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VAM Fungi in *Ginkgo biloba* Roots: Their Interactions at Cellular Level

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Abstract

The pattern of vesicular-arbuscular mycorrhizal (VAM) colonization in *Ginkgo biloba* L. roots was investigated using light and electron microscopy.

Different species of *Glomus* – inoculated under controlled conditions – show a similar pattern of large coils in rhizodermic cells, rare intercellular hyphae and vesicles and abundant intercalary arbuscules in the cortical cells. The endophytes rarely colonize the innermost cortical layer, whose radial walls are surrounded by a lignified thickening, the phi layer. At the ultrastructural level, the endophytes possess an electron-dense wall, which becomes progressively thinner towards the inner cortex. Their cytoplasm contains bacteria-like inclusions, electron dense granules and a developed vacuolar system. The arbuscule-infected cells are the only ones to show a clear reaction to fungal penetration. The plasmalemma increases in length, the starch disappears, and the central vacuole splits into many small vacuoles.

Our observations suggest that the pattern of VAM colonization in *G. biloba* is rather different from that in the Angiosperms studied so far, and that the changes are host-controlled. On the other hand, the ultrastructural organization of different spp. of endophytes and their interactions with this host are alike.

We conclude that the host cell – regardless of the systematic position of the plant – acts as a constant environment for the fungus. To reach this biotic niche, the fungus must pass through the host cell wall, whose nature differs greatly in plants. This barrier can force the fungus to modify its growth pattern in different ways.

Key words: Cell wall, symbiosis, ultrastructure of *Ginkgo biloba* roots, VA mycorrhizae.

1. Introduction

Vesicular-arbuscular mycorrhizae are symbiotic associations between the roots of numerous plants and a restricted number of soil fungi. The beneficial effects of these associations on plant growth are due mainly to an improved balance of mineral nutrition (Harley and Smith, 1983).

The understanding of this balance may come from the study of partner interactions at the cellular level. This is perhaps the reason that in the last ten years the morphological features of VA mycorrhizae of Angiosperms have been described in considerable detail (for recent reviews, see Carling and Brown, 1982; Scannerini and Bonfante-Fasolo, 1983; Bonfante-Fasolo, 1984).

In contrast, morphological observations of VAM fungi living in Gymnosperm roots are surprisingly few (Strullu et al., 1982). They are in fact limited to *Taxus baccata* (Prat, 1926; Strullu, 1978) and to two *Sequoias* (Mejstrik and Kelley, 1979) and were obtained by studying samples collected in the field.

The aim of the present paper was to determine whether the pattern of infection described for Angiosperms can be extended to Gymnosperms. *Ginkgo biloba* was chosen because it is a tree of great interest from evolutionary and taxonomic points of view and, moreover, it is able to form VA mycorrhizae under controlled conditions (Fontana, 1985).

2. Material and Methods

Seeds of *Ginkgo biloba* L. were surface sterilized by shaking them for 30 min in 4°/₀ silver nitrate; they germinated 20-30 days later on moist filter paper. Uniformly sized seedlings were planted in perforated plastic bags containing sterilized soil and inoculated with the following inocula:

- roots of *Vitis vinifera* L. infected by *Glomus fasciculatum* Gerdemann and Trappe, from the Botanical Gardens of Torino;
- roots of *Allium porrum* L. infected by *Glomus macrocarpum* Tul. and Tul. and *G. monosporum* Gerdemann and Trappe, from the "Facultè de Foresterie et Geodesie", Laval University, Québec;
- roots of *Ginkgo biloba* growing in the Botanical Gardens of Torino and infected by an endophyte (Fontana, 1985), tentatively identified as *G. caledonium*;
- spores of *Glomus versiforme* (Karsten) Berch (= *G. epigaeum* Daniels and Trappe), obtained from Dr. Daniels.

Other seedlings were grown without VAM inocula.

The seedlings were raised in a constant environment room at 21°C (16 h photoperiod, 80% humidity), watered daily with distilled water and after two months transferred to a green house.

Sampling for cytological studies was taken six months later and was repeated bimonthly for two years (1981-1983). Mycorrhizal and non mycorrhizal roots were fixed in 2.5% glutaraldehyde (GA) in 0.1 M cacodylate buffer for 3 h and postfixed in 1% OsO_4 in the same buffer for 2 h at room temperature, dehydrated in an ethanol series and embedded in Durcupan ACM.

Thick sections of 1 μm were stained with 1% toluidine blue in Na_2CO_3 0.1% and observed with a light microscope, while the thin sections were poststained

with uranyl acetate and lead citrate and observed with Philips 300 Electron Microscope.

