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Ultrastructure of wall layers in crushed spores of *Acaulospora tuberculata* (Diversisporales, Glomeromycota)

Ultraestructura de los componentes de pared en esporas rotas de *Acaulospora tuberculata* (Diversisporales, Glomeromycota)

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Palabras clave: Acaulosporaceae, pared arosariada, pared amorfa, taxonomía

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ABSTRACT

Spores of two accessions of *Acaulospora tuberculata* Janos & Trappe (CNPAB 17 Embrapa-Agrobiologia, Rio de Janeiro, Brazil and BR 103-6 from International Vesicular-Arbuscular Mycorrhizal Fungi Collection, INVAM) were used to determine advantages of spore crushing and desiccation before fixation and embedding for studying the ultrastructure of the spore wall layers. All the spore wall components previously reported for this species were observed and ultrastructurally described, but an additional innermost layer, the eighth in *A. tuberculata* spores, was observed which results novel in this species. That innermost layer in many spores showing an innermost germinal group with central coriaceous or amorphous components discovered under the light microscope. This layer seems to be a characteristic of several, if not all species belonging to Acaulosporaceae. It might be a fixed character for many genera with internal germinal wall, such as *Scutellospora*, *Pacispora* and *Ambispora*.

RESUMEN

Fueron empleadas esporas de dos registros de *Acaulospora tuberculata* Janos & Trappe, (CNPAB 17 de la Embrapa-Agrobiologia, Río de Janeiro, Brasil y BR 103-6 procedente de la Colección Internacional de Hongos Micorrizógenos Vesículo-Arbusculares, INVAM) con el fin de estudiar las ventajas de romper y desecar las esporas antes de fijarlas y embeberlas para estudiar la ultraestructura de sus componentes de las paredes. Además de la existencia de los siete componentes de las paredes descritos para dichas esporas, también se descubrió una capa adicional hacia el interior, lo cual resulta novedoso para esta especie. Esta capa más interna ha sido observada en muchas esporas que muestran un grupo germinal más interno con un componente central coriáceo o amorfo cuando las observaciones son hechas en el microscopio compuesto. Este componente más interno parece ser una característica de varias especies pertenecientes a Acaulosporaceae. Puede ser un carácter fijo para varios géneros con paredes germinales internas, tales como *Scutellospora*, *Pacispora* y *Ambispora.*

INTRODUCTION

Glomerospores term was established by Goto and Maia (2006), denomination to be used only for the spores of Glomeromycota. Maia *et al*. (1993a, b) exhaustively studied the utility of various techniques for fixation, dehydration and embedding of glomerospores for transmission electron microscopy (TEM). Some useful results have been published on the Transmission Electron Microscope (TEM) analysis of spore wall composition using entire and broken spores of *Rhizoglomus intraradices* (N.C. Schenck & G.S. Sm.) Sieverd., G.A.

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Silva & Oehl (Rosendahl *et al*., 1994; Maia and Kimbrough, 1993a, 1994), *Funneliformis geosporum* (T.H. Nicolson & Gerd.) C. Walker & A. Schüssler (Rosendahl *et al*., 1994) and *Rhizoglomus proliferum* (Dalpé & Declerck) Sieverd., G.A. Silva & Oehl (Declerck *et al*., 2000).

Similar studios were developed also with *Glomus clarum* (T.H. Nicolson & N.C. Schenck) Sieverd., G.A. Silva & Oehl (Walley and Germida, 1996), *Funneliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker & A. Schüssler (Filippi *et al*., 1998), *Glomus sp*. L906 (Maia and Kimbrough, 1993a, 1998) *Acaulospora morrowiae* Spain & N.C. Schenck and *Acaulospora scrobiculata* Trappe (Maia and Kimbrough, 1993a, 1993b), *Gigaspora albida* N.C. Schenck & G.S. Sm. (Maia and Kimbrough, 1993a; Maia *et al*., 1993), *Fuscutata heterogama* Oehl, F.A. Souza, L.C. Maia & Sieverd. (Jeffries *et al*., 2007) and others.

However, when glomerospores are entire, it is sometimes difficult to recognize in TEM images the whole complex of spore wall layers after fixation and embedding independent of the fact of being or not broken under liquid nitrogen before post-fixation with osmium tetroxide. This is particularly important for spores of *Acaulospora* spp., *Archaeospora* spp., *Entrophospora* spp. and *Scutellospora* spp., whose spore wall is commonly composed by more than one spore wall and posses multiple layers that remain tightly adherent in intact spores. On the other hand, there are no TEM analyses of spore wall layers in spores crushed before the fixation process. Such a type of analysis might be advantageous to clarify the ultrastructure of spore wall layers that are out of their original position, i.e., equaling their characteristics when they are observed in the compound microscope. When TEM is used to analyze those free wall layers, their ultrastructural characteristics might explain how they react physically and also explain, for instance, why beaded walls retract to dislodge in rounded free to aggregated granules (beads). The ultrastructure of those free wall layers could also explain the causes of physical transformations shown by other spore wall components.

Working with crushed spores would also be possible to confirm at the TEM the number and characteristics of spore wall layers observed under the compound microscope. For example, spores of species such as *Acaulospora denticulata* Sieverding & Toro, *A. scrobiculata*, *A. tuberculata*, *Kuklospora kentinensis* (C.G. Wu & Y.S. Liu) Oehl & Sieverd. and others form spores which have been reported (INVAM: http://invam.caf.wvu.edu) to exhibit seven wall layers. In many species such as *A. bireticulata* Rothwell & Trappe, *A. colossica* Schultz, Bever & Morton, *A. denticulata*, *A. dilatata* Morton, *A. foveata* Trappe & Janos, *A. laevis* Gerdemann & Trappe, *A. mellea* Spain & N.C. Schenck, *A. morrowiae*, *A. myriocarpa* Spain, Sieverding & Schenck, *A. rehmii* Sieverding & Toro, *A. rugosa* Morton and *A. scrobiculata*, the spore wall is composed by three outer layers (SW = L1 + L2 + L3). While inwards two germinal walls are observed, e.g. *Acaulospora*, *Scutellospora*, *Cetraspora*, *Dentiscutata* and *Ambispora* species, both are composed by two thin membranes (L1 + L2) (Stürmer and Morton, 1999). We will refer to the outermost germinal wall as GW1 and to the innermost germinal wall as GW2. Up to now, GW2 has been described as joining from outward to inward, a beaded membranous and a somehow thick, often amorphous wall layer, if all the variants for all the previously mentioned species are considered.

