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Branched absorbing structures (BAS): a feature of the extraradical mycelium of symbiotic arbuscular mycorrhizal fungi

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SUMMARY

The present work describes the morphogenesis and cytological characteristics of 'branched absorbing structures' (BAS, formely named arbuscule-like structures, ALS), small groups of dichotomous hyphae formed by the extraradical mycelium of arbuscular mycorrhizal (AM) fungi. Monoxenic cultures of the AM fungus *Glomus intraradices* Smith & Schenck and tomato (*Lycopersicum esculentum* Mill.) roots allowed the continuous, non-destructive study of BAS development. These structures were not observed in axenic cultures of the fungus under different nutritional conditions or in unsuccessful (asymbiotic) monoxenic cultures. However, extraradical mycelium of *G. intraradices* formed BAS immediately after fungal penetration of the host root and establishment of the symbiosis. The average BAS development time was 7 d under our culture conditions, after which they degenerated, becoming empty septate structures. Certain BAS were closely associated with spore formation, appearing at the spore's substending hypha. Branches of these spore-associated BAS (spore-BAS) usually formed spores. Electron microscopy studies revealed that BAS and arbuscules show several ultrastructural similarities. The possible role of BAS in nutrient uptake by the mycorrhizal plant is discussed.

Key words: Arbuscular mycorrhizas, branched absorbing structures (BAS), external mycelium, *Glomus intraradices* Smith & Schenck, monoxenic culture.

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi are obligate symbionts which colonize the roots of over 80% of land plants, improving their nutrition, growth and disease resistence (Harley & Smith, 1983; Smith & Read, 1997). The functionality of the symbiosis is based on a bi-directional nutrient exchange. Carbohydrates, synthesized by the plant's photosynthetic system, are released by host root cells and taken up by the fungus as its major carbon source. In return, soil mineral nutrients acquired by the fungal extraradical mycelium are transported to the plant, where they are released and actively taken up by the root cells. In most plants, the AM fungus colonizes the root cortex intercellularly and intracellularly, developing arbuscules, the main feature of the AM symbiosis inside the root.

Arbuscules are complex, profusely branched structures formed inside the host root cortical cell wall, but outside its protoplast (Alexander *et al.*, 1988, 1989). They are greatly extended apices of the fungal colony within root tissues (Gianinazzi-Pearson *et al.*, 1995), and considered to be a dead-end in the growth of AM fungi inside the plant (Bonfante & Perotto, 1995). Owing to the increased contact surface area, and the fact that almost no physical barrier remains between plant and fungal plasmamembranes at the arbuscular branch level, arbuscules have been proposed as the preferential site for plant-fungal nutrient exchange (Bonfante-Fasolo, 1984; Smith & Smith, 1990). In this sense, arbuscules may be considered as modified haustoria (Gerdemann, 1968) which fulfil the role of C uptake structures. However Gianinazzi-Pearson *et al.* (1991) put forward the

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hypothesis that a certain compartmentation exists in P and C transfer processes, with some carbon acquisition suggested to take place through intercellular hyphae.

Arbuscules are also considered essentials for qualifying a given AM symbiosis as 'functional' (Bécard & Piché, 1989; Gianinazzi-Pearson *et al*., 1995). Gianinazzi-Pearson *et al*. (1995) demonstrated that pea mutants which induce aborted arbuscule formation, lack H^+-ATPase activity in root-cell membranes surrounding the aborted structures. This provides further cytochemical evidence that plant–AM fungal interactions are not functionally efficient in the absence of well developed arbuscules. Moreover, in the absence of arbuscules AM fungi seem to be unable to complete their life cycle (Bécard & Piché, 1989).

Besides the intraradical phase, AM fungi have an extraradical phase comprising a network of hyphae which ramify within the soil, exploring and exploiting it for mineral nutrients. It has been shown that these hyphae actively take up P, N, and several micronutrients (George *et al*., 1992; George, Marschner & Jakobsen, 1995; Harrison & van Buuren, 1995; Bago *et al*., 1996*b*) which are afterwards transferred to the host plant, thus improving its mineral nutrition. In contrast to the relatively abundant information on morphogenesis and hyphal cytology of the intraradical phase, little is known about the specific features of the extraradical mycelium of AM fungi. This is mainly due to the difficulty in designing experimental systems that allow extraradical mycelium development to be followed in a non-destructive manner. Soil-based systems (usually used in AM research) can conceal the development of specific structures associated with extraradical hyphae, making them difficult to study.

Mosse & Hepper (1975) reported the first successful attempt to establish *in vitro* AM fungi–root–organ cultures in agar-based substrates (monoxenic AM cultures) between clover (*Trifolium* ssp.) and the AM fungus *Glomus mosseae*. In their description of the post-colonization events they stated that the extraradical mycelium produced 'a form of branching strongly reminiscent of arbuscules (…) which occurred extensively in some successful cultures'. These 'arbuscule-like branches' (Mosse & Hepper, 1975) have since been observed in other AM monoxenic cultures: transformed carrot (*Daucus carota*) roots with *Gigaspora margarita* (Bécard & Fortin, 1988), *Glomus intraradices* (Mosse, 1988; Chabot, Bécard & Piché, 1992) or *Glomus versiforme* (Declerck, Strullu & Plenchette, 1996); and non-transformed tomato (*Lycopersicon esculentum*) roots with *Glomus intraradices* (Chabot *et al*., 1992; Bago, Azcón-Aguilar & Piché, 1998). Mosse & Hepper (1975) considered it 'interesting (…) that finely branched, thin-walled structures very similar to arbuscules can develop outside the root'. However, to the best of our knowledge, there has been no further investigation into these structures which seem to be, paraphrasing Bonfante & Perotto (1995), a dead-end in the growth of AM fungi *outside* the plant.

Recently, Bago *et al*. (1998) highlighted AM monoxenic cultures as an appropriate tool for studying the extraradical phase of AM. They used this culture technique to study external hyphal morphogenesis, formation of associated structures and developmental dynamics of the AM fungus *Glomus intraradices*. In this work, the authors described the formation of arbuscule-like branches (which they referred to as 'arbuscule-like structures', ALS) along the hyphae.

The aim of the present work was to study, under monoxenic conditions, the morphogenesis and ultrastructural cytology of the above mentioned 'arbuscule-like branches' or 'structures', formed by the extraradical mycelium of AM fungi after establishment of symbiosis. These structures will be now renamed as 'branched absorbing structures', BAS.

MATERIALS AND METHODS

Biological material and culture conditions

The AM fungus *Glomus intraradices* Smith & Schenck (DAOM 197198, Biosystematic Research Center, Ottawa, Canada) was grown either monoxenically with tomato (*Lycopersicon esculentum* Mill. cv. Vendor) roots, or axenically.

Monoxenic cultures. Monoxenic cultures were established as described by Chabot *et al*. (1992). Briefly, 20 ml of sterilized (121 °C, 20 min) minimal (M) medium (Chabot *et al*., 1992) were poured into 9-cm Petri dishes. Non-transformed tomato roots obtained from a previous root organ culture were grown together with small cubes of medium from a previous monoxenic *G. intraradices*/tomato culture. The cubes contained *c*. 50 AM fungal spores, as well as mycorrhizal root fragments and external AM hyphae. Six replicates were prepared. The plates were incubated in the dark at 25 °C for 14 wk.