Other roots were prepared for scanning electron microscopy. After a fixation in 2.5% GA for 2 h, they were carefully washed, dehydrated in an ethanol series followed by a graded ethanol – amyl acetate series. Subsequentally they were dried using the critical point method in A Denton Vacuum DCPI. Specimens were coated with gold platinum in a Sputtering Hummer II (Technics) and examined using an Etec Autoscan SEM at 20 KV. In order to obtain internal views, other GA fixed roots were cut using a freeze microtome, treated with sodium hypochlorite for 5 min to remove host protoplasm and then processed as described above.

3. Results

All the different species of *Glomus* heavily colonize roots of *Ginkgo biloba* and improve the vegetative development of the host plants as previously described by Fontana (1985). The endophytes colonize only the third order roots (2-6 mm thick and 5-10 mm long; Fig. 1), after forming a loose weft of extramatrical mycelia around the whole rootlet (Fig. 2). Penetration points are found only in the differentiated root regions.

The pattern of root colonization by the different species of *Glomus* is constant and so is their interaction with the host cells at ultrastructural level. Therefore morphological observations are presented together, without making comparisons between the different species.

The endophytes penetrate the root by passing through the external rhizodermic cells of the host and forming intracellular coils. In the inner cortex the hyphae differentiate into arbuscules and more rarely, into vesicles. They never colonize the stele (Fig. 3).

Coils

The external hyphae pass below the sloughing and rhizodermic cells (Figs. 2, 4). The hyphae, characterized by a thick wall and a large diameter (from 3.5 μm in *G. fasciculatum* to 5.5 μm in *G. monosporum*) penetrate cells of the first living layer, then enlarge up to 10 μm in diameter and acquire a coiled shape. Up to four successive coils can be observed (Fig. 5). The hyphae usually pass into the successive inner layer at the middle point of the long side of the cell. The coiled hyphae show an electron-dense wall with a lamellar structure (Fig. 6). The thickening of the wall varies in different endophytes (from 0.4 μm in *G. fasciculatum* to 0.2 μm in *G. versiforme*, whose wall appears more electron transparent than that of the other endophytes). Nuclei are minute, mitochondria are abundant as are glycogen particles, ribosomes and lipid globules (Figs. 6, 9). A peculiar feature is the presence of two different types of electron-dense granules: the first type (0.1 μm in diameter) occurs inside large vacuoles, often in a peripheral position, while the second type is closely surrounded by a membrane

unit (Figs. 8, 9). Bacteria-like inclusions about 0.4 μm in size, often showing signs of division, are present in the fungal cytoplasm (Fig. 6, inset).

The fungus is consistently enveloped by the host plasmalemma (Figs. 6, 7, 8), that becomes invaginated around the endophyte. The interfacial area between the fungal wall and the host plasmalemma is filled with interfacial material that appears continuous with the host cell wall near the point of penetration (Fig. 8).

The ultrastructural organization of the host cell containing the coils is similar to the uninfected cell both in the same root and in an uninoculated root. The cytoplasm has a peripheral distribution with occasional organelles, a small and roundish nucleus and a large central vacuole; amyloplasts are usually present (Fig. 6). Cell walls are thickened and dark in the flattened cells of the outermost layers, then they become thin and electron transparent.

Cell to cell passage and intercellular hyphae

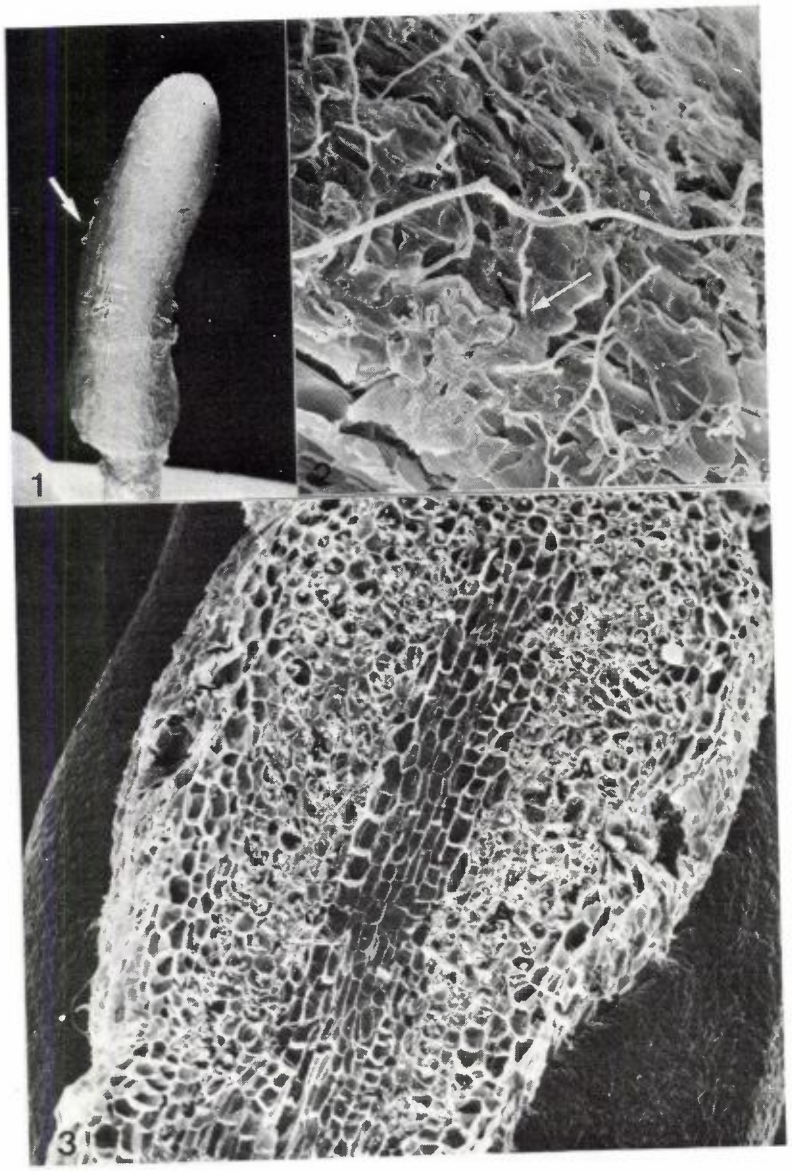
Deeper in the cortex, the coiled hyphae penetrate inside other cortical cells, where they branch, giving rise to the arbuscules (Figs. 3, 4).