Acaulospora tuberculata originally described by Janos and Trappe (1982) was mentioned to have three spore walls (outer wall and GW1+GW2). Nevertheless, more recent descriptions of new *Acaulospora* species suggest that this fine innermost layer underlying two outer layers of GW2 exists (Oehl *et al*., 2012; 2014). All the same, the existence of this layer in *Acaulospora* has still been questioned, e.g., also for *A. tuberculata*.

It has been observed that sometimes descriptions differ according to the criterion used by the author. This is the case of *Diversispora spurca*, where the occurrence innermost flexible third wall layer has been discussed. The species was originally described (Pfeiffer *et al*., 1996) as showing this innermost layer. However, in a recently emended description Kennedy *et al.* (1999) mentioned that this layer does not exist. The visibility of this layer might depend on the stage of the spore ontogeny, and it is well possible that the observations made already by Pfeiffer *et al.* (1996) were correct.

According to our observations, three layers showing a sandwich-like structure form the mentioned GW2 in *Acaulospora denticulata*, *A. scrobiculata*, *A. tuberculata*, *Entrophospora kentinensis*, other Acaulosporaceae and in some species of *Scutellospora*. This sandwich-like layers or wall (endospore) shows an outermost beaded layer, and an innermost very thin layer that may wrinkle profusely; both enclose a somehow thick, unit to finely laminate, often coriaceous layer that shows a limited to large amorphous expansion in PVLG.

In this paper, we analyzed the usefulness of observing the ultrastructure and physical reactions of wall layers in crushed glomerospores. A deeper analysis of the beaded and amorphous walls is also presented. Finally, based on the ultrastructure of *Acaulospora tuberculata*, it is demonstrated the occurrence of a sandwich-like inner wall (endospore). This last character might be shared by many

other taxa of Acaulosporaceae and *Scutellospora,* where three instead of two wall layers, as reported elsewhere, constitutes the innermost germinal wall indeed.

MATERIALS AND METHODS

Origin of the accessions of *Acaulospora tuberculata* and spore extraction

Two accessions of *A. tuberculata* were analyzed: (a) accession CNPAB 17 Embrapa-Agrobiologia (Rio de Janeiro, Brazil) and (b) accession BR 103-6 from INVAM (West Virginia, USA) were obtained among 1999 and 2000, from both mentioned sites.

Ultrastructural analysis

Glomerospores were extracted from soil by wet sieving and decanting followed by centrifugation in a 2M sucrose – water density gradient (Sieverding, 1991). The spores were then mounted in distilled water on a glass slide under a coverslip, and carefully pressed down to be crushed; still under the coverslip, they were left to dehydrate at room temperature. After 2h, the glomerospores were carefully taken from the glass slide and fixed 24h at room temperature in Karnovsky's (2% glutaraldehyde plus 2% formaldehyde prepared from paraformaldehyde in 0.2 M sodium cacodylate buffer). After that, spores were washed, post-fixed in 1% osmium tetroxide, rinsed with the same buffer, and dehydrated in a distilled water-ethanol series (15, 25, 35, 50, 75, 95 and 100%, 1h each bath). The next step was embedding them in resine-ethanol baths to pure resin (acrilic resin, LR White medium grade, 4:1, 3:2, 2:3, 1:4, and pure resin, two times each bath, 1 hour each). After embedded, the glomerospores were kept for 18 hours in gelatine capsules for polymerization at 65°C.

Ultra thin sections were stained with Toluidine Blue and observed at a TEM (Philips CM 100 electron microscope at 60 kV) and in a compound microscope (Olympus BH2). The ultrastructure of wall components was analyzed and general characteristics of the spore wall layers were identified, using thin sections at magnifications ranging from 7 000 to 85 000 times.

Ultrastructural analysis of *Acaulospora tuberculata* pot-cultured at INVAM

Spores of *Acaulospora tuberculata*, BR 103-6 were recovered from a trap culture established at the INVAM (International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi, University of West Virginia, Morgantown) as described previously. After extraction, approximately 150 spores were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7) overnight. Later the spores were separated from the glutaraldehyde solution, washed three times (each 10 min.) with pure cacodylate buffer followed by fixation with 1 % osmium tetroxide (OsO_4) during 2h Next step consisted in eliminating the OsO_4 solution and washing three times (each 15 min.) with iced distilled water.

Dehydration proceeded using an ethanol series (50, 70, 80, 90 and 100%, 30 min. each bath), followed by incubation in propylene oxide (100%) during 30 min. Spores were subsequently embedded in propylene oxide: resin series (1:1 and 2:1, each during 2h) followed by pure resin Med Cast for 24h. Finally, the spores were transferred to capsules holding the pure resin for polymerization at 60°C during 48 hours.

Blocks were thin sectioned (60 µm) on an ultra-microtome. The sections were collected on copper grids (300 µm mesh), post-stained for 40 minutes in 2 % (w/v) aqueous uranyl acetate plus 5 min. in Reynolds (1963) lead citrate, and observed on a Philips CM 100 electron microscope at 60 kV.

RESULTS

Ultrastructure of the accession *Acaulospora tuberculata* CNPAB17:

The dehydration of crushed spores allows the spores to be clean off the whole cytoplasm so that the spore oily material do not interferes with the action of the resin embedding in the wall components. TEM analyses corresponding to *A. tuberculata* spores CNPAB 017 accession are showed in Figures 1 and 2. We mention wall layers components in Arabic numbers to make clear the explanations. The outermost layer 1 remains surrounding the tubercles and it is electronically translucent. Indeed, the ornamentation of *A. tuberculata* seems to be composed by almost capitates, centrally empty projections that somehow interwove, and in a transverse view of the spore seems to be composed by straight to curved bacula-like or clavae-like structures (**Fig. 1a**). When separately analysed, projections are regularly polygonal at their apex, measuring 1.0 – 1.5 µm width. However, below the apex they are extremely irregular and thin, presenting bases 0.5 – 0.8 µm width.