Axenic cultures. To test the putative ability of *G. intraradices* to form BAS asymbiotically, axenic cultures of the fungus were prepared. Spores of *G. intraradices* were collected from a previous monoxenic culture by means of a Pasteur pipette, and transferred to Petri dishes (9-cm diameter) containing 20 ml of either sterilized water–agar medium (WA treatment, 0·8 % Bacto-Difco® agar in distilled water) or M medium (M treatment). Five replicates per treatment were prepared, each containing five spores. Plates were incubated at 25 °C in the dark for 7 wk.

BAS morphogenesis

Ten BAS and ten BAS which appeared in close association with spores (spore-associated BAS, 'spore-BAS') were chosen at the beginning of their development, in order to study their morphogenesis. Structural and cytological changes were observed every 2 d using a Leitz® Laborlux 12 microscope, equipped with a Wild® photoautomat MPS 55. Different stages of BAS morphogenesis were recorded (Kodak® 160 ISO films).

BAS ultrastructural cytology

Four wk after the beginning of the experiment, one randomly chosen Petri dish from the monoxenic cultures was used to carry out BAS ultrastructural and cytological studies. The fungal material was fixed by filling the Petri dish with a mixture of glutaraldehyde (2%) and formaldehyde (2%) in 0·1 M cacodylate buffer (pH 7·2), and incubating at 4 °C overnight. After fixation, five small agar cubes containing non-septated BAS were cut and rinsed in cacodylate buffer solution for 3–12 h. The buffer was changed three times. Samples were then postfixed with 1% osmium tetroxide in the same buffer for 2 h, dehydrated in ethanol and embedded in Epon® 812. Serial sections of the specimens were taken at different levels along the length of the BAS (see Fig. 4). Observations were carried out with a JEOL® 1200X electron microscope.

Fungal and root growth measurements

At the end of the experiments (14 wk and 7 wk for monoxenic and axenic cultures respectively) total root and hyphal length were measured, and the total number of extraradical fungal structures (BAS and spores) was determined. Measurements were carried out under a Wild M3Z stereomicroscope. Axenic and extraradical monoxenic hyphal development were measured as described by Marsh (1971) using a 2 × 2-mm grid. Root length in monoxenic cutures was measured in a similar way, using a 1 × 1-cm grid. To assess and quantify AM fungal root colonization, roots from monoxenic cultures were removed from the medium and stained (Phillips & Hayman, 1970). Intraradical colonization was estimated by the gridline-intersect method (Giovannetti & Mosse, 1980). Mean confidence intervals were calculated at the 5% significance level.

RESULTS

Monoxenic culture development

One d after transfer to the monoxenic cultures, all roots exhibited a normal growth rate. However, 2–3 d later a reduced growth rate was observed in three of the six replicates. These finally stopped growing *c*. 1 wk after transfer. The roots of the other three replicates developed normally, growing extensively within the medium. This low rate (50%) of successful root growth in monoxenic cultures is in the range usually achieved when working with non-transformed tomato roots (20–60% successful cultures, B. Bago, unpublished; A. Coughlan pers. comm.).

After 1 wk, irrespective of the degree of root development, all monoxenic cultures showed AM fungal growth from the inoculum cubes. These hyphae, which were thick and poorly branched, grew

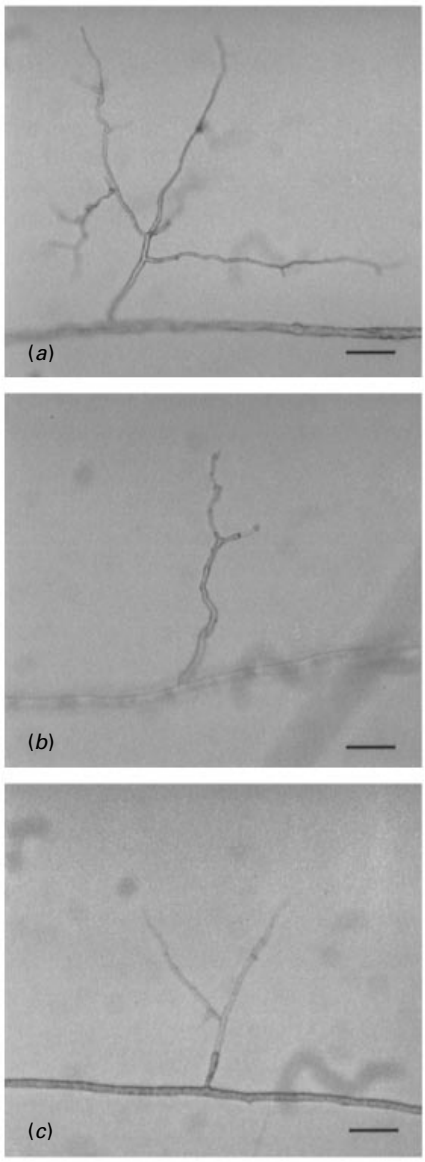

Figure 1. Short ramifications formed by *Glomus intraradices* in the different cultures assayed. (*a*) Dichotomously branched short ramification (branched absorbing structure, BAS) developing on a runner hypha in a monoxenic symbiotic culture. (*b*) Short ramification formed by a runner hypha in a monoxenic asymbiotic culture. (*c*) Short ramification formed by a spore germ tube (axenic culture). Note the aborted dichotomous branching pattern of short ramifications in (*b*) and (*c*). Bars, 50 μm.

Table 1. *Developmental parameters in the different monoxenic and* Glomus intraradices *cultures assayed*

Culture	Total root length (cm per plate)	Root colonization (%)	Extraradical hyphae length (cm cm^{-2})	BAS (no. cm^{-2})	Short branches (no. cm^{-2})	Spores (no. cm^{-2})
Monoxenic						
Symbiotic	71·6 (21·5)[a]	74·6 (10·3)	30·0 (13·3)	246·8 (136·1)	—	78·2 (57·5)
Asymbiotic	27·6 (17·6)	0·0	4·6 (2·9)	0·0	8·7 (5·0)	0·0
Axenic						
M medium	—	—	0·8 (1·1)	0·0	n.d.[b]	0·0
WA medium	—	—	3·9 (1·1)	0·0	n.d.[b]	0·0

[a] Figures in parentheses express the mean confidence interval ($P < 0{\cdot}05$).
[b] Not determined. In axenic culture 'short branches' were rare and irregularly distributed.

mainly around the inoculum cube, producing occasional thick branches, which grew radially into the medium.

Four wk after the beginning of the experiment, monoxenic cultures in which roots continued growing normally (monoxenic 'symbiotic' cultures) exhibited a considerable amount of AM fungal hyphae, which extensively colonized the medium. In these cultures the hyphae showed a growing pattern similar to that described by Bago *et al*. (1998): straight, thick runner hyphae which branched (*c*. 45°) producing progressively thinner runner hyphae. Some of these thinner runner hyphae exhibited numerous short ramifications at intervals of 25–300 μm, which ended in dichotomously BAS (Fig. 1*a*). At this stage of growth *G. intraradices* produced isolated spores.

After 4 wk of culture, Petri dishes in which roots had stopped growing (monoxenic 'asymbiotic' cultures), showed a much poorer AM hyphal development. Thick runner hyphae grew around the inoculum cubes, branching occasionally to produce thick hyphae which grew into the medium. Some of these hyphae displayed short branches at intervals of *c*. 900 μm. These short branches showed an aborted dichotomous branching pattern, with cytoplasmic retraction and septation in the apices (Fig. 1*b*).

