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Endomycorrhizas in the Gentianaceae. II. Ultrastructural Aspects of Symbiont Relationships in *Gentiana lutea* L.

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Abstract

The ultrastructural aspects of natural and control-infected (with *Glomus mosseae* or *G. intraradices*) mycorrhizal plants of *Gentiana lutea* L. have been investigated using cytochemical techniques for the localisation of polysaccharides, proteins and alkaline phosphatase. Cellular relationships between the mycorrhizal associates during the different stages of fungal development in host tissues are described. Initial penetration of root cells appears to involve some degradation of host wall material. Subsequent fungal development is entirely intracellular, although intercellular spaces exist in *G. lutea* roots. Infection is limited to a simple unbranched hypha in the outer cell layers of the root, while in the parenchymal cortical cells hyphal coils develop and give rise to arbuscules. The intracellular interactions occurring between symbionts during infection development are discussed in relation to those described for typical vesicular-arbuscular mycorrhizal associations.

Keywords: endomycorrhiza, *Gentiana lutea*, *Glomus mosseae*, *G. intraradices*, ultrastructure, cytochemistry

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1. Introduction

The identification of mycorrhizal fungi associated with members of the Gentianaceae has been a problem because of the particular morphology of the fungal symbiont in roots of these plants. Neumann (1934) likened endomycorrhizas of gentianaceous species, including *G. lutea*, to those formed by members of the Orchidaceae. Several studies have since pointed out the morphological similarities between gentian mycorrhizas and the vesicular-arbuscular type of endomycorrhiza (Jacquelinet-Jeanmougin, 1982; Gay et al., 1982; Kuhn and Weber, 1986), and inoculation experiments with *G. lutea* and *Centaurium dubium* have confirmed that the mycorrhizas are formed by VA mycorrhizal fungi (Jacquelinet-Jeanmougin and Gianinazzi-Pearson, 1983; McGee, 1985). However, although vesicles and arbuscules are present in the roots of gentianaceous species, the form and behaviour of fungal hyphae differ from that in typical VA mycorrhizal host plants (McGee, 1985; Jacquelinet-Jeanmougin et al., 1986) and for some authors gentian mycorrhizas still can not be considered as vesicular-arbuscular (Tester et al., 1987). There have been numerous studies of the ultrastructural features of VA mycorrhizal associations (see Scannerini and Bonfante-Fasolo, 1983; Bonfante-Fasolo, 1984; Gianinazzi-Pearson, 1984 for references) but there is no information about the fine structure of endomycorrhiza in the Gentianaceae. The aim of the present work was to define the ultrastructural organisation and some cytochemical features of endomycorrhizas of *G. lutea* formed under natural and controlled conditions, in order to determine whether they really differ from typical VA endomycorrhiza at the cellular level.

2. Materials and Methods

Six-month old naturally infected plants of *Gentiana lutea* L. were collected from a low altitude (210 m) experimental plot at Fauverney, Côte d'Or (INRA, Dijon). For controlled infections, plants were inoculated with *Glomus mosseae* (Nicol and Gerd) (LPA5) or *G. intraradices* (Schenck and Smith) (LPA8) as described previously (Jacquelinet-Jeanmougin and Gianinazzi-Pearson, 1983), and grown for 12 weeks in a controlled environment cabinet (day/night temperature 20/15° C, photoperiod 16 hr at 26 $W.m^{-2}$).

Five mm long pieces of mycorrhizal root were fixed in 2.5% gluteraldehyde in 0.1 M cacodylate buffer, pH 7.2, overnight at 4° C, rinsed, cut into 1 mm segments and postfixed 1 hr at room temperature in 2% OsO_4 in the same buffer. After fixation, specimens were rinsed, dehydrated through an acetone series and progressively embedded in Epon (Epikote) 812. Samples were cut

with a diamond knife ultramicrotome (Reichert Ultracut E). Thick sections (0.5 μm) were stained with 1% basic toluidine blue in 3% Na_2CO_3 for light microscope observations.

Thin sections (80 nm) were treated as follows:

1. poststained in uranyl acetate and lead citrate,
2. submitted to the PATAg test for the localisation of 1–4 polysaccharides (Thiéry, 1967); control sections were prepared by replacing thiocarbohydrazide with 20% aqueous Na borohydride,
3. submitted to the silver reaction for the detection of cystine-containing proteins (Swift, 1968); control sections were treated 4 hr with pepsin (50U) before staining.

For the localisation of alkaline phosphatase, mycorrhizal root pieces were only prefixed for 1 hr and enzyme activity was revealed as described by Gianinazzi et al. (1979); enzyme specificity was controlled by incubating specimens in the presence of 4 mM KCN.