The cell to cell passage is the same in all the rhizodermic and cortical layers. When a growing hypha reaches the host cell periphery, the host plasmalemma, which envelops the fungus, becomes continuous with the host plasmalemma adhering to the host cell wall (Fig. 7). In this area there are many polyvesicular bodies, which seemingly contribute to plasmalemma fusion (Fig. 7, inset). The constricted hypha crosses the wall by lysing the middle lamella and stretching the primary wall. At the same time the host plasmalemma of the newly penetrated cell is pushed inside and proliferates to follow the fungal branch (Fig. 8). When the fungus reaches an intercellular space, it develops into a large intercellular hypha, the ultrastructural organization of which corresponds to that already described for coiled hyphae (Fig. 9).

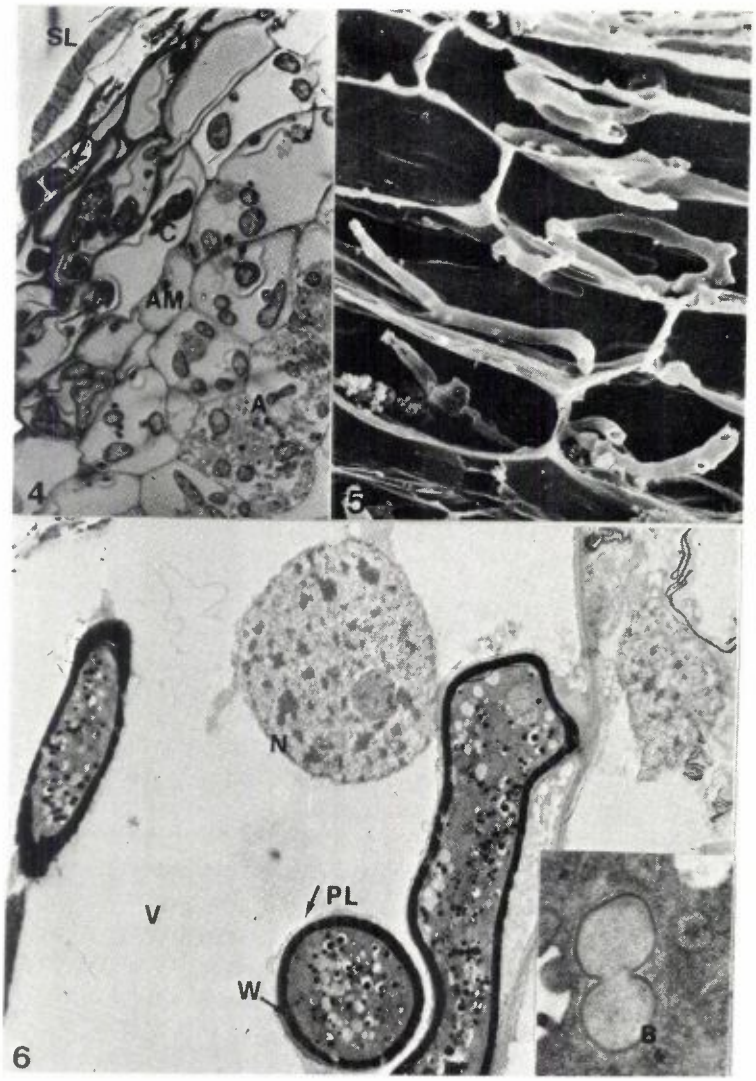
Arbuscules

Arbuscules are abundant in the cortical zone: when the infection is well established nearly every cell contains an arbuscule (Fig. 3). In *G. biloba* the arbuscules can be defined as intercalary, in relation to the mother hypha. The latter – in continuity with a coil or with an intercellular hypha – shows a coiled shape, passes from one cell to another in the same layer and forms lateral branches, i.e. arbuscules in each cell (Figs. 10-13).