In order to compare extraradical and intraradical developmental features of the fungus, roots of one replicate of the 4-wk-old monoxenic symbiotic and of the monoxenic asymbiotic cultures were stained, and fungal colonization ascertained. Monoxenic symbiotic cultures exhibited a 6% intraradical colonization. Pre-infection structures (fan-like structures, Giovannetti *et al*. (1994)) and appressoria were observed on the root surface. Intraradical colonization consisted of intercellular hyphae and coil-like structures. No arbuscules were observed at this stage. In the monoxenic asymbiotic culture neither external hyphal attachments nor internal root colonization were found. After 14 wk, monoxenic symbiotic cultures showed a well developed extraradical mycelium. The main features of the culture are shown in Table 1. Hyphae had intensively colonized the agar medium, and a high density of BAS per cm^2 was observed. Many spores were also found at this stage of development. Root staining revealed heavy fungal colonization (74·6%), with a high density of arbuscules.

By contrast, monoxenic asymbiotic cultures showed a developmental pattern similar to that observed after 4 wk (Table 1). Neither well developed BAS nor fungal spores were found in these cultures. Staining revealed neither internal colonization, nor hyphal attachment to the root surface.

Axenic culture development

After 7 wk of axenic culture, 52% of *G. intraradices* spores had germinated in the water-agar (WA) treatment. By contrast, only 36% of the spores had germinated in the M medium (M). Mycelia produced in both media showed similar developmental features. After 4 wk of culture, germ tubes arising from spores usually showed a localized (2-mm diameter) radial network of hyphae around them. Some straight, thick runner hyphae developed from this, branching occasionally at *c*. 90°, and forming new thick runner hyphae. Short branches were rarely observed on the runner hyphae, however when present they were always close to the spore. Although showing an incipient dichotomous-branching pattern, these short branches stopped growing shortly after initiation, their cytoplasm began to retract and septa appeared in the hyphal tips (Fig. 1*c*).

After 7 wk, *G. intraradices* germ tubes had stopped growing in both treatments, and all hyphal apices were septate. Hyphal development was significantly greater in the WA than in the M medium (Table 1). Total hyphal development was significantly lower when the fungus grew axenically as opposed to monoxenically in the symbiotic cultures, although no significant differences were found between axenic and monoxenic asymbiotic cultures. No BAS or spores were formed in either axenic or monoxenic asymbiotic cultures.

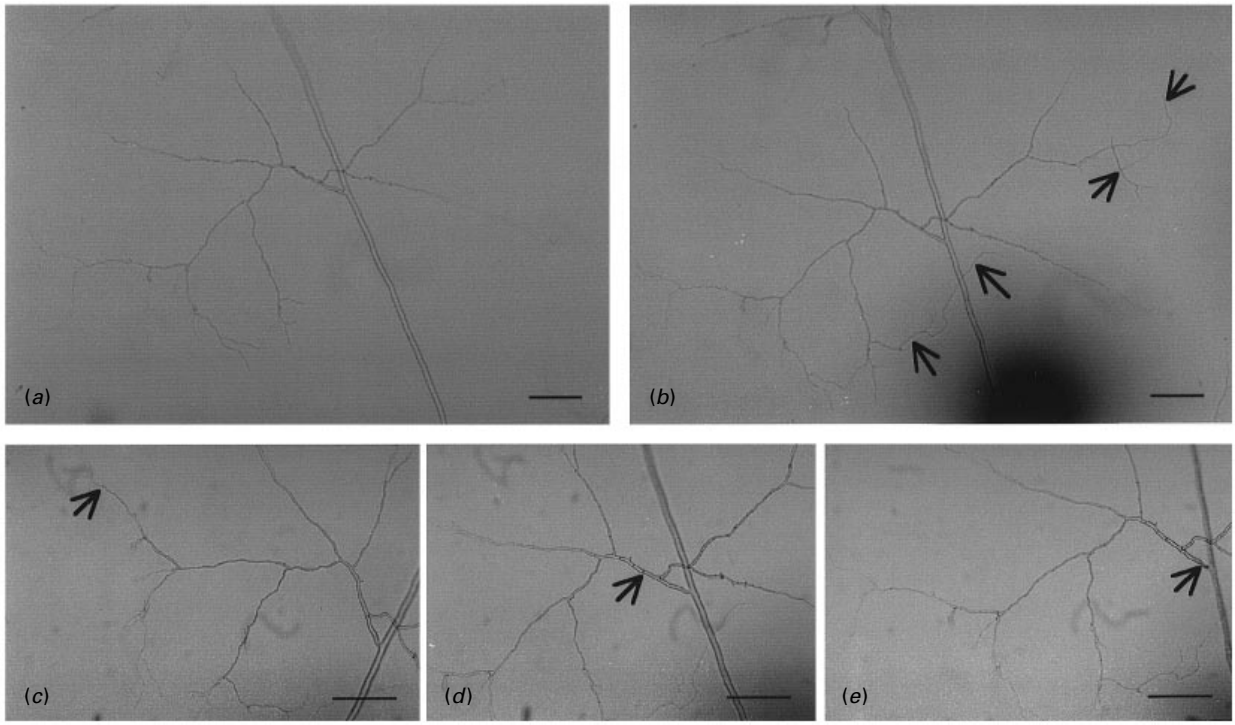

Figure 2. Morphogenesis of a branched absorbing structure (BAS). (*a*) BAS almost completely developed ($t = 0$ d). (*b*) Progressively thinner hyphae are formed through dichotomous branching (arrows) ($t = 2$ d). (*c*) After reaching its full development, BAS enters a degeneration process: the cytoplasm retracts from their thinnest branches, which become septated (arrow) ($t = 11$ d). (*d*) Cytoplasm retraction and septa formation extends to the rest of the BAS branches (arrow) ($t = 24$ d). (*e*) The whole BAS becomes empty and septated, including BAS trunk (arrow) ($t = 65$ d). Bars, 70 μm.

BAS morphogenesis

BAS were formed on either side of thin runner hyphae in monoxenic symbiotic cultures, developing initially as short ramifications which then branched dichotomously, producing progressively thinner hyphae (Fig. 2*a*). BAS formation took *c.* 7 d from the first dichotomous ramification of the short branch to the formation of the thinnest branches (*c.* 1·5 μm, Fig. 2*b*, arrows). When fully developed, no septa were apparent in the BAS. However, shortly after, a continuous process of cytoplasm retraction and septa formation occurred. This process began in the thinnest BAS branches (Fig. 2*c*, arrow), extending progressively to the rest of the branches (Fig. 2*d*, arrow) and finally the BAS trunk (Fig. 2e, arrow). The full process of BAS septation took *c.* 5 wk, although the trunk generally retained its cytoplasm for longer (*c.* 3 months).

Some BAS were associated with spores or groups of spores (Fig. 3*a*). These spore-BAS usually appeared on spore subtending hyphae, and exhibited a longer lifespan than BAS. Spore-BAS remained apparently unchanged for up to 3 wk. Thereafter, they did not retract their cytoplasm, but conserved it until spores started to form on them (Fig. 3*b*, arrow). At the end of this process, spore-BAS branches had thickened, becoming subtending hyphae for groups of spores (Fig. 3*c*).

BAS ultrastructural cytology

Figure 4 shows a schematic view of a representative BAS with the approximate position of the ultrathin sections obtained (Fig. 4, levels 1–5). Photomicrographs of these sections are shown in Figures 5 and 6.