Layer 2 seems to be constituted by two indivisible regions A and B (**Fig. 1a**). The whole layer (SW-L2) is electronically intermediate. The outermost half (A) is amorphous and the second half (B) inwards is formed by lamellae (about 8) each one being constituted by apparently arched fibrils, which are also an artefact. In region B, this lamellae tend to be thinner outward and inward (**Figs. 1a** and **1b**), with the central ones being thicker. In light microscopy (LM), SW 2 is commonly brown and we were not able to observe its sub-layers or laminations.

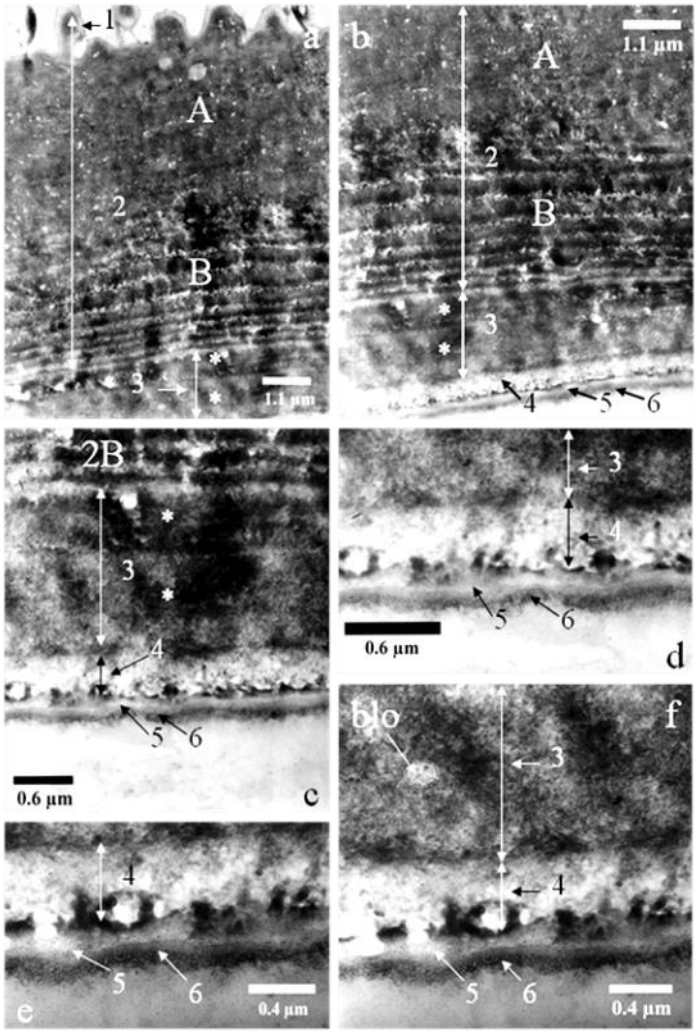

Figure 1. Ultrastructure of the spore wall layers of *Acaulospora tuberculata*, accession EMBRAPA, Rio de Janeiro. a to c, Layers 1 to 6: in a the surface projections appear surrounded by layer 1; layer 2 show the granulose and amorphous region A and the lamellate region B; note in B that lamellae are thicker to the central zone of the region; component 3 show its two lamellae (marked with asterisks) showing a certain longitudinal organization of fibrils or being amorphously disorganized; layer 4 appear as desegregated amorphous granules due to spore crushing; layer 5, being electron translucent is subjacent to layer 4; wall material corresponding to layer 6 remains attached to the innermost side of layer 5. d to f, A larger magnification of components 3 to 6 is showed.

Figura 1. Ultraestructura de la capas de la pared de la espora de *Acaulospora tuberculata*, cepa de la EMBRAPA, Rio de Janeiro. a to c, Capas de la 1 a la 6: en a, la superficie de las proyecciones aparece rodeada por la capa 1; capa 2 muestra la región granulosa y amorfa A y la región laminada B; note en B que las láminas son mayores en la zona central de la región; componente 3 muestra sus dos láminas (marcadas con asterisco) revelando una cierta organización longitudinal de las fibrillas o estando desorganizadas de forma amorfa; capa 4 aparece como gránulos amorfos desorganizados, debido a la rotura de la espora; capa 5 siendo electrónicamente translúcida subyacente a la capa 4; material de pared correspondiente a la capa 6 permanece unido a la cara más interna de la capa 5. d a f Se muestra un mayor aumento de los componentes 3 al 6.

Layer 3 is also electronically intermediate and amorphous (**Figs. 1a** to **1d**, **1f**, **2a** and **2c**). Two inseparable lamellae (marked with asterisks in **Figs. 1a**, **1b** and **1c**) are commonly observed, the upper one, subjacent to layer 2, being thinner. In addition, layer 3 is the innermost in the spore wall. This layer appears to be composed by longitudinal fibrils what supposedly favours its desegregation into two to several false layers when spores are crushed and observed under the LM. However, in the same layer the fibrils may appear amorphously disorganized, showing its fragility.

Subjacent to layer 3 are the remaining components of the endospore-like structure. The outermost endospore layer (4) is a granulose, apparently electronic dense component, which amorphously retract when the spore is crushed and desiccated (**Figs. 1b** to **1f**, and **2a** and **2c**). Next layer inwards is (5) being electronically translucent and seems more resistant than layer 4. Layers 4 and 5 remain in the same group even when spores are mounted in PVLG, crushed, and observed at the LM. In figures where layer 5 is present, a granulose layer, electronically intermediate, is subjacent to its innermost side. As observed below, these granules are probably detached from component 6 during spore crushing.

