Storage and secretion processes in the spore of *Gigaspora margarita* Becker & Hall as revealed by high-pressure freezing and freeze substitution

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SUMMARY

Spores of *Gigaspora margarita* Becker & Hall, an arbuscular mycorrhizal fungus, were cryofixed using high-pressure freezing and prepared for transmission electron microscopy by freeze substitution. The cryotechniques improved the ultrastructural preservation of the spore cytoplasm and revealed storage and secretion processes in the germinating spores of *G. margarita*. The storage structures in the hydrated and/or germinating spores have been identified as belonging to three different classes: (i) lipid droplets limited by a membrane, (ii) protein-like bodies inside specialized vacuoles, and (iii) glycogen particles. During germ-tube differentiation, an abundant membranous system was observed. It consists of rough ER, tubules and balloon-like Golgi equivalents, which produce vesicles associated with electron-dense granules. The granules seem to be incorporated into the wall. The results suggest that secretion and storage occur in the fungal spore during events comparable to those in yeasts or plant cells.

Key words: Spore, arbuscular mycorrhizal fungi, high-pressure freezing, freeze substitution, membranous system.

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi are ancient fungi, the origin of which has been dated about 350 million years ago (Simon *et al.*, 1993). They live in close association with the roots of many land plants and accomplish their life cycle by the establishment of a symbiotic relationship which provides them with a continuous flow of nutrients (Bonfante & Perotto, 1992). In addition to the intraradical structures, AM fungi produce spores which develop outside the host plant. They are of paramount importance for the survival of the species and for the completion of the fungal life cycle.

Spore morphology is the foundation of AM fungal taxonomy. The recent creation of the new order of Glomales (Morton & Benny, 1990) and the list of 130 species therein (Walker, 1992) are principally based on the spore structure. However, studies providing

detailed morphological observations are limited (Mosse, 1970; Sward, 1981*a, b*; Bonfante & Bianciotto, 1994 for a review) because good preservation of samples for EM observations (Meier & Charvat, 1992; Maia, Kimbrough & Erdos, 1993) is difficult: complex and thick cell walls rich in hydrophobic substances (Bonfante & Vian, 1984) seem to create a barrier to the fixative.

The present paper investigates the different types of storage structures, as well as the vesicle populations, in hydrated and germinating spores of *Gigaspora margarita* Becker & Hall, after high-pressure freeze fixation. This experimental approach has already been demonstrated to be superior to chemical fixation in the preservation of parasitic structures (Welter, Muller & Mendgen, 1988).

Figures 1–4. Morphology of *Gigaspora margarita* spores 15 d after hydration.

Figure 1. Light microscope section. The wall (W) is laminated, while the cytoplasm is filled with lipids (L) and vacuoles with protein bodies (arrows). Bar = 30 μm . **Figure 2.** Blue Coomassie staining clearly reveals protein bodies (arrows) inside the vacuoles (V) in light microscope sections. L, lipids. Bar = 10 μm . **Figure 3.** Ultrathin section showing the contact area between the germination plate (GP) and the remnants of the storage area. Vacuoles are electron-transparent. Bar = 5 μm . **Figure 4.** Ultrathin section through the germination plate. Many nuclei (N) are present, surrounded by a dense cytoplasm rich in ribosomes and membrane systems. Bar = 5 μm .

Figures 5–6. Ultrastructural features of storage structures. Bars = 1 μ m.

Figure 5. Lipid globules (L) are present as electron-transparent bodies, while small vacuoles (V) possess granular contents. The clear regions (G) are identified as glycogen masses on the basis of their staining properties (cf. Fig. 11). **Figure 6.** Lipid globules at higher magnification (L). Endoplasmic reticulum membranes and ribosomes are close to the lipid membrane (arrows). The vacuoles (V) contain electron-dense bodies.

MATERIALS AND METHODS

Fungal material

Spores of *G. margarita* Becker & Hall (HC/Fungi E10) were recovered from pot cultures of *Trifolium repens* L. by wet sieving (Gerdemann, 1963), stored at 4 °C and sterilized before use. The isolated spores were surface-sterilized with 4% chloramine T and 300 ppm of streptomycin for 30 min, and rinsed with sterile distilled water twice for 10 min. They were kept in distilled water (pH 6.5) in Petri dishes (10 spores per dish) at 30 °C in the dark for hydration. A high percentage (70–80%) of germination was obtained within 15 d.