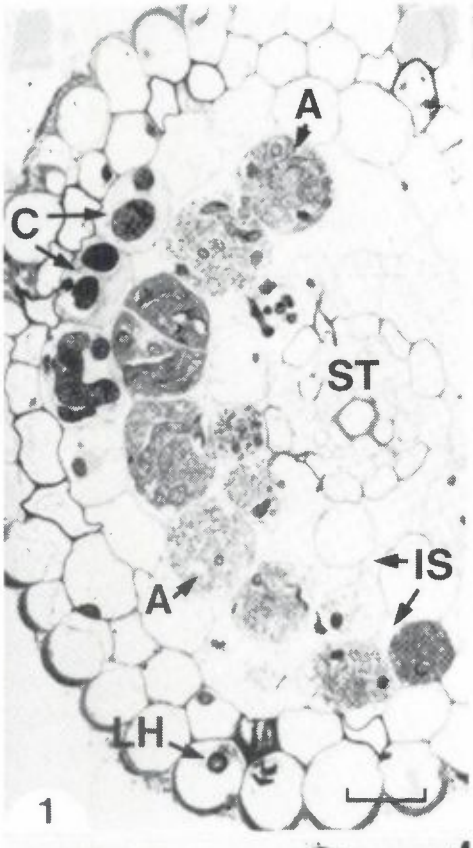
Thin sections were examined using a Hitachi 600 electron microscope at 75 Kv.

3. Results

No differences were observed in infection morphology and ultrastructual features of naturally or control-infected mycorrhizal roots of *G. lutea* and the following observations are pertinent to either.

General structure of infected roots of G. lutea

In transverse sections, *G. lutea* roots had two distinct outer cell layers and three to four layers of large parenchymal cells surrounding the central stele (Fig. 1). The latter was never colonised by fungal hyphae. Intracellular hyphae were observed in the two outer layers of cells but they did not spread laterally in the root tissue. These hyphae were distinguishable by only one cross section per cell, indicating that they crossed these cells linearly without branching. In contrast, when the fungus reached the large parenchymal cortical cells, the infection spread intensely from cell to cell and as many as 40% of parenchymal cells in anyone section could be colonised. In this root tissue, two intracellular hyphal formations could be distinguished in the light microscope: coils, represented by several sections of large hyphae, developed in some cells, while others were entirely filled by numberous hyphae of different diameters, indicating the development of much-branched arbuscular structures. Hyphae never developed in the intercellular spaces of *G. lutea* roots.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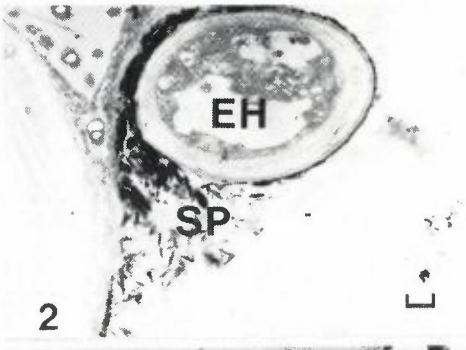
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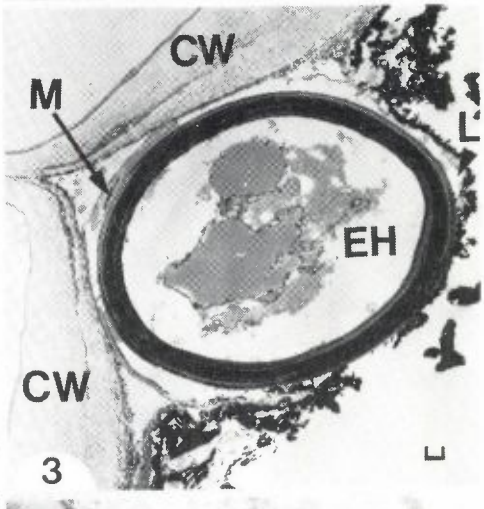
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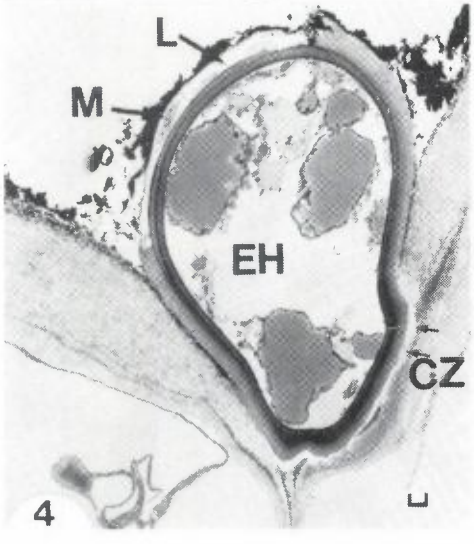
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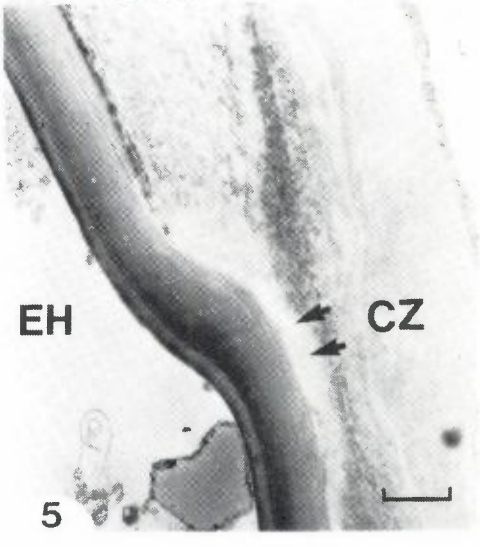
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