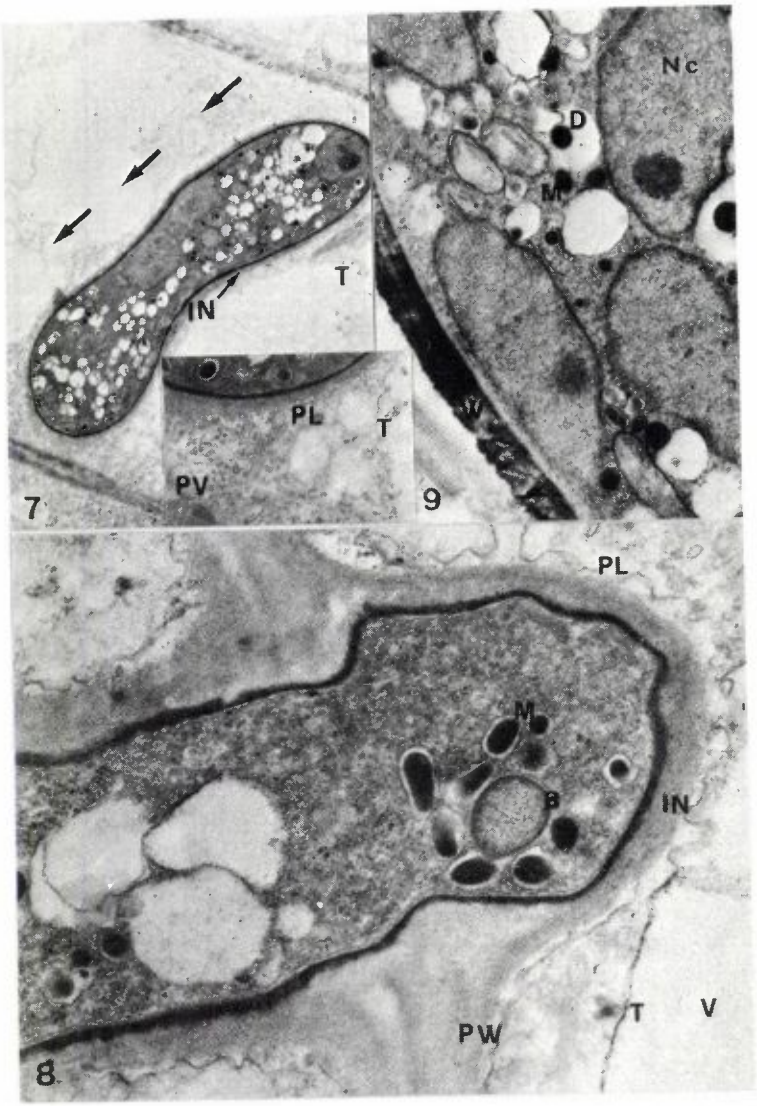
The branches of the arbuscules are progressively narrower ultimately reaching 0.2 μm in diameter. Their ultrastructural organization varies at different stages of development. In the mother hypha the cytoplasm is similar to that of the coiled and intercellular hyphae, while the smaller branches show thinner walls (up to 0.02 μm thick) and a vacuolated cytoplasm free of dense granules. The hyphae then develop membrane whorls, as usual after autolysis and become empty and