Cryofixation and freeze substitution

Spores of *Gigaspora margarita* were vacuum-infiltrated in 20 mM MES buffer solution (pH 5.5) containing 2 mM CaCl_2 , 2 mM KCl and 0.2 M sucrose 15 d after hydration.

Single samples were then placed in an aluminium

holder as described in Mendgen *et al.* (1991) and immediately frozen at high pressure using the Balzer HPM 010 apparatus (Muller & Moor, 1984). Frozen samples were stored in liquid nitrogen until further processing. The medium for the freeze substitution consisted of 4% osmium tetroxide in acetone which had been dried by molecular sieve. Samples were freeze substituted in this medium from –90 °C to 0 °C over 3 d, changing the substitution medium once at –20 °C. Subsequently the samples were washed 3 times in dry acetone, slowly raising the temperature to +10 °C.

Samples were infiltrated with an Epon/Araldite–acetone mixture from 5% to 100% resin over 4 d while raising the temperature to +20 °C. Samples were then placed in fresh resin and polymerized at 60 °C for 10 h.

Transmission electron microscopy

Thin sections were stained for 20 min in uranyl acetate followed by 10 min in basic lead citrate. Some sections were subjected to the periodic acid–

Figures 7–10. Ultrastructural features of the storage structures.

thiocarbohydrazide-silver proteinate test (PATAg) for a general visualization of polysaccharides (Roland, 1978). The preparations were examined with a Philips CM 10 transmission electron microscope operated at 80 kV.

Staining for light microscopy

Sections. Sections cut for light microscopy (0.5–1.0 μm) were stained routinely with 1% toluidine blue O in 1% aqueous sodium tetraborate for general histological observations.

Proteins. Coomassie brilliant blue (0.25%) in 7% acetic acid for 10 min at 50 °C (O'Brien & McCully, 1981) was used. Controls involved incubating sections in 0.5% pepsin in 0.1% HCl at 60 °C for 48 h, rinsing well with distilled water, and staining as above.

Polysaccharides. Semithin sections were treated with 1% periodic acid for 10 min followed by Schiff's reagent for 30 min. Controls consisted of omitting periodic acid prior to staining.

RESULTS

Spores exhibit a thick laminated wall. During the hydration phase the cytoplasm redistributed into storage areas rich in lipid droplets and vacuoles surrounded by smaller regions containing nuclei and mitochondria (Figs 1, 2). In the course of germ-tube development, a dense cytoplasmic area (the germination plate, according to Sward, 1981*a, b*) became evident. It was rich in nuclei and was clearly separated from the storage zone, mostly consisting of electron-transparent vacuoles (Figs 3, 4).

In spores where storage areas were predominant, lipid droplets (from 1 to 7 μm) were found as discrete electron-transparent organelles limited by a very thin membrane (Fig. 5). They were often associated with endoplasmic reticulum (ER) membranes decorated by numerous ribosomes as well as with pleiomorphic vacuoles (Fig. 6).

Vacuoles ranged in size from minute (0.30 μm) to huge (13 μm) as well as in content, which was finely granular (Figs 5, 6) or homogeneously dense (Fig. 7). The vacuoles were limited by the tonoplast and often contained bacteria-like organisms (Figs 8, 9), isolated or in groups. Some small vacuoles contained membrane remnants, while others displayed compact electron-dense bodies embedded in a dense matrix. These organelles were morphologically similar to the protein bodies of the storage tissues in

vascular plants (Figs 6, 9) and were strongly stained by the Coomassie blue. They very often presented a black core.

Some vacuoles had a granular content which condensed to form a central electron-dense body (Fig. 6). In some smaller vacuoles the electron-dense body filled up the whole lumen (Fig. 10). By contrast, in the spores with a long germ tube, the number and the size of the protein-like bodies seemed to decrease and the vacuoles were often electron-transparent (Fig. 3).