Observations of serial sections of the BAS hyphal tip (Fig. 4, level 1a) revealed the ultrastructural organization of the apical zone of these structures (Fig. 5*a–e*). Total hyphal diameter increased progressively with distance from the hyphal tip (1·5 μm, Fig. 5*a*, to 1·9 μm, Fig. 5*e*). The cell wall consisted of a 50-nm-thick outer wall, which remained constant in thickness and appearance throughout the whole BAS; and an inner wall, which was thick and multilayered close to the hyphal tip (Fig. 5*b*, white arrow), becoming progressively thinner and less complex thereafter (180 nm, Fig. 5*c* to 100 nm, Fig. 5*e*). Initially an electron-dense layer occured between the outer and inner walls (Fig. 5*a*, ML), however this eventually merged into the inner wall (Figs 5*d*, *e*). At the hyphal tip the inner wall displayed some electron-transparent patches (Fig. 5*a*, arrows) and channels (Fig. 5*b* arrows). Some regions of the cell wall started to exhibit a loosely woven fibrillar appearance *c.* 3 μm from the hyphal tip, (Fig. 5*d*, *e*, arrows). Fungal cytoplasm in BAS apical zones was characterized by the presence of

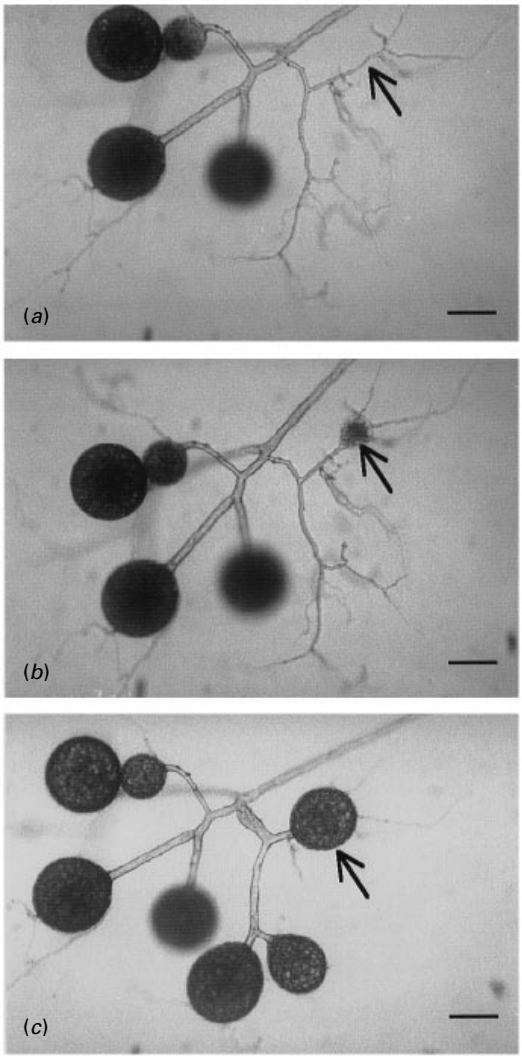

Figure 3. Morphogenesis of a spore-associated BAS (spore-BAS). Arrows indicate the same site on the BAS through time. (*a*) BAS appears associated to the substending hyphae of a group of spores. (*b*) The same spore-BAS 13 d later. A new spore is being formed intercalarly in one of the BAS second-order ramifications (arrow). (*c*) Fifty-four days later each BAS second-order ramification has formed a new spore. Bars, 50 μm.

numerous glycogen deposits and electron-transparent vacuoles (Fig. 5*c–e*). Electron-dense granules, either closely delimited by a membrane (Fig. 5*c*, DG) or inside a larger electron-transparent vacuole (Fig. 5*c*, dg), and membrane curls (Fig. 5*e*) were also evident.

Diameter of third order BAS branches (Fig. 4, level 1b) was $1{\cdot}8 \times 2{\cdot}1$ μm (Fig. 5*f*). At this level cell-wall structure and thickness were similar to that shown by the last sections of BAS apical zones. Deposition zones of possible cell-wall components were noted on the inner wall (Fig. 5*f*, arrow). The cytoplasm contained small vacuoles, an increasing number of mitochondria and a nucleus.

At second order branches (Fig. 4, level 2) the hyphal diameter ranged from 2·0 to 2·5 μm. The loosely woven appearance of the cell wall was evident over the whole perimeter (Fig. 5*g*). Some fibrils extended from the inner and outer walls into the surrounding agar (arrows). The inner wall (120 nm) was single-layered and fibrillar in texture. The cytoplasm was rich in mitochondria, glycogen deposits, polyvesicular bodies, dense granules and small vacuoles. Other vacuoles resembling the membrane-bound crystals described by Sward (1981), were also noted (Fig. 5*g*, Cr).

First-order branches (Fig. 4, levels 3a, 3b; Fig. 6*a*, *b*) exhibited diameters of between 2·5 and 3·0 μm. Their inner wall (200 nm) consisted of two distinct layers (Fig. 6*a*): an electron-dense outer layer (L1) and an innermost layer (L2), which appeared less organized. Deposition of cell-wall material was often observed in the inner wall at this level. Large regions of the outer and inner walls displayed the loosely woven appearence mentioned above. Closer to the first order ramification (Fig. 4 level 3b; Fig. 6*b*) the cell wall appeared unravelled, and thick fibre-like material (white arrow) was observed. The fungal cytoplasm displayed a similar content to that shown by second-order branches.

At the BAS trunk level (Fig. 4, level 4; Fig. 6c) the hypha appeared flattened ($3{\cdot}5 \times 8$ μm). Total cell-wall thickness was *c.* 300 nm (inner wall, 250 nm), and similar in structure to that of first order branches, except for the now stratified innermost layer. The loosely woven appearance of the cell wall became less evident along the BAS trunk. Cytoplasmic features were similar to that of second and first order branches, with an increased number of small vacuoles.

Total hyphal diameter of the thin runner hypha from which BAS developed (Fig. 4, level 5) was 3·5 μm (Fig. 6*d*). The cell wall consisted of the constant, 50-nm outer wall, and a double-layered, fibrillar 275-nm inner wall. The fungal cytoplasm showed an increased vacuolation.

DISCUSSION

Arbuscular mycorrhizal fungi and land plants have co-evolved since the Devonian period (Simon *et al.*, 1993; Taylor *et al.*, 1995). Fossil evidence suggests that the ancestors of present land plants had poorly developed roots or rhizoids. Association with organisms capable of supplying mineral nutrients might have been essential, allowing them to overcome the inherent restrictions to growth in the terrestrial environment. In return, ancestors of AM fungi would have found plants optimal partners allowing them to overcome the limitations of their heterotrophic condition. It is not surprising that after 400 Myr of successful cooperation, both organisms have reached a high degree of structural, physiological and genetic integration and interdependence (Azcón-Aguilar & Bago, 1994). During this time, it is possible that some former intrinsic