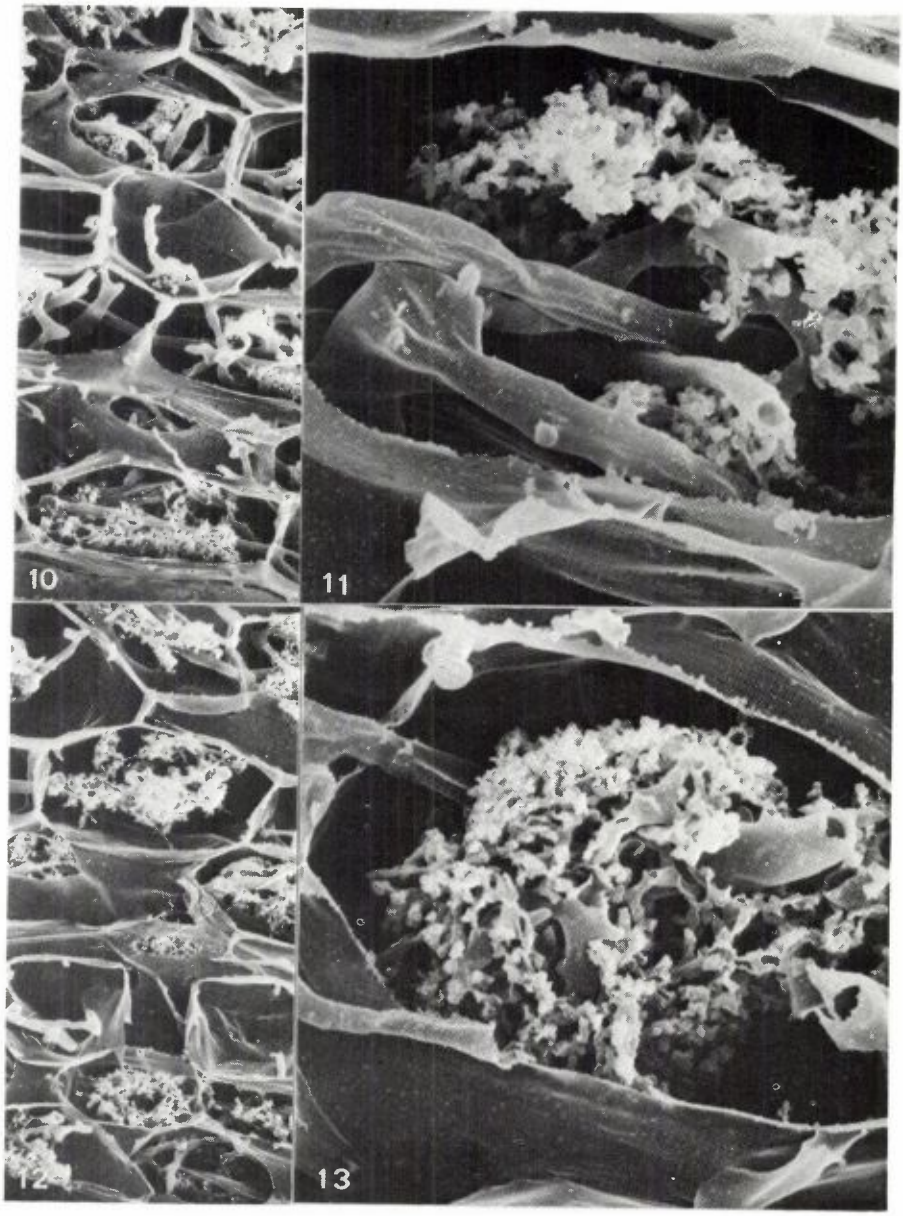
Figures 1-3. Scanning electron micrograph (SEM) of a third order root of *Ginkgo biloba*. Arrow indicates extramatrical mycelium. ×20. 2) SEM of the extramatrical mycelium of *Glomus caledonium* on the root surface of *Ginkgo*. The runner hyphae pass below the sloughing rhizodermic cells (arrow). ×200. 3) View of a whole root in an almost median section. Arbuscules (A) of *G. caledonium* are easily observed in the cortical layers, while they are absent in the stele. ×100.



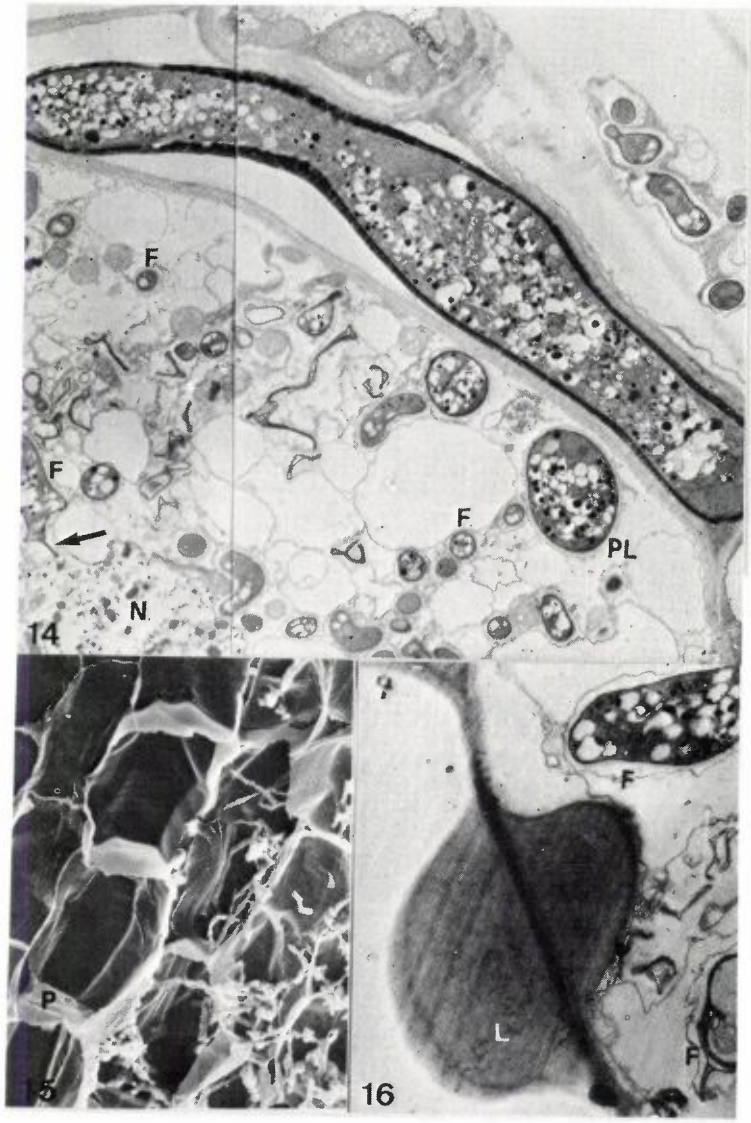
Figures 4-6. 4) Cross section of a root, as seen in light microscope. External hyphae of *G. macrocarpum* (arrow) pass below the sloughing cells (SL), acquiring a coiled shape (C) in the rhizoderm. Amyloplasts (AM) are abundant only in the rhizoderm. The intercellular spaces are small and are filled with electron-dense material. Arbuscules (A) occur only in the innermost layers. ×800. 5) SEM of a root showing a succession of coils of *G. caledonium* in the rhizodermic cells. ×520. 6) Transmission electron micrograph (TEM) showing a coil of *G. fasciculatum* formed by hyphae with electron-dense walls (W) and abundant granules. The hyphae are surrounded by the host plasmalemma (PL). The host cell displays a roundish nucleus (N) and a large vacuole (V). ×4800. Inset: Magnification of a dividing bacteria-like inclusion inside the fungal cytoplasm (B). ×48000.