Between the lipid globules and the vacuoles, some electron-transparent particles were observed (Fig. 5). After the PATAg reaction, they appeared as huge glycogen masses organized in rosettes (Fig. 11). In the light microscope, these areas were PAS-positive (results not shown).

In spores with a clearly defined germination plate (Figs 3, 4), nuclei with condensed electron-dense chromatin were observed (Fig. 12). The most striking feature in this area was the abundance of ribosomes, often associated with the endoplasmic reticulum (ER) membranes (Figs 13–15). In addition, a complex membranous system consisting of tubules and ballooned vesicles with a strong electron-dense content (Fig. 15) formed a network within the germination plate. The vesicles appeared to be associated with an abundant population of electron-dense granules (Fig. 13). Granules with a similar morphology were usually seen near the thick and laminated wall, which constantly presented an electron-dense layer between the inner and the outer wall (Fig. 16). The granules seemed to be incorporated into the electron-dense layer (Fig. 16 and inset).

DISCUSSION

This study describes specific processes of differentiation in the germinating spores of *Gigaspora margarita*. They include the building up of storage molecules, vesicle production and secretion events. High-pressure freezing and freeze substitution were used to improve the fine structure of the involved organelles. Previous reports have provided information limited to cell-wall architecture (Bonfante & Bianciotto, 1994 for a review of data), while only a few have investigated the cytoplasmic structures (Meier & Charvat, 1992; Maia *et al.*, 1993). According to these last authors, an adequate preservation of the spore cytoplasm or organelles has not yet been achieved. So far the best results date back to Sward (1981*a, b*), who treated spores with 0.5%

Figure 7. Massive, electron-dense vacuoles (V) are surrounded by smaller lipid globules (L). Bar = 1 μm . **Figure 8.** Group of bacteria-like organisms (B) occurs inside a vacuole (V). The bacterial wall is stained by the PATAg reaction. T, tonoplast. Bar = 0.3 μm . **Figure 9.** An electron-dense body (P) and a rod bacterium-like organism (B) are lying together inside a vacuole. Bar = 0.3 μm . **Figure 10.** An electron-dense body filling the lumen of a vacuole. The protein-like body (P) is surrounded by numerous ribosomes. Bar = 0.3 μm .

Figures 11. A massive aggregation of glycogen (G) is stained by the PATAg reaction, revealing the typical rosette organization. Bar = 0.5 μ m. **Figures 12.** Two nuclei close together (N) in the germination plate. Bar = 0.5 μ m.

sodium hypochlorite before fixation. In his detailed study he described the cytoplasm of *Gigaspora margarita* as a fine network of granules and particles interlaced between large oil droplets.

In the present study the storage structures in the hydrated and/or germinating spores can be identified as belonging to three different classes: (i) lipid droplets limited by a membrane, (ii) protein-like bodies inside specialized vacuoles and (iii) glycogen particles.

Lipids correspond to the oleosomes consisting of triglycerides and phospholipids and often found not only in fungal spores (Weete, 1981), but also in seeds and pollen grains. By contrast, the presence of electron-dense bodies, which are stained by Coomassie blue revealing a proteinaceous nature and which are morphologically similar to the protein bodies of higher plants, is less usual. Turnau, Kottke & Oberwinkler (1993) found electron-dense bodies with a low P content and a high N composition in *Paxillus involutus* (Batsc.: Fr.) Fr. during the mycorrhizal phase and suggested that they could be protein bodies. Electron-dense globules which were labelled by a fucose-gold probe were found in

Endogone pisiformis Link. hyphae (Jabaji-Hare & Charest, 1987): however, the meaning of the finding was not discussed. Vacuoles as reserve structures are very common in seed tissues, where protein bodies are found in the parenchyma cells of endosperm or in cotyledon cells. The following events are traditionally identified in a seed parenchyma cell: storage proteins are synthesized on the rough ER, transported to the Golgi apparatus, packaged into Golgi-derived vesicles and transported to the vacuole/protein body compartment. After fusion with the tonoplast, the vesicle content is discharged into the vacuolar lumen (Vitale & Chrispeels, 1992). The morphological similarities between the plant protein bodies and those observed in *Gigaspora* suggest that a pathway of protein secretion involving vacuoles is active in AM fungi, as already demonstrated in yeasts (Valls, Winther & Stevens, 1990). However, nothing is known about spore-specific proteins in AM fungi and therefore the absence of specific probes prevents, at least for the moment, the carrying out of immunocytochemical localization experiments.