Figures 2b, **2d** and **2e** show the characteristics of the remaining innermost components. They perhaps constitute the genuine germinal endospore layers. Actually, **Fig. 2b** shows a new report of a TEM image of a "beaded" layer. Apparently, this layer retracts during spore crushing or due to physical or chemical properties of the mounting fluids (light microscopy) or fixation and for embedding reagents (TEM). Apparently, two types of granules constitute the "beads": some electronically dense and mostly located at the bead's surface, and others electronically intermediate and located towards the bead's central core (**Figs. 2d** and **2e**). The physical properties of these two regions, being electronically contrasting and reacting as mucopolysaccharides, are the cause for the retraction of this layer, forming single or clumped beads, which are also able to dislodge out. The word moniliform instead of beaded should be used to mention layers as the one here described.

Layer 7, subjacent to layer 6 is electronically translucent. It is a thick layer being somewhat plastic and probably equivalent to a "coriaceous" wall (**Fig. 2b**). We found an eighth layer, which is really the innermost one. This layer is electronically dense (black) and commonly flaked into pieces with angular edges during cutting of the spores (**Fig. 2b**).

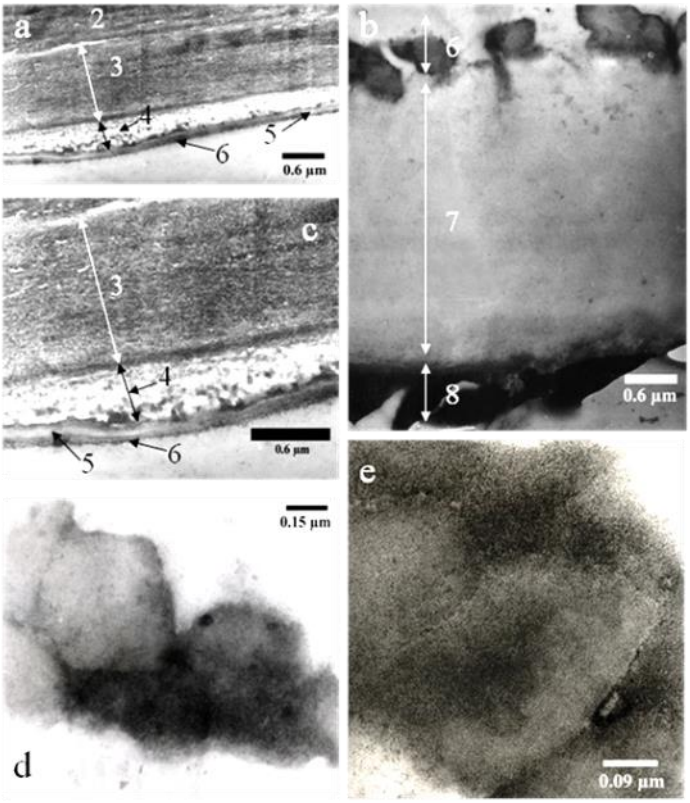

Figure 2. Ultrastructure of the innermost layers of the spore wall of *Acaulospora tuberculata*, accession EMBRAPA, Rio de Janeiro. a to c, Layers 2 to 6: the internal structure of component 3, composed by longitudinal fibrils is showed though lamellae are not observed as in **Fig. 1;** explanation for the remaining components as in **Fig. 1. b**, ultrastructure of the innermost germinal group showing the outermost electronically intermediate moniliform layer 6 being dislodged into beads, the electronically translucent coriaceous and much thicker layer 7 and the electronically very dense innermost layer 8. d and e, ultrastructure of layer 6 beads, showing to be composed by electronically translucent inward and electronically intermediate outward.

Figura 2. Ultraestructura de las capas más internas de la pared de la espora de *Acaulospora tuberculata,* cepa de la EMBRAPA de Rio de Janeiro. De a hasta c, capas de la 2 a la 6: Se muestra la estructura interna del componente 3, compuesto por fibrillas longitudinales aunque las láminas no se aprecian como en la **Fig. 1**; la explicación del resto de los componentes como en la **Fig. 1. b**, ultraestructura del grupo germinal más interno mostrando la capa mas externa moniliforme correspondiente con la capa 6 electrónicamente intermedia, y estando dislocada en "cuentas", la capa 7 coriácea, electrónicamente translúcida mucho más gruesa y la capa mas interna 8 electrónicamente muy densa. d y e, ultraestructura de la capa 6 "arosariada", mostrando estar compuesta por una zona electrónicamente translúcida hacia adentro y electrónicamente intermedia hacia afuera.

Reaction of layers to tolluidine blue stain and wall layer thickness at TEM:

The staining reactions of the spore wall components of *A. tuberculata* to tolluidine blue (TB) are variable as their apparent ultrastructure. The outermost layer 1 stained dark purple, while layers 2 and 3 were light violet when in tolluidine blue. Layer 4, electronically dense stained dark violet with TB, while layer 5, electronically translucent stained light purple. Layer 6, which is moniliform in PVLG and at the TEM seems to be electronically composed by dense and translucent granules, stains light violet, while component 7, the coriaceous layer is light purple. The innermost layer 8, electronically very dense, stains light violet.

The thickness of components 2 to 8 (**Fig 1** and **2**) are (in µm) as follows: 2: 6.88, 3 (both lamellae): 1.24 – 1.63; 4: 0.34 – 0.39; 5: 0.09 – 0.17; 6: 0.78; 7: 3.39; and 8: 0.52. The thickness of component 5 is probably overestimated, as the measurement corresponded to a region distended during spore crushing.

Ultrastructure of the accession *Acaulospora tuberculata* BR103-6

The ultrastructure of layers corresponded with those described for the EMBRAPA's accession of *A. tuberculata*. Ornamentation is formed by hollow to solid projections, which are somehow interwoven. Upper projections appear commonly surrounded by rest of layer 1 (**Figs. 3a** to **3d**, and **4a**). As observed in these figures, wall material of the hollow projections is continuous with the one at region A of component 2, being granulose and electronically intermediate.

As shown in **Figs. 3b**, **4a** and **4b**, 18 to 27 lamellae composed by arched fibrils were found in the region B of layer 2 in the spores belonging to the INVAM's cultotype of *A. tuberculata*. The former number of lamellae is higher that the observed at the same region B of layer 2 in the EMBRAPA's accession.