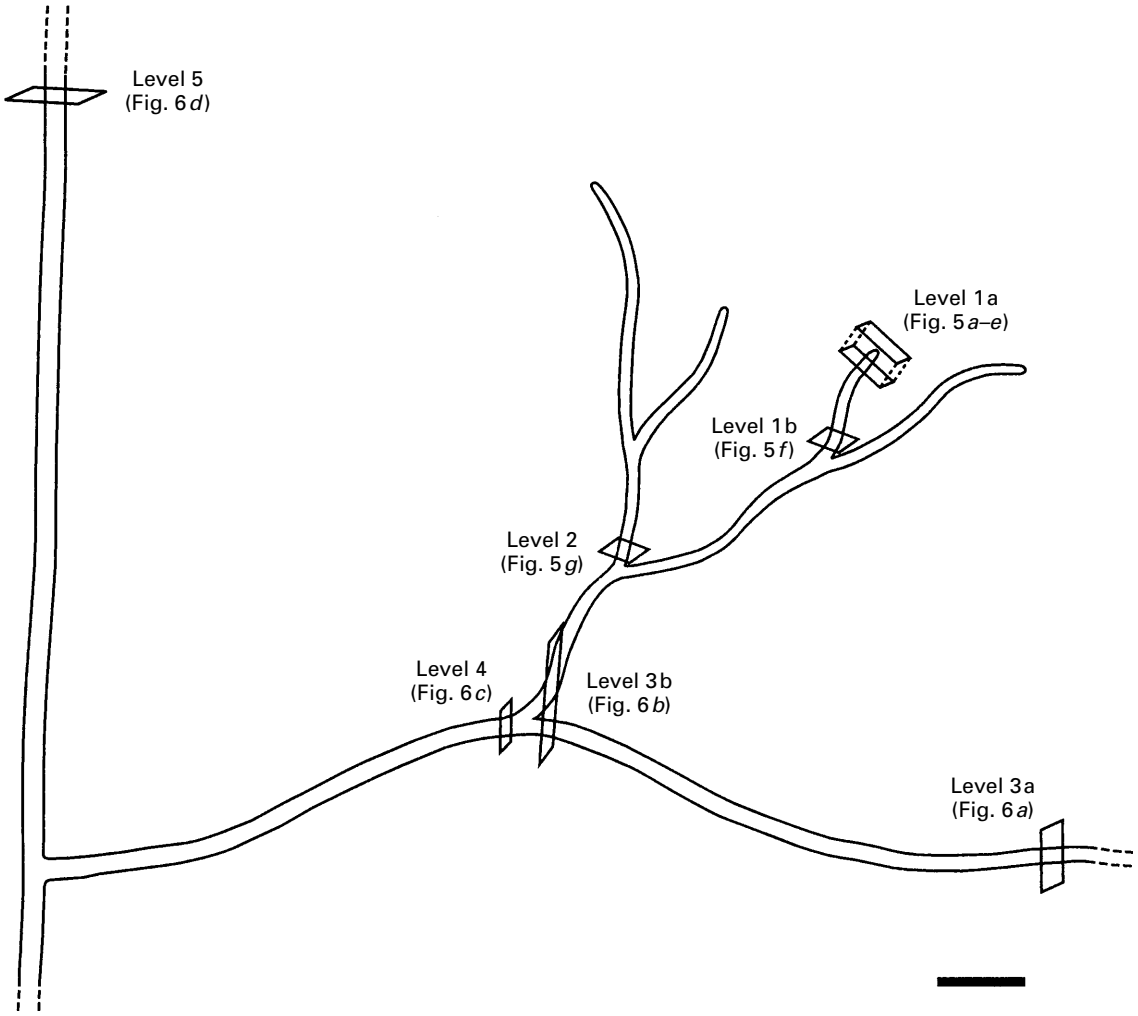

Figure 4. Schematic view of the BAS used for the cytological study showing the different levels at which ultrathin sections were taken. Level 1a, BAS hyphal tip; level 1b, third order branches; level 2, second order branches; level 3a and 3b, first order branches; level 4, BAS trunk; level 5, thin runner hypha. Bar, 15 μm.

capabilities have changed or even been lost. This could explain the inability of AM fungi to complete their life cycle in the absence of a host root. Amongst the hypotheses that have been put forward to explain this failure (reviewed by Azcón-Aguilar & Barea (1994)), the loss of part of the genetic material involved in the free-living growth of the fungus, and the inability of AM fungi to form their feeding structures (i.e. arbuscules) in the absence of a host root seem to be the most feasible.

The present study has shown that *G. intraradices* is incapable of forming the dichotomous-branched structures formerly named 'arbuscule-like structures, ALS' (Bago *et al.*, 1998) and now renamed branched absorbing structures (BAS), unless an intimate, intraradical contact between the fungus and a living host root has been established. BAS must not be confused with other branched structures (e.g. pre-infection or 'fan-like' structures), formed by the AM fungi. When suitably elicited these occur even in the absence of any physical contact with the host root (reviewed by Giovannetti *et al.* (1994, 1996); Nagahashi *et al.* (1996)). BAS were not formed under axenic conditions, even when the culture medium was supplemented with different mineral nutrients, or in monoxenic conditions when successful establishment of the symbiosis failed. They could, therefore, be considered as distinctive morphological markers of successful AM establishment.

Recently, Bago *et al.* (1998) suggested that what they named ALS, now BAS, might play an important role as preferential sites for external mycelium-mediated AM nutrient uptake. The present work, which expands on these observations, provides new data concerning BAS ontogenic, morphological, developmental and cytological characteristics. Interestingly, some of these features seem to be shared by both BAS and arbuscules.

Ontogenic and morphological features

This study has shown that BAS are formed on thin (3·5 μm), extraradical runner hyphae, at intervals of 25–300 μm. Arbuscules develop along intraradical, intercellular 'runner' hyphae or intracellular coils