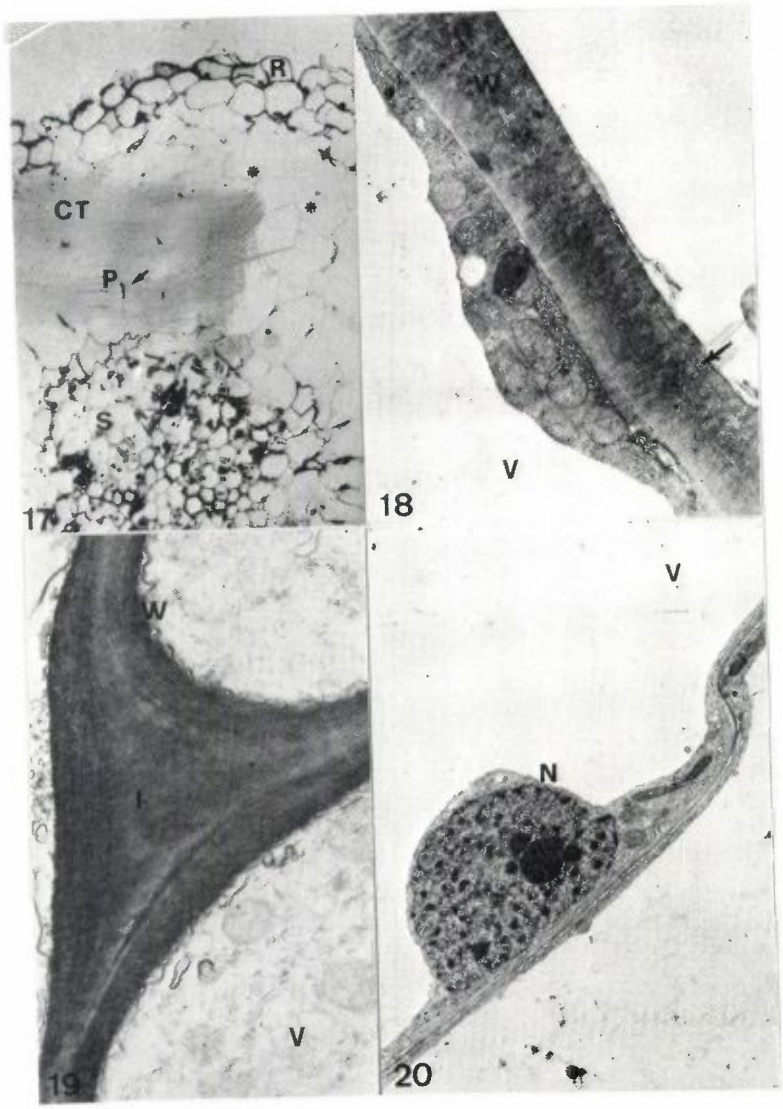
Figures 7-9. 7)A hypha of *G. macrocarpum* passes through a rhizodermic cell. Arrows indicate the direction of penetration. ×5600. Inset: Magnification of the zone in which the host plasmalemma surrounding the fungus (PL) proliferates to forming polyvesicular bodies (PV). Interfacial material (IN) is well evident as well as is the tonoplast (T) lining the vacuole. ×16,000. 8) A hypha of *G. macrocarpum* crosses the host cell wall, stretching the primary wall (PW) and pushing out the host plasmalemma (PL). A bacteria-like inclusion (B) is surrounded by granules enveloped by a membrane (M); (interfacial material (IN)). ×31000. 9) Details of an intercellular hypha of *G. fasciculatum*. Dense granules inside the vacuoles (D) and others closely enveloped by membrane (M) are abundant and surround three small nuclei (Nc). ×16000.