In both accessions, lamellae in the central zone of region B of the layer 2 were thicker (0.52 – 0.56 µm) and in both inward and outward the central part of the region was thinner (0.13 – 0.17 µm). Arched fibrils might be considered as electronically dense. However, their

bow-shaped structure with separations gives an image electronically intermediate. On the other hand, we do not have an explanation for the occurrence of irregular strips electronically dense (black) commonly subjacent to component 2 (**Figs. 3b**, **4a** and **4d**).

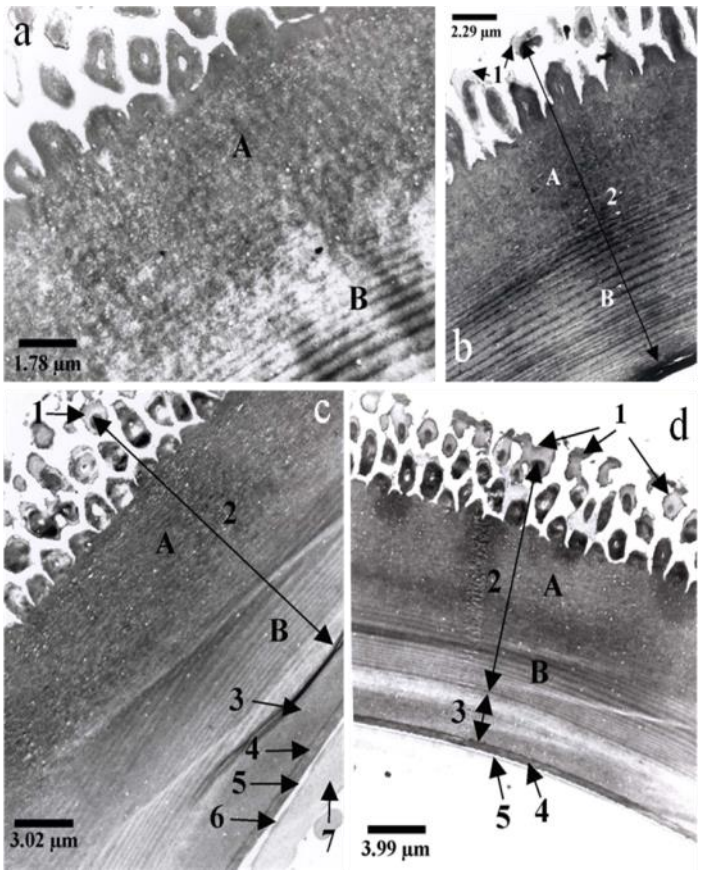

Figure 3. Ultrastructure of the spore wall layers of *Acaulospora tuberculata*, accession INVAM BR 103-6. a and b Hollow projections being surrounded by rest of layer 1 and showing their continuity with the granulose material of region A of layer 2; the lamellar composition of region B in the same component is observed. c and d, Layers 1 to 7 in an apparently immature spore; lamellae in region B of component 2 are generally very thin and arched fibrils are not observed; component 3 do not show its paired lamellar composition but the occurrence of fibrils positioned longitudinally is observed; the innermost components 5 to 6 are very thin and particularly 6 is almost indistinguishable.

Figura 3. Ultraestructura de la capas de la pared de la espora de *Acaulospora tuberculata,* cepa procedente del INVAM BR 103-6. a y b Proyecciones huecas rodeadas por resto de la capa 1 mostrando su continuidad con el material granuloso de la región A de la capa 2; se observa la composición laminada de la región B en el mismo componente. c y d, capas de la 1 a la 7 en una espora aparentemente inmadura; láminas en la región B del componente 2 son generalmente muy finasy no se observan las fibrillas arqueadas; el componente 3 no muestra su composición lamelar apareada, pero se observa la aparición de fibrillas colocadas longitudinalmente; los componentes más internos 5 y 6 son muy finos y el 6 practicamente no se puede distinguir.

The ultrastructure of layer 3 is composed both by fibrils organized longitudinally (**Figs. 3c** and **3d**), and amorphously disorganized fibrils (**Figs. 4a** to **4c**). As showed in these figures, two lamellae can be distinguished in component 3, being the innermost much thicker than the others as in the EMBRAPA's accession are.

Figs. 3c and **3d** show two images belonging probably to an immature spore. Lamellae in region B of component 2 are extremely thin and arched fibrils are not observed. On the other hand, in the same figure the paired lamellae of layer 3 are not observed, and component 6 is almost indistinguishable. The paired lamellae of layer 3 are clearly visible in **Figs. 4b** and **4c**. In **Fig. 4c**, layers 4, 5 and 6 are easily observed, while layer 7 is apparently being formed. **Fig. 4d** show the innermost endospore layers 4 to 7 totally developed in a mature spore. Layer 4 is always much thicker than layers 5 and 6. While layer 4 is electronically intermediate and granulose, layer 5 is electronically translucent, and layer 6 seems to be constituted by the deposition of electronic dense particles. Finally, an innermost layer 7 is electronically translucent and its thickness remembers the coriaceous wall layer.

According to the images in **Figs. 3** and **4**, components 2 to 7 thickness are (in µm) as follows: 2, 14.5 – 19.0 (including ornamentation), 3 (both lamellae), 1.04 – 3.02; 4, 0.29 – 0.70; 5, 0.10 – 0.47; 6, 0.07 – 0.19 and 7, 1.18 – 1.70. A layer 8 was observed in this EMBRAPA's accession, but did not appear in the INVAM's accession from 0.5 – 1.00 µm thick.

Updating the ultrastructure of *Acaulospora scrobiculata*

At the time when Maia and Kimbrough (1993) published their results about TEM analysis of *Acaulospora scrobiculata* the study of glomeromycotan spore wall components were much useful but still inconclusive. In this respect, a significant progress occurred when Morton (web site) made it available a large amount of images showing well-described glomeromycotan spore wall layers and inedited original species descriptions. Thanks to such advance, we can today rebuild the TEM result showed by Maia and Kimbrough (1993) for the spore wall layers of *A. scrobiculata*.