In the spores of *G. margarita*, vacuoles with other

Figures 13-16. Ultrastructural features of secretory structures in the germination plate. Bars = 0.3 μ m.

roles may also exist: some of them contain bacteria-like organisms (BLOs) with a rod shape and with a thick wall. Similar micro-organisms are commonly associated with AM fungi (Scannerini & Bonfante, 1991 and references therein; Meier & Charvat, 1992; Schüßler *et al.*, 1994). By using high-pressure freezing and freeze substitution, their appearance slightly changes. Following chemical fixation BLOs have been described as lying within the cytoplasm; in contrast, our findings point to their vacuolar location, often associated with the protein-like bodies. This location could be the result of better membrane preservation or alternatively perhaps suggests the presence of two different bacterial populations living inside the spores of AM fungi (Scannerini & Bonfante, 1991).

Notwithstanding their pleiomorphic features, all the vacuoles possess a slightly granular content. This morphology has been described as a characteristic ultrastructural feature of the vacuoles in freeze-substituted fungal cells (Hoch, 1991; Orlovich & Ashford, 1993).

Lipids and protein-like bodies make the spores of *G. margarita* very similar to a plant storage cell, but a feature unique to the spores is the presence of glycogen. In the more advanced stages of germination, when some of the reserves are mobilized, glycogen becomes more important, as also occurs in rust fungi (Mendgen, 1973).

In addition to the various vacuoles, a Golgi-equivalent was also detected in this study in the area corresponding to the germination plate and previously identified by Sward (1981a, b) within the spore. The interconnected system of tubules and ballooned vesicles with a strong electron-dense content can be readily equated with a Golgi system. To the best of our knowledge, dictyosomes have never been described in AM fungi. The structures seen in *G. margarita* are similar to those described as Golgi-equivalents in *Uromyces appendiculatus* (Pers.) Unger (Welter *et al.*, 1988; Hoch, 1991). Accumulation of their associated dark electron-dense granules in rows along the plasma membrane suggests incorporation into the cell wall. Similar events have been observed in the lobes of *Micrasterias denticulata* Breb. (Meindl, Lancelle & Hepler, 1992). We suggest that the electron-dense granules contain melanin in addition to other wall constituents (Gripiolo & Bonfante, 1984). This material has been demonstrated to produce an electron-dense layer which reinforces the spore walls of *Glomus versiforme* (Karst.) Berch (Gripiolo &

Bonfante, 1984), and to play a role in defence against soil micro-organisms.

We conclude that the AM spore is a unique cell. Two areas with different roles can be identified in this huge spore (200 μm): the first is rich in nuclei in mitosis (Sward, 1981b; Becard & Pfeffer, 1993) as well as in membranous systems. The second consists of rough ER, tubules and balloon-like Golgi equivalents producing vesicles and granules directed towards the wall. The second area can be described as a storage compartment, where lipids, protein bodies and glycogen accumulations are found.

These observations reflect an intense metabolism; the spore of an AM fungus produces energy which supports mitosis and growth of the germinating mycelium (Bianciotto & Bonfante, 1993). This is the basis for the subsequent differentiation of infection structures and penetration into the host.

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Figure 13. Low magnification showing vacuoles with granular contents (V), abundant polysomes, labyrinthine membranous systems (M) and electron-dense granules (DG). **Figure 14.** Membrane systems (M) are saccate with electron-dense contents. **Figure 15.** Close juxtaposition between the membrane system and the electron-dense granules (DG). **Figure 16.** Electron-dense granules are seen near the membrane, closely related to the thick wall (W), characterized by an electron-dense layer (asterisk). Inset: the contact between a granule and the electron-dense layer. N, nucleus.

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