Figures 10-13. SEM view of arbuscules of *Glomus caledonium*. Note the cell to cell passage of the mother hypha, and its coiled shape. The arbuscules are in intercalary position, originating as lateral branches of the mother hypha. Figs. 10 and 12: ×400; Figs. 11 and 13: ×2000.



Figures 14-16. 14) TEM view of an arbuscule of *G. monosporum*. Fungal branches (F) of different size and in different stages of development. They are surrounded by the plasmalemma (PL). Arrow indicates a collapsed hypha. A large hypha occupies an intercellular space. ×5800. 15) SEM of the inner cortical layer, showing the phi thickenings (P) in a longitudinal section. ×420. 16) TEM of phi layer showing the electron-dense lignin thickening (L) and the branches of a terminal arbuscule (F) of *Glomus caledonium*. ×8100.



Figures 17-20. 17) Transverse section of an uninoculated root, observed in light microscope. Rhizoderm (R), Cortex (CT), Stele (S), Phi Layer (P) with thickenings (arrow). Well-developed air spaces are found between the cortical cells (asterisks). ×400. 18) TEM of a rhizodermic cell in the outer layer of an uninoculated root. The wall (W) is thick and formed by densely packed lamellae (arrow; vacuole (V)). ×12500. 19) Lamellar structure of the wall (W) in a rhizodermic cell. The intercellular space (I) is filled with dense material (vacuole (V)). ×24,000. 20) TEM of a cortical cell in an uninoculated root, showing a large central vacuole (V), the peripheral nucleus (N) and cytoplasm. ×3200.

collapse, while a septum delimits the empty collapsed apical zone from the active one. Finally the isolated collapsed hyphae form a large clump (Fig. 14).

Throughout the different stages of arbuscule development, the host plasmalemma surrounds all the arbuscular branches. In addition to the proliferated plasmalemma, the cortical cell shows striking changes in comparison with that of the uninfected root (Fig. 20). The nucleus is large and lobed, plastids lack starch and show plastoglobules, endoplasmic reticulum is well developed, the vacuole is split into many vacuoles. Moreover, the interfacial material becomes progressively thinner around the terminal fungal branches (Fig. 14), as previously described (Scannerini and Bonfante-Fasolo, 1983).

In the innermost cortical layer, around the stele, the cells are surrounded by a typical thickening (Fig. 15), forming the so-called phi layer (Russow, 1875; Peterson et al., 1981). The lignified thickenings which occur in the radial wall as oval, electron-dense bodies (Figs. 15, 16) are autofluorescent in U.V. light and give a positive reaction to phloroglucinol staining. They block the passage of the intracellular hyphae from cell to cell laterally within the same layer in such a way that arbuscules do not occur in the innermost cortical layer (Fig. 15). If present, each arbuscule is formed by a hypha coming from the previous infected cells or from the intercellular spaces. The hypha branches give rise to arbuscules in a terminal rather than intercalary position (Fig. 16).

Uninfected root

The anatomy of uninfected roots is similar to that of infected ones: the central cylinder is surrounded by the endodermis, the cortical cylinder, whose innermost layer shows the phi thickenings, and a rhizoderm of three to four layers of cells (Fig. 17). The outer walls of the rhizodermic cells are strongly UV autofluorescent and, at TEM level, show a structure with electron-dense lamellae (Fig. 18) similar to those described as typical of suberin (Peterson et al., 1978). Like in mycorrhizal roots, intercellular spaces of the rhizoderm display electron-dense deposits (Fig. 19), while between the cortical cells there are well-developed air spaces (Fig. 17). Uninfected cortical cells are characterized by a large central vacuole and a peripheral protoplasm (Fig. 20).

4. Discussion

All the observations made on the roots of *Ginkgo biloba* colonized by different VA endophytes under controlled conditions offer new information on the host/endophyte cellular relationships during the mycorrhizal symbiosis. The first observation is that the pattern of VAM colonization of the root in *G. biloba* – characterized by numerous large coils, a reduced number of intercellular hyphae and arbuscules with the so far undescribed intercalary position – presents only some features common with the VAMs already described in Angiosperms. The presence of large intracellular coils has been reported in some Angiosperms for example in

Liriodendron (Kinden and Brown, 1975), in *Vitis vinifera* (Bonfante-Fasolo and Grippiolo, 1982), or *Gentiana* (Jacquelinet-Jeanmougin and Gianinazzi-Pearson, 1983). In *G. biloba* as in *Taxus baccata* (Strullu, 1978), intercellular hyphae are rarer than in Angiosperms such as Liliaceae or Leguminosae where intercellular hyphae running parallel to the long root axis are very abundant (Casana and Bonfante-Fasolo, 1982; Cox and Sanders, 1974; Abbott, 1982).