Seven layers as shown in INVAM (2012) constitute the spore wall (SW) of *A. scrobiculata*. These wall layers, from

outside inwards are in the first group: a mucilaginous thin layer (SW-L1); a laminated thicker layer (SW-L2) and a "unit"-like thin layer (SW-L3). In the second group: a first germinal wall, formed by two thin flexible membranes (GW1= L1 + L2), and more internally a second and last germinal wall, composed by a "beaded" layer and an innermost thicker, somehow "coriaceous" layer (GW2, L1 + L2).

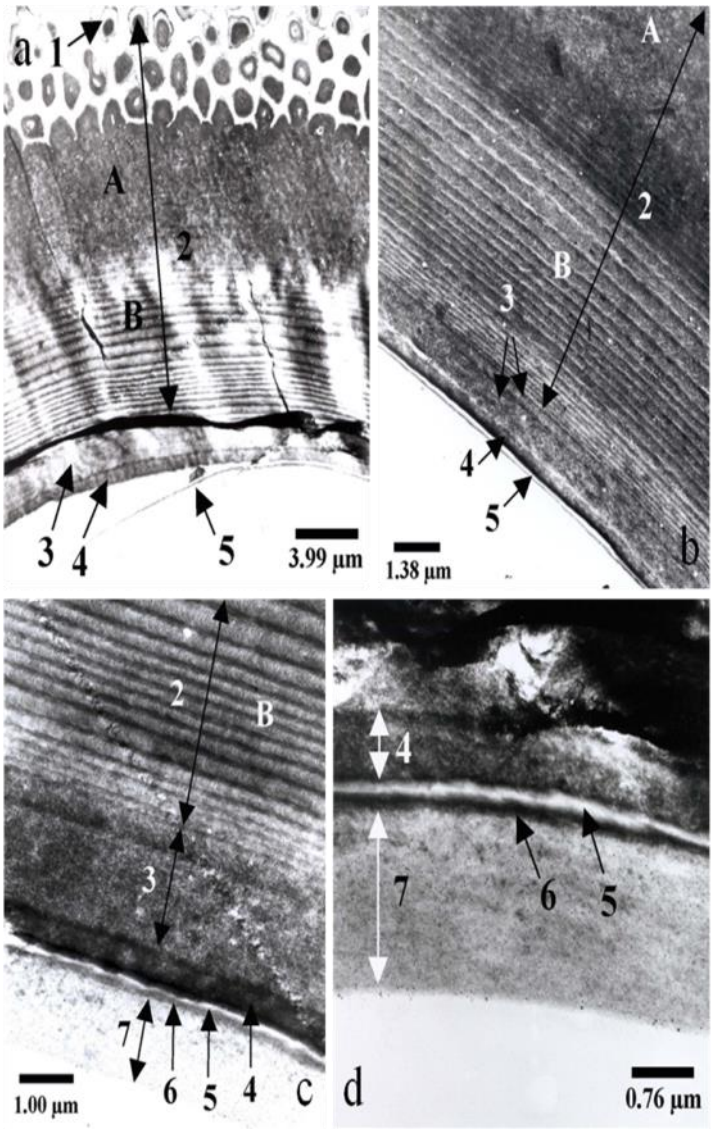

Figure 4. Ultrastructure of wall layers of a mature spore of *Acaulospora tuberculata*, accession INVAM BR 103-6. a, Ultrastructure of components 1 to 5: as in the previous figure, hollow projections ornamenting the spore surface are observed surrounded by rests of layer 1; causes originating the striking black irregular strip are unknown; layer 3 do not show its paired lamellar composition. b, Arched (bow-shaped) fibrils integrating lamellae are observed in region B of component 2; observe the thickness of lamellae in the region B, being thinner outwards, to the contact with region A, and inwards, to the contact with component 3, this last showing its paired lamellar composition and the occurrence of disorganized fibrils (see also 4c). c, Observe components 4 to 6 totally developed, while component 7 is being formed. d, Ultrastructure of the innermost endospore layers in a mature spore; in this case, an innermost electronically very dense layer subjacent to component 7 was not observed.

Figura 4. Ultraestructura de las capas de la pared de una espora madura de *Acaulospora tuberculata*, cepa del INVAM BR 103-6. a, Ultraestructura de los componentes 1 a 5: como en la figura anterior, se observan proyecciones huecas que adornan la superficie de la espora rodeadas por restos de la capa 1; las causas que originan la llamativa franja negra irregular son desconocidas; la capa 3 no muestra su composición laminarr apareada. b, se observan fibrillas arqueadas (en forma de arco) que integran laminillas en la región B del componente 2; observe el grosor de las láminas en la región B, que es más delgada hacia el exterior, al contacto con la región A, y hacia el interior, al contacto con el componente 3, este último muestra su composición laminada apareada y la aparición de fibrillas desorganizadas (véase también 4c). c, observe los componentes 4 a 6 totalmente desarrollados, mientras que el componente 7 se está formando. d, ultraestructura de las capas más internas de la endospora en una espora madura; en este caso, no se observó una capa muy densa más interna electrónicamente subyacente al componente 7.

Maia and Kimbrough (1993) did not refer to SW-L1. However, they mentioned SW-L2 (zone A) as "composed by a series of lamellae (up to 20) with an arched, bow-shaped appearance (Fig. 9). The inner lamellae of zone A are thinner that the outer ones, which suggest that the wall develops new lamellae from the protoplasm outwardly…." "…The arched appearance diminishes gradually towards the inner region of zone A, at which point they are no longer discernible (Fig. 9)." We agree totally about the correspondence between the zone A (component 1) of Maia and Kimbrough (1993) and the component SW-L2 of *A. scrobiculata* spores.

The authors (Maia and Kimbrough, 1993) referred to the assembly of the remaining subjacent layers as the zone B, and mentioned that they were able to identify three layers numbering them 2 to 4. Indeed, zone B, in Figs. 9 and 11 of the mentioned paper, is composed by 5 layers. According to our experience (see below), Fig. 11 shows a miss-interpreted image being the fibrils apparently distributed radially probably an artifact, and the mentioned layer being actually more inwards than the "unit" component subjacent to the SW-L2.