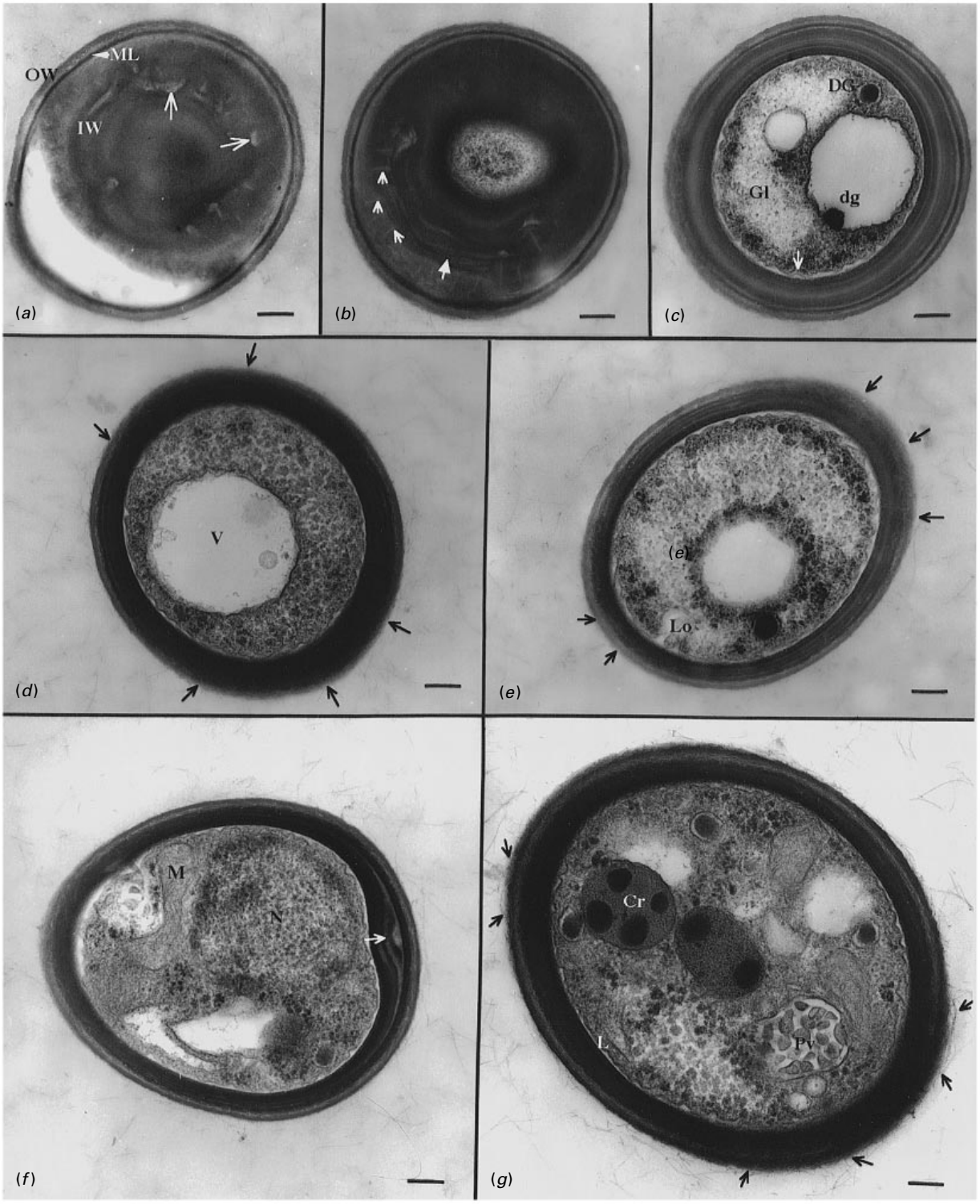

Figure 5. BAS ultrastructural cytology (I). (*a*)–(*e*) sequential micrographs of a BAS apex (level 1a). (*a*) Section throughout the outermost extremity of the hyphal tip. Only cell-wall components are revealed. OW, outer wall; ML, medium electron-dense layer; IW, inner wall. Some electron-transparent zones are apparent in the inner wall (arrows). (*b*) The second section of the series, showing the cytoplasm's granular texture. The inner wall displays a multi-layered appearance (full arrow). Electron-transparent zones are still visible, as are clear channels (arrows). (*c*) The third section of the series, showing two types of electron-dense granules: an osmophilic globule enclosed by a tight-fitting membrane (DG), and an opaque granule inside an electron-transparent vacuole (dg). Some glycogen deposits (Gl) are also evident, and the plasmamembrane (arrow) is easily recognizable. (*d*) The fourth section of the series shows a prominent, electron-transparent vacuole (V) in the cytoplasm. Note the loosely woven appearance of the cell wall (arrows). (*e*) Final section of the apex series. Lo, lomasome-like invagination (membrane curl). Arrows indicates the zones in which the cell wall shows a loosely woven appearance. (*f*) Level 1b, ultrathin section through third order branching hyphae at

(3–4 μm, Bonfante-Fasolo (1984) Brundrett & Kendrick (1990); Widden, (1996)), their number being proportional to the amount of intercellular hyphae growing along the internal cortex of the host root (Arum-type arbuscular mycorrhiza, Bennett, (1986); Toth *et al*. (1990)). It is interesting to note that the formation of both structures is repeated along fungal hyphae, growing either intraradical or extraradically. This seems to suggest that the formation of branched structures at regular intervals on the runner hyphae is an inherent capability of AM fungal mycelium, which can only be accomplished when symbiotic with a host root. This is further supported by the fact that axenically and monoxenically, but asymbiotically-cultured, AM fungi have the potential to form short branches with an incipient dichotomous pattern. The abortion of these structures shortly after initiation suggests that the fungus lacks a signal which is provided by living roots, and which is needed for the differentiation process.

A host 'switch-on' has been hypothesized as necessary for AM fungi to accomplish their life cycle (Bécard & Piché, 1989; Azcón-Aguilar & Barea, 1994; Bonfante & Perotto, 1995; Bago *et al*. 1996*b*). Bécard & Piché (1989) suggested that this stimulation was linked to arbuscule development and C acquisition by the AM fungus. However, results presented here show that fungal invigoration (and BAS formation) occurs after intraradical root colonization and coil formation, but before arbuscule formation. Consequently, the 'switch-on' should occur after initial hyphal attachment to the root and the first stages of root colonization, but before arbuscule formation. This trigger seems capable of unlocking the AM fungal cell cycle, allowing extensive fungal growth and resulting in the formation of branched intraradical (arbuscules) or extraradical (BAS) structures. The AM fungal life cycle is completed with a sporulation event occuring after extensive colonization of the substrate by runner hyphae and BAS (Bago *et al*., 1998). Spore formation has been suggested to be a process controlled by the release of signals through AM fungal cytoplasm (Bago *et al*., 1998). The present study shows that some BAS do not degenerate, but survive longer eventually forming spores, which possibly occurs following the reception of such a cytoplasmic signal. The longer lifespan of spore-BAS has been related to the role of such BAS in spore formation via preferential nutrient uptake processes and subsequent nutrient transfer towards the spore (Bago *et al*., 1998).

The dichotomous branching pattern of BAS and arbuscules is somehow similar, although BAS branching is more diffuse. In both structures the diameter of the branches decreases with increased branching order. However, the smallest arbuscular branches are thinner (1–0·3 μm, Bonfante-Fasolo (1984)) than the smallest BAS branches reported here (1·5 μm). Arbuscules are formed in a very specific and constant environment (Smith & Read, 1997), the apoplast between the protoplast and the cell wall of host plant cortical cells, where they encounter specific physical, chemical and biotic conditions. These conditions are quite different from those experienced by BAS in the agar-based, monoxenic culture. It is possible that the growth pattern of these branched structures is adapted according to the different environmental constraints. In the case of arbuscules, host plant cells must play an important role in fungal growth and morphogenesis, whereas the characteristics of the substrate and the soil microbiota probably influence BAS development.

Developmental features

BAS are ephemeral structures of the extraradical mycelium with an average development time of 7 d. Thereafter, BAS degeneration occurred, characterized by cytoplasm retraction and hyphae septation. Arbuscule lifespan has been estimated to be between 2·5 d (Alexander *et al*., 1988, 1989) and 10 d (Cox & Tinker, 1976). Thereafter these structures deteriorate, becoming septate, and collapse (branches collapse before trunk, Toth & Miller (1984)) forming clumps of degenerated material (reviewed by Bonfante-Fasolo (1984)). This material is further lysed and absorbed by the plant, allowing the previously colonized host cell to revert to its normal state and appearance (Toth & Miller, 1984). In our monoxenic cultures the process of BAS degeneration took 2–3 months, after which empty, septate BAS cell-wall skeletons remained still attached to the thin runner-hyphae. This slow degenerative process probably occurs due to the highly controlled, soil micro-organisms free, *in vitro* conditions. BAS growing in a soil environment are probably degraded more rapidly.

Cytological features

To the best of our knowledge the present work describes for the first time the cytological features of

subapical BAS zones. M, mitochondria; N, nucleus. An inner wall deposition core (arrow) is distinguishable. (*g*) Micrograph of an ultrathin section at level 2, showing ultrastructural features of BAS second order branches. Pv, polyvesicular bodies; Cr, membrane-bound crystals; L, innermost cell-wall layer. Note the unravelled appearance of the cell wall, and the extracellular fibrils which extend in the surrounding agar (arrows). Bars, 200 nm.