However the most important difference is seen in the pattern of arbuscule formation. In Angiosperms arbuscules are usually described as originating from intercellular hyphae which penetrate the cortical cells and branch dichotomously (Carling and Brown, 1982; Harley and Smith, 1983; Bonfante-Fasolo, 1984). Conversely, in *G. biloba*, the hyphae that spread the infection are already intracellular and maintain a coiled shape, before giving rise to lateral branches which form intercalary arbuscules. Only in the innermost layer does the arbuscule have the usual terminal position. This model strongly resembles the infection in Pteridophytes, according to the drawings by Boullard (1979).

The pattern of colonization described here is alike in all the endophytes belonging to different species of *Glomus* that we used. However, some of the same fungi may display a different behaviour in plants other than *Ginkgo biloba*: for example *G. fasciculatum* does not form coils in maize roots (Kariya and Toth, 1981) or it can form coils, but not intercalary arbuscules in *Vitis vinifera* (Bonfante-Fasolo and Grippiolo, 1982).

Although generalization to other Gymnosperms should be avoided until new data on different species are obtained, the present observations clearly suggest that the pattern of VAM colonization in *Ginkgo biloba* is different from those described in Angiosperms.

The second point concerns the cellular organization of the two partners which are "identical" to those of all the mycorrhizal systems described so far: the fungus always displays a cytoplasm which contains bacteria-like inclusions, electron-dense granules considered polyphosphate granules (Strullu et al., 1981) and a wall that becomes thinner as it penetrates into the root cortex. This constancy of the fungus morphology can be explained by a low degree of speciation exhibited by VAM endophytes during their evolutionary history (Pirozynski, 1981; Bonfante-Fasolo, 1984). The reproductive mechanisms of VAM endophytes-based on asexual multiplication – is thought to favour nearly identical progeny already selected for their mutualistic properties (Law and Lewis, 1983).

In *Ginkgo biloba* the clear separation between coils and arbuscules enables identification of two distinct types of host response to fungal infection. In the coil-bearing cells the host does not show any change, while in the arbuscule-infected cells the response of the host is evident. Here the host plasmalemma increases its length, enveloping the fungus, while the interfacial material produced by the host appears to be continuous with the host wall and is progressively reduced. This response is similar to that observed in other intracellular mycorrhizal symbioses (for example in the ericoid ones, Bonfante-Fasolo et al., 1984) and can be

interpreted as a compatibility response, in which the host increases its metabolic activity, allowing the fungal development to take place (Cox and Tinker, 1976; To'h and Miller, 1984). It is probable that the fungal growth is controlled by the host plasmalemma, perhaps this represents a "... sequestration of symbionts" that "may offer some form of control ..." (Smith, 1979). If so, in VAM the mechanism of this control appears to be very conservative in evolutionary terms, since the host/fungus interactions inside cortical cells are similar in all plants, irrespective of their systematic position.

The two conclusions (differences in the pattern of fungal colonization, constancy of partner interactions at the cellular level) seem to be contradictory. However, this apparent contradiction can be understood by assuming that the single host cell represents a biologically constant environment for the fungus, while the multicellular system (i.e. the root) introduces some variable factors that can regulate fungal growth. The host cell wall could act as one of these factors, representing the most important barrier for the fungus in reaching the host cell. McNeil and his colleagues (1984) assume that "the constituents that make up the wall of the different plants must be different – at least quantitatively, in different species" and "... the complex carbohydrates of the walls of the different cells in individual organs and tissues of a single plant are also different". If so, the variability of the patterns exhibited by VAM endophytes in the different plants could be related to the different compositions of their cell walls. Even though biochemical analysis of cell wall composition in *G. biloba* is not available, to our knowledge, morphological observations enable us to recognize different components in the cell wall of the various tissues, as well as a different behaviour of the endophyte. The fungus does not cross the suberized wall of the external rhizodermic cells, but grows among the sloughing cells. It is able to cross the primary walls of the rhizodermic cells, but is blocked by the dark material of the intercellular spaces similar to that considered to be phenolic material in *Allium cepa* (Peterson et al., 1978). In addition, the fungus easily grows among the thin primary walls of the cortex but it is completely blocked by lignin deposits in the phi layer.

In conclusion we hypothesize that the host wall and its different constituents represent a constraint modulating the pattern of colonization of VAM fungi.

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