In the upper part **of Fig. 9,** just when the arrowhead of zone A is pointing up, we see the start of the layer SW-L3, which thickness finishes where the arrowhead of zone B is pointing down. Subsequently, upwards in the image (inwards in the spore wall), a light layer (almost white) seems to be GW1-L1 and the next layer (darker in the image) might be the GW1-L2. More inwards, an almost black granulose layer is perhaps the first report of a TEM view of a "beaded" layer (GW2-L1) and the innermost thicker, grayish and granulose layer (numbered 4 in Fig. 9) might be the thicker layer GW2-L2.

If we rebuild the characteristics of the mentioned wall components in the former paragraph, we might tell that: SW-L3 is amorphous, granulose and electronically intermediate; GW1-L1 is electronically translucent; GW1-L2 is electronically intermediate; GW2-L1 is amorphous, granulose and electronically dense; and the innermost GW2-L2 is also amorphous, granulose and electronically intermediate.

On the other hand, according to Morton (INVAM, 2002) the spore wall of *A. tuberculata* is constituted by seven wall layers when observed at light microscopy: SW-L1 (Component 1): Hyaline, 0.9-3.75 µm thick, remains after the tubercles on L2 (below) have been formed in some spores, but it is not observed except from a side view of L2.

SW-L2 (Component 2): A laminate layer that thickens initially by formation of red-brown sublayers followed by synthesis of polygonal (4-5 sides) projections (tubercles) 1.4 – 3.5 µm high and 1.0 – 1.3 µm wide. Total thickness of this layer (sublayers + tubercles) ranges from 8.5 – 11.8 µm (mean = 9.24 µm). At maturity, the pore between the spore and the saccule neck is bridged by sublayers of this layer (without tubercles) resembling an "endospore".

SW-L3 (Component 3): A yellow-brown to red-brown layer, which appears discrete in some spores due to separation from the spore wall, but which otherwise, appears to be inner sublayers of L2. It can be undetected in some spores (completely adherent to L2), appear as a pair of thin adherent flexible layers, or appear as a wide range of layers due to folds, ranging from 0.5-4.0 µm when measurable.

GW1-L1 & GW1-L2 (Components 4 and 5): Two tightly adherent hyaline layers are formed, each measuring 0.5 – 0.8 µm thick. GW2-L1 & GW2-L2 (Components 6 and 7): Two adherent hyaline layers that together measure 5.0 – 10.6 µm when measured in PVLG. L1 is hyaline, 0.8 – 1.8 µm thick with granular excrescences (or "beads") that tend to become dislodged and float away when pressured. L2 is hyaline and somewhat plastic (probably equivalent to a "coriaceous" wall), 1.2 – 2.0 µm thick in PVLG-based mountants.

DISCUSSION

In spite of the two *A. tuberculata* accessions being cultivated under very different conditions, they show very similar wall composition and ultrastructure. As mentioned by Morton (INVAM: http://invam.caf.wvu.edu), the outermost layer 1 remains surrounding the tubercles and at TEM it is electronically translucent. In layer 2, which is formed by two indivisible regions (A and B), while the outermost half (A) is amorphous; the second half of layer (B) is somehow similar to the SW-L2 described by Maia and Kimbrough (1993) for *A. scrobiculata*. Region A of layer 2 at LM tends to be darker than region B when mounted in PVLG, while in PVLG-Melzer occur the opposite (see Morton's web pages).

In layer 3, two inseparable lamellae, commonly observed, are equivalent to SW-L3 and SW-L4 as mentioned by Morton (web pages see above). Subjacent to this layer, are the remaining components of the endospore-like structure (mentioned by Morton as germinal wall layers GW). Layers 4 and 5 remain in the same group even when spores are mounted in PVLG and crushed, like are observed at the LM. They correspond to the inner wall GW1 (L1 + L2) according to Morton (web pages).

The remaining innermost components perhaps constitute the genuine germinal endospore layers. The outermost layer, 6, is the one mentioned by Morton (web site) as being "beaded". Layer 7, subjacent to layer 6, corresponds to that mentioned by Morton, when describing GW2-L2. It is probably equivalent to a "coriaceous" wall. According to Morton this would be the innermost component. However, we found an eighth layer, which is really the innermost one. It is very similar to the innermost ones reported for *Glomus proliferum* (Declerck *et al*., 2000), *R. intraradices* (Rosendahl *et al*., 1994; Maia and Kimbrough, 1993a, 1994), *G. clarum* (Walley and Germida, 1996) and *Glomus* sp. L906 (Maia and Kimbrough, 1993a, 1998). In addition, this eighth layer is similar to the one subjacent to the outermost layer that constitutes the germination tube of *Glomus* sp. L906.

The occurrence of an innermost eighth layer in *A. tuberculata* indeed, we have commonly observed the sandwich-like endospore in many species of Acaulosporaceae and *Scutellospora*. This sandwich-like endospore always holds a thin, coriaceous (limited expansion) to amorphous (large expansion) layer, surrounded by a moniliform (beaded) layer (which may

also appear as a straight or sinuous membrane) and enclosing an innermost thin membrane which may appear straight, folded or wrinkled.

We believe that these innermost layers are the called germinal walls being probably similar to the germinal membranes appearing in *Gigaspora* spp. just before germination. The germinal membrane (layer 8) of *A. tuberculata* is not always present, as commonly occurs in *Acaulospora denticulata*, *Quatunica erythropus* (Koske & C. Walker) F.A. Souza, Sieverd. & Oehl and others (personal observations based on LM and TEM).

In addition, if the former hypothesis is true, one might suppose that in *R. intraradices* and probably also in *G. clarum*, the innermost laminae being one to several successively alternate in TEM images, with alternate electronically dense and intermediate sublayers (Rosendahl *et al*., 1994; Maia and Kimbrough, 1993a, 1994). Perhaps the number of electronically dense (black) layers in *R. intraradices* represents the number of times that spore prepared for germination and stopped it due to environmental factors. If this phenomenon is true, we might suppose that it is functionally and genetically similar to the ability of germ tubes to form septa and retract the cytoplasm when host roots are not available (Giovannetti *et al*., 2000; Giovannetti and Sbrana, 2001; Logi *et al*., 1998) if well, the capacity of germinate more than once has been only demonstrated in Gigasporaceae.