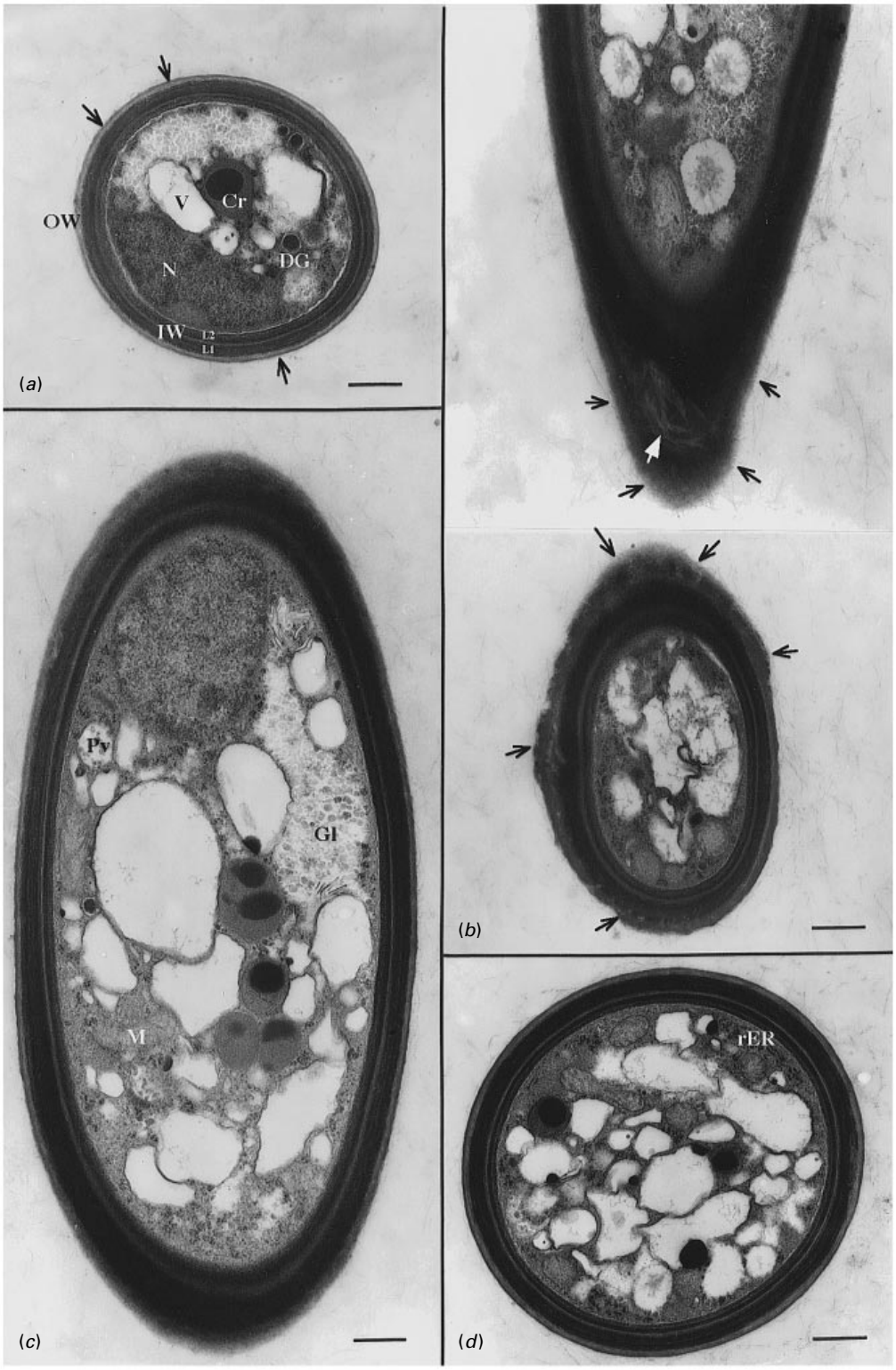

Figure 6. BAS ultrastructural cytology (II). (*a*) Level 3a, BAS first order branches. N, nucleus; V, vacuole; DG, dense granule; Cr, membrane-bound crystals; OW, outer wall; IW, inner wall; L1, outermost inner wall layer; L2, innermost inner wall layer. Arrows indicate the loosely woven cell wall and extracellular fibrils. (*b*) BAS hyphae close to the first order branching (level 3b). Note the highly unravelled appearence of the cell wall (arrows) and fibre-like structures (full, white arrow). (*c*) BAS trunk (level 4). M, mitochondria; Gl, glycogen deposits; Pv, polyvesicular bodies. (*d*) Ultrastructural features of the thin runner hypha from which the BAS developed (level 5). rER, rough endoplasmic reticulum. Bars, 500 nm.

BAS. These metabolically active structures, in accordance with the coenocytic condition exhibited by AM fungi, display a changing organelle and cell-wall pattern along their hyphal length. Figure 7 offers a schematic view of the progressive cellular changes occurring along BAS. As already described

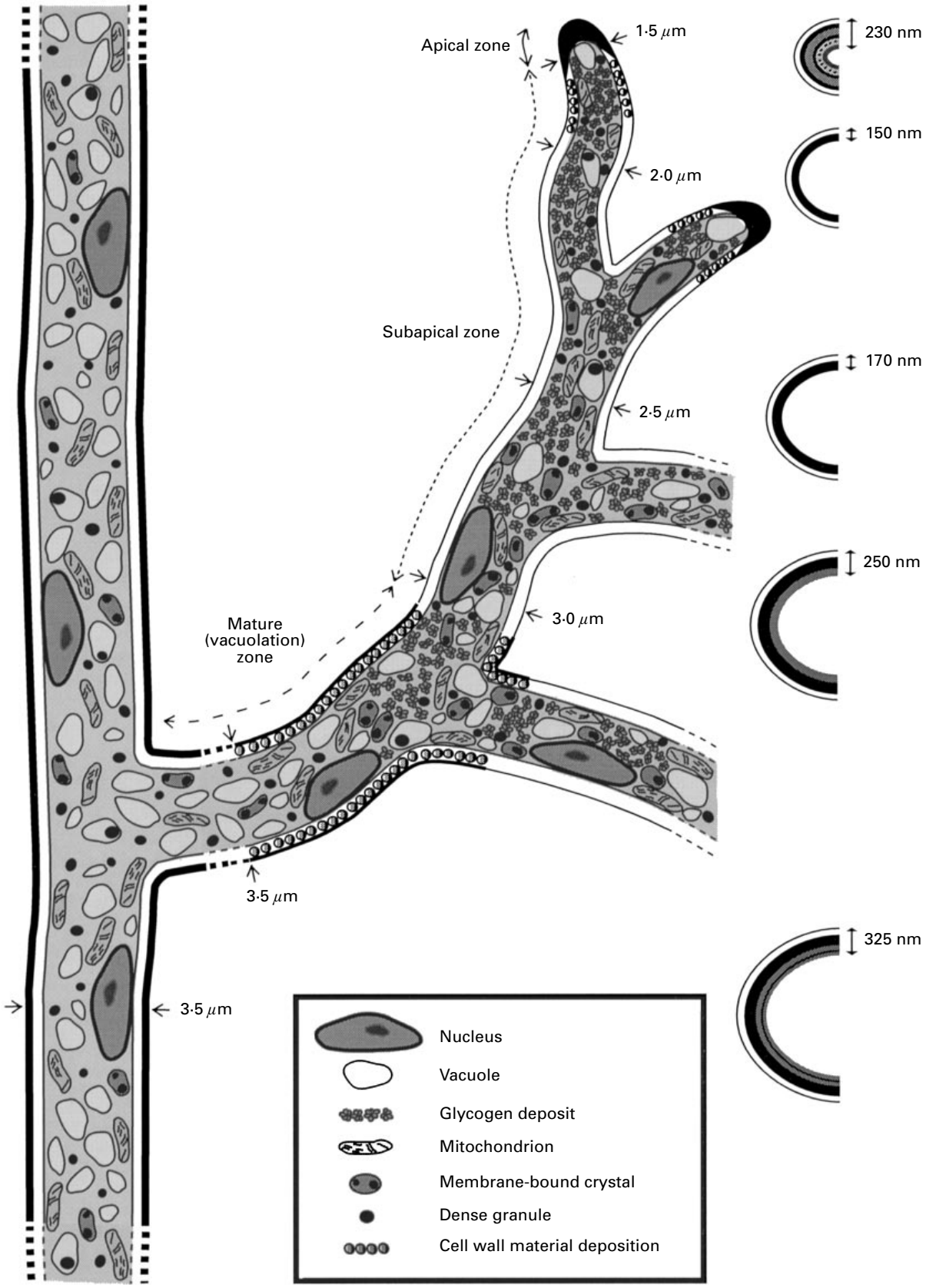

Figure 7. Schematic representation of BAS cytological features. The schema reflects the gradual changes in cell-wall structure and organelle content from the BAS tip to the thin runner hypha from which the BAS originated.

for many fungal hyphae (Grove & Bracker, 1970; Sward, 1981) BAS hyphae can be divided into three developmental zones: (i) apical zone; (ii) subapical zone; and (iii) mature (vacuolation) zone.

