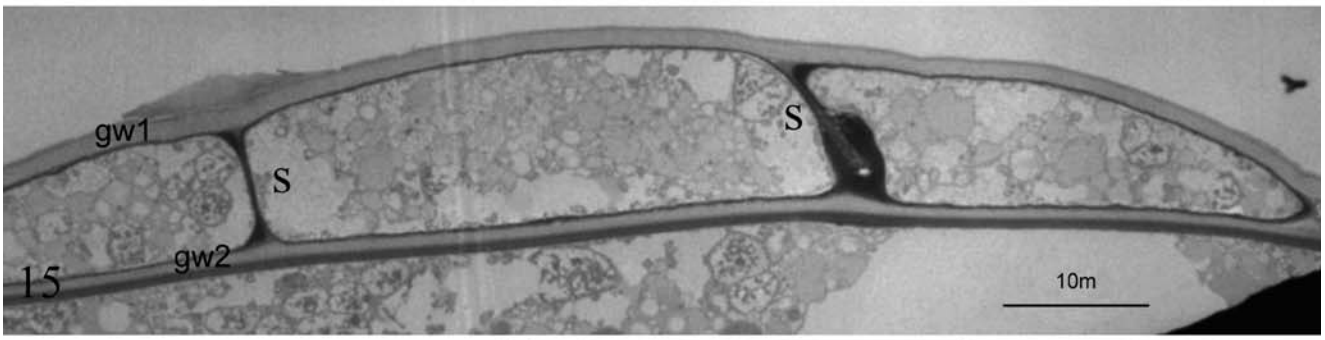
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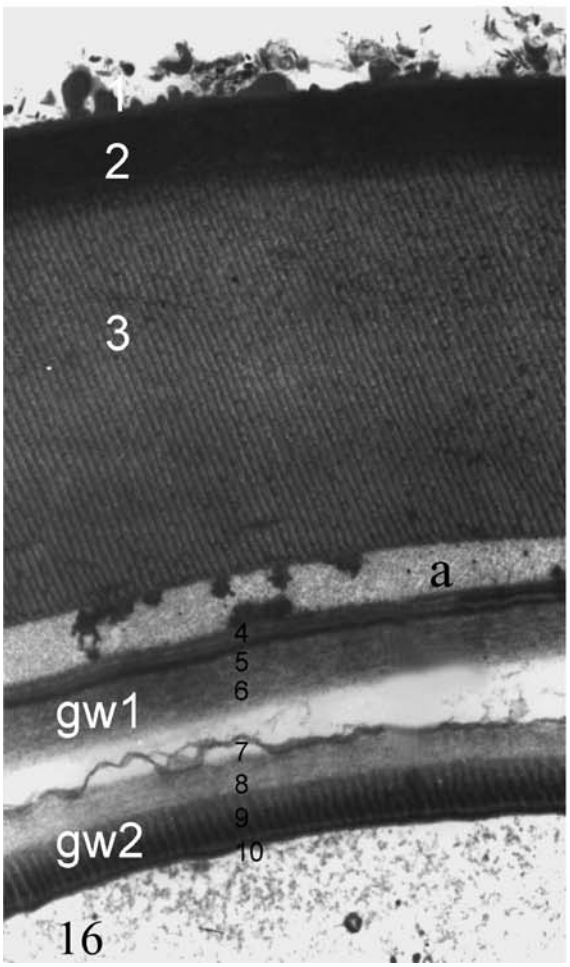
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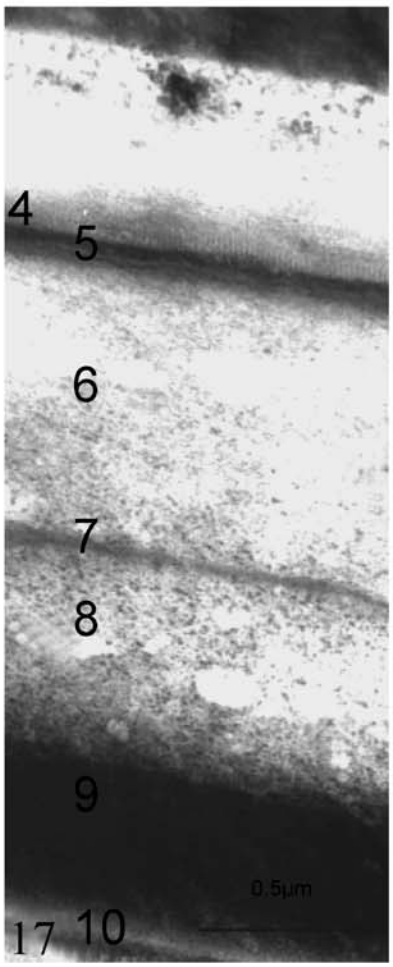
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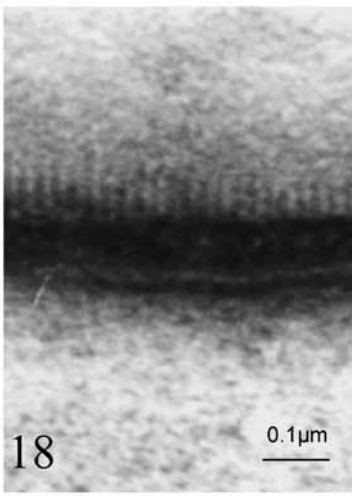
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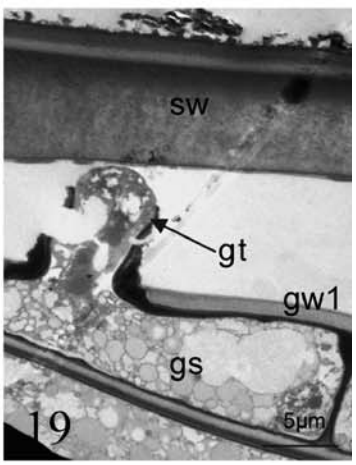
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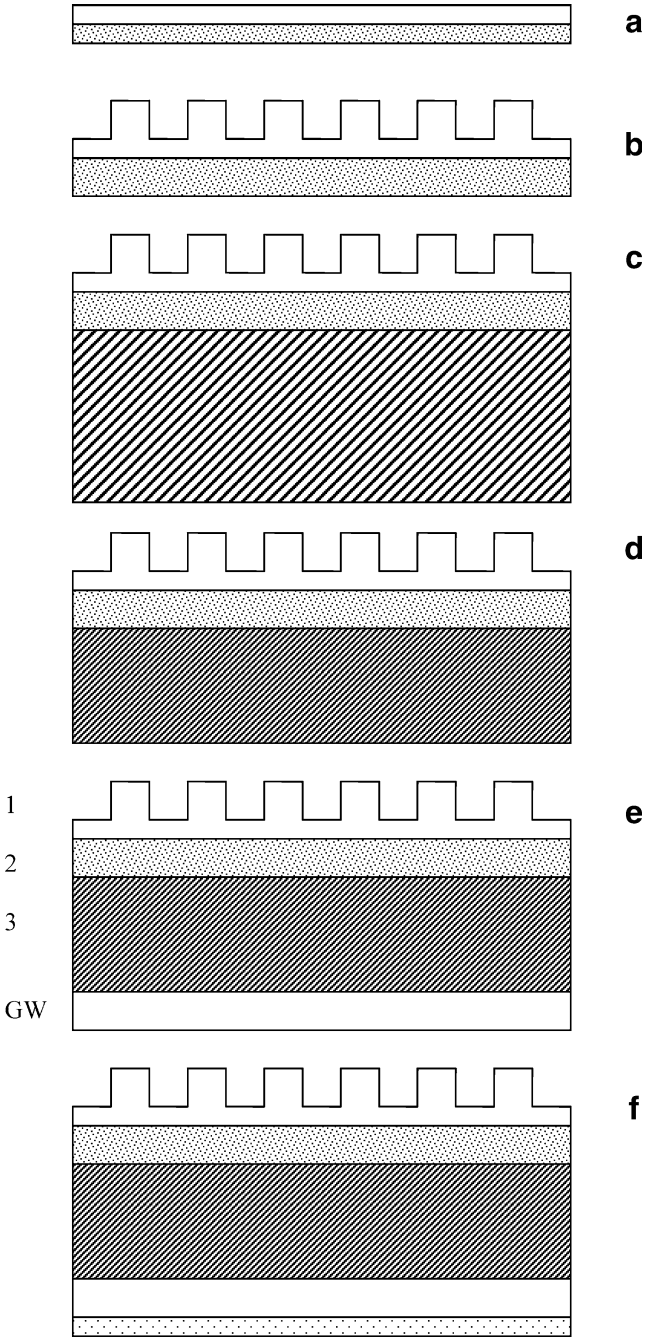

Fig. 20 Diagrammatic representation of formation of spore wall (layers *1*, *2* and *3* in **e**) and initiation of germinal walls (*GW* in **e**). **a** The spore of *Scutellospora heterogama* BEG35 initially has two wall layers contiguous with those of the hyphal attachment. **b** The outer spore wall layer develops ornamentation. **c** A new expansive spore wall layer (indicated by *diagonal lines*) develops. **d** The new layer contracts and rigidifies. **e** The germinal wall develops as a single homogeneous layer, which then becomes electron dense adjacent to the spore contents (**f**)

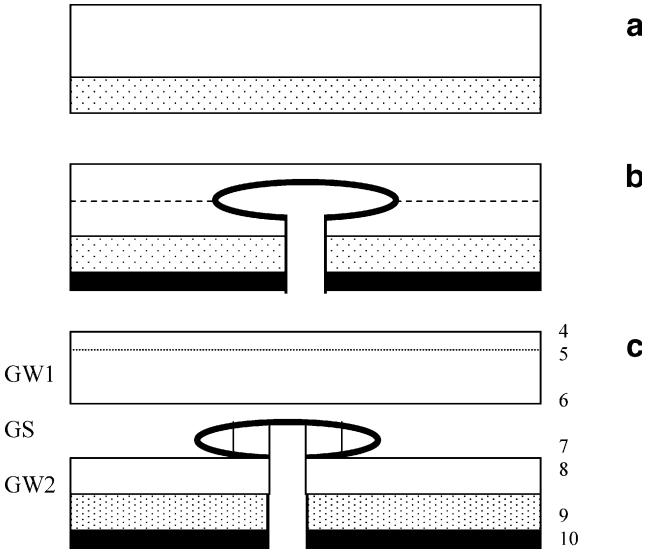

Fig. 21 Diagrammatic representation of the germinal wall (*GW1*, *GW2*) and germination shield (*GS*) development. **c** represents the mature germinal wall structure with the wall layers numbered to correspond with those in the text and in the photographs in Figs. 16 and 17. **a** The developing germinal wall has two regions of differential electron density. **b** The germination shield forms within the electronlucent zone of the developing germinal wall; it is surrounded by a new wall layer developed within the spore. The zone where the germinal walls will separate is delimited by a thin electron-dense layer (*dotted line*). **c** The two germinal walls are separated, and germinal wall 1 has developed the striate layer. The mature germination shield is now septate

Gigaspora margarita), which match those described in *Glomus* spp. (Miller and Jeffries 1994) and are typical of a 'laminated' wall *sensu* Walker (1983). This does not necessarily mean that *S. heterogama* does not have this type of wall architecture, as visualisation of the arcuate structure is very dependent on the angle of sectioning and the orientation of the microfibrils. The poor preservation of the inner spore wall layer, as evidenced by knife microchatter, also means that visualisation of any arcuate structure would be difficult.

Germinal walls also begin development as a simple structure (Fig. 20e), which develops regions of differential electron density (Figs. 20f and 21a). The outer region of electronlucent appearance hosts the developing germination shield (Fig. 21b) and then later separates to form two distinct germinal walls. Once the two walls are separated, other complex sub-layers develop within the walls as they mature (Fig. 21c). For example, there is a distinct, striate layer (layer 5) within germinal wall 1. This feature was also observed in *S. nigra* (Old et al. 1973), *A. laevis* (Mosse 1970) and *A. scrobiculata* (Maia and Kimbrough 1993). By careful observation at the light microscope level, Franke and Morton (1994) observed that germinal wall 1 comprised a thin, outer layer and a thicker, inner layer. This presumably correlates with the division across the striated, membranous zone observed in thin sections. Franke and Morton (1994) also observed two thin adherent layers in germinal wall 2, but ultrastructural observations again indicate that the layering is more complex. At least three zones develop, the inner one appearing similar to that of the expanding layer of the spore wall. This inner zone is

formed as a more electron-dense region during the initiation of the germinal wall complex before separation of the two germinal layers. The thin and flexible properties of germinal walls may be evidence that they are not true 'walls' in a protective sense, but instead are specialised layers necessary for the formation and development of the germination shield. As Franke and Morton (1994) pointed out, the inner walls in *Scutellospora* have no counterpart in fungal groups outside Glomeromycota. The ultrastructural observations also show an additional wall is formed on the inside of the germinal wall during development. This is distinguished here from the germinal walls as it continues to develop separately to surround the developing germination shield, and then ultimately forms the wall of the germ tube.

Germination shields develop very rapidly, as intermediate stages of development were seldom observed. Our observations extend those of Franke and Morton (1994) and show that the germination shield differentiates within the inner wall layers as they are developing rather than as a final stage of spore maturity (Fig. 21b). This was clear both from electron micrographs of the germinal wall complex, wherein the germination shield could be seen before separation of germinal wall 1 from germinal wall 2, and from light microscope observations of germination shields in dark orange spores. These observations support the hypothesis that the germinal walls are functionally linked with germination shield formation rather than in protecting the spore contents. These observations support the formal usage of the term 'germinal walls' that appear to function solely in the germination process.

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