The fact that this germinal membrane may or not be present, depending upon spore ontogeny and maturity might justify why spores of *D. spurca*, *F. mosseae*, *Claroideoglomus etunicatum* (W.N. Becker & Gerd.) C. Walker & A. Schüssler and other taxa an innermost membrane is not always observed. This last argument would explain why Pfeiffer *et al.* (1996) described *D. spurca* with an innermost membrane, while Kennedy *et al.* (1999) emended the description to eliminate it.

What is apparently accurate is that species like *Paraglomus occultum* (C. Walker) J.B. Morton & D. Redecker, *P. brasilianum* (Spain & J. Miranda) J.B. Morton & D. Redecker, *G. aggregatum* N.C. Schenck & G.S. Sm., *G. microaggregatum* Koske, Gemma & P.D. Olexia, and some others show a relatively thick innermost membrane which is not as flexible and thin as the ones showed by, e.g. *D. spurca*, but they are as thick as one of the laminae in *R. intraradices* and *G. clarum*.

Considering that through TEM analysis was demonstrated that they might be considered as germinal layers- independently from their electronic density we proposed that glomeromycotan spores might be actually considered as monospored sporangia as shown in species of Archaeosporaceae, Acaulosporaceae and Gigasporaceae.

The staining reactions of the spore wall components of *A. tuberculata* to tolluidine blue (TB) are variable. It seems that there is not a high correspondence between staining with TB and the electronical density of a layer. Further research is needed to identify the biochemical components of the wall layers.

Considering Morton's description (web site) the thickness of components 2 to 7 are (in µm) as follows: 2: 8.5-11.8 (mean = 9.24); 3: (both lamellae), 0.5 – 4.0; 4 and 5, each: 0.5 – 0.8; 6: 0.8 – 1.8; and 7: 1.2 – 2.0, though 6 and 7 might reach 5.0 – 10.6. Thickness of wall layers 3, 4 and 5 in our measurements are under the limits given by Morton (web site) for the same components and we might conclude that it is probably due to the higher resolution in TEM than in light microscopy. Layer 2 was also under the limits described by Morton (web site).

The number of lamellae found in the region B of layer 2 in the spores belonging to the INVAM's cultotype of *A. tuberculata* is higher that the observed at the same region B of layer 2 in the EMBRAPA's accession. However, we believe that this difference might be explained by the genetic variability among strains of a same species or different spores belonging to the same strain and/or population.

When compared with the EMBRAPA's accession, the INVAM spores did not show an innermost electronically dense layer. However, we think that absence of this last layer is due to the spore inmaturity. According to our experience this layer subjacent to coriaceous and amorphous components in Acaulosporaceae and *Scutellospora spp*. spores, is not always present, but it is a fixed character. It is probably the germinal layer, which will constitute the future germ tube wall. The fact that this last innermost layer may or may not be present suggests that it is probably the same phenomenon occurring in *Gigaspora* spp. spores, where the germinal membrane showing germinating papillae is not always present. Besides, the occurrence of a thin innermost layer being always subjacent to coriaceous or amorphous components suggests that both, coriaceous or amorphous, should be deeply studied in order to determine their exact composition , i.e. they are probably the same wall layer type. We observed species, e.g. *Entrophospora kentinensis* with populations that produce spores showing amorphous layers in their innermost germinal group that sometimes do not expand in PVLG and appear to be coriaceous.

As previously mentioned, thickness of layer 2 was over the limits, while thickness of layers 3 to 7 corresponded with the limits reported by Morton (web site). A layer 8, present in spores of Morton's accession, was not observed in the EMBRAPA's accession probably because this layer is formed just before germination. Therefore, it is not always observed in the spores. This layer has been observed many times even for *A. tuberculata* and many other taxa of Acaulosporaceae and *Scutellospora*. Several examples can be observed at Morton's web site, where the eighth layer of the spore wall is commonly subjacent to the seventh one and reacts much darker than other layers when Melzer´s reagent is used.

Regarding the ultrastructure of *Acaulospora scrobiculata*, at the time when Maia and Kimbrough (1993) published their results about TEM analysis of spores of this species, the study of glomalean spore wall components were much useful but still inconclusive. In this respect, a significant progress occurred when Morton (web site) made it available a large amount of images showing well described glomeromycotan spore wall layers and inedited original species descriptions. Thanks to such advance we can today rebuild the TEM result showed by Maia and Kimbrough (1993) for the spore wall layers of *A. scrobiculata*.

Seven layers as shown in INVAM (2012) constitute the spore wall (SW) of *A. scrobiculata*. Maia and Kimbrough (1993) did not refer to SW-L1. However, they mentioned SW-L2 (zone A) as "composed by a series of lamellae (up to 20) with an arched, bow-shaped appearance (Fig. 9). The inner lamellae of zone A are thinner that the outer ones, which suggest that the wall develops new lamellae from the protoplasm outwardly…." "…The arched appearance diminishes gradually towards the inner region of zone A, at which point they are no longer discernible (Fig. 9)." We agree totally about the correspondence between the zone A (component 1) of Maia and Kimbrough (1993) and the component SW-L2 of *A. scrobiculata* spores.

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In the upper part of Fig. 9, just when the arrowhead of zone A is pointing up, we see the start of the layer SW-L3, which thickness finishes where the arrowhead of zone B is pointing down. Subsequently, upwards in the image (inwards in the spore wall) a light layer (almost white) seems to be GW1-L1 and the next layer (darker in the image) might be the GW1-L2. More inwards, an almost black granulose layer is perhaps the first report of a TEM view of a "beaded" layer (GW2-L1) and the innermost thicker, grayish and granulose layer (numbered 4 in Fig. 9) might be the thicker layer GW2-L2.

If we rebuild the characteristics of the mentioned wall components in the former paragraph we might tell that: SW-L3 is amorphous, granulose and electronically intermediate; GW1-L1 is electronically translucent; GW1-L2 is electronically intermediate; GW2-L1 is amorphous, granulose and electronically dense; and the innermost GW2-L2 is also amorphous, granulose and electronically intermediate.

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