(i) Apical zones of BAS extend 4–5 μm back from the hyphal tips. At this level the BAS exhibits a thickened, multi-layered cell wall. This morphological feature is uncommon for fungal apical regions, which usually show a thinner, more plastic and porous cell wall than at any other part of the mycelium (Ruiz-Herrera, 1992; Jennings, 1995). However, hyphal tips stopped growing once the BAS was fully developed. It is also documented that cell-wall deposition occurs at fungal apices (Ruiz-Herrera, 1992). Our observation of polyvesicular bodies and membrane curls (related to wall deposition processes, Marchant, Peat & Banbury, 1967; Sward, 1981) indicates that wall deposition was taking place at BAS tips. Cell wall deposition, without growth, would explain the cell-wall thickening observed at BAS tips.

As already described for germ tube apices of other AM fungi (Sward, 1981), BAS apical zones are dominated by vacuoles and vesicles to the exclusion of most other organelles. In this study, glycogen deposits were also found at this level. This typical fungal carbohydrate reserve (Kendrick, 1992) has been found in association with lipidic droplets in axenically-cultured germ tubes, where it is presumed to be a breakdown product of lipids, previously stored within the fungal spore (Sward, 1981). Glycogen has been also found in AM fungal hyphae when the fungus is host-associated (Bonfante-Fasolo, 1984; Shachar-Hill *et al*., 1995). In this case the glycogen was suggested to be at least partially derived from the host plant (see Sward (1981) for references). Since we did not observe lipidic droplets in BAS and extraradical hyphae have been found unable to take up C (Pfeffer *et al*., 1996), the glycogen deposits observed might also be of a similar plant-derived origin. Isotopic ^{14}C, and ^{13}C-NMR studies could further clarify this suggestion.

(ii) Hyphal subapical zones are defined as the regions which contain the electrogenic pumps allowing nutrient uptake by fungi (Ruiz-Herrera, 1992). In this region the cell wall is not yet mature, allowing maximal extensibility (Jennings, 1995). Taking this into account, BAS subapical zones extend from 4–5 μm behind the apex (3rd-order branches) back to 1st-order branches, where numerous membrane curls appear, and where the immature subapical cell wall becomes more similar in structure and thickness to that of thin runner hyphae. Enlarged subapical zones of BAS suggest once more that these structures might be preferential sites for nutrient uptake by the AM extraradical mycelium.

Along the apical and subapical BAS zones, the cell wall often showed a loosely woven appearance, with some extracellular fibrils transversing the inner and outer walls, and extending into the surrounding agar. This was most evident close to first order ramifications. Hyphae of some pathogenic, saprophytic and symbiotic fungi have been observed to develop an extracellular fibrilar sheath (Nicole, Ruel & Ouellette, 1994; Smith & Read, 1997), which can coat several fungal structures including appresoria, haustoria, conidia and penetrating hyphae (Nicole *et al*., 1994). Extracellular sheaths could play different roles, e.g. enabling fungal structures to bind to plant surfaces, enhancing plant cell-wall degradation, or as protection of the fungal cell from unfavourable environmental conditions. Although already described for ericoid mycorrhizas (see Smith & Read (1997) for references), as far as we know, there are no reports of the existence of such structures in AM fungi. Further research should clarify its composition and function in BAS.

Cytological features of BAS subapical zones are indicative of a metabolically active cell, with numerous mitochondria, some nuclei, an increased number of small electron-transparent vacuoles, glycogen deposits, and an increased number of dense granules. The latter osmophilic globules appeared either bound to a close-fitting membrane (DG) or inside larger electron-transparent vacuoles (dg). The former have been proposed to contain cell-wall material (Sward, 1981), however their appearance suggests that they could be microbodies responsible for different fungal enzymatic activities. Cytochemical studies should confirm their role. Some dense granules inside electron-transparent vacuoles (dg) have been shown to contain P and Ca (White & Brown, 1979), suggesting that they might be polyphosphate deposits formed by the fungus as a means of transferring P taken up from the soil to other parts of the fungus–plant system. However, Orlovich & Ashford (1993) found that similar dense granules present in the extraradical mycelium of an ectomycorrhizal fungus were, in fact, fixation artefacts. Therefore, caution is necessary when evaluating the existence and function of such osmophilic inclusions.

Further research is needed to explain the presence, extent and function of vacuoles similar to those described as membrane-bound crystals (Mosse, 1970; Sward, 1981), which appear in subapical zones of the BAS. Membrane-bound crystals were considered to be protein storage units (Mosse, 1970; Sward, 1981; Meier & Charvat 1992).

(iii) The mature (vacuolation) zone consists of the BAS trunk, whose ultrastructural features (multilayered cell wall, increase in vacuole number, etc.) reflect the transition between the ephemeral BAS and the thin runner hypha from which they are produced.

Also at the cytological level, BAS and arbuscules seem to share several cytological characteristics. The cytoplasm of arbuscular branches contains numerous

nuclei, mitochondria, glycogen deposits, lipid globules, abundant polyvesicular bodies and electron-dense granules inside small vacuoles (Bonfante-Fasolo, 1984). Vacuoles are also dominant in thinner arbuscular branches (Bonfante-Fasolo, 1984). Both BAS and arbuscule cell walls become thinner, and their structure simplified, from the trunk on. However, the minimal thickness of the BAS cell wall (150 nm) is still thicker than that of the arbuscule cell walls, which for the thinnest branches is 20 nm (Bonfante-Fasolo, 1984). As already discussed, arbuscule environmental conditions are very specific and different to those endured by BAS, and therefore host influence on morphogenesis of fungal intraradical hyphae must be considered (Bonfante-Fasolo, 1987).

According to the present results, arbuscules and BAS seem to be formed along AM fungal hyphae (either intracellular or extracellular) following a switch-on signal provided by the host plant. Considering their shared morphological, developmental and cytological features, it would be tempting to consider them as homologous structures, their final morphology depending on the surrounding environment. BAS morphogenesis should be a process under fungal control, e.g. through variations in spatial distribution of wall synthesis (Wessels, 1994) or fungal chitin-synthase (Bago *et al*., 1996*a*) self-regulation, whereas both, the fungus and the host plant, would be involved in controlling arbuscule development and duration by these same and/or other mechanisms, e.g. plant chitinases (Bonfante-Fasolo, 1987) and other lytic enzymes (Lambais & Mehdy, 1993).

To date, BAS have been described only in monoxenic cultures. However, Mosse & Hepper (1975) stated that 'in soil, remnants of similar fine, much-branched, rhizoid-like structures are also found, often attached to particles of organic matter'. Most studies on AM extraradical mycelium usually employ destructive hyphal extraction methods (Dodd, 1994; Bago *et al*., 1998) which probably discard thinner AM fungal structures including BAS. Further studies are needed to confirm the putative production of BAS in soil, their developmental dynamics under natural conditions, as well as their role in the physiology and ecology of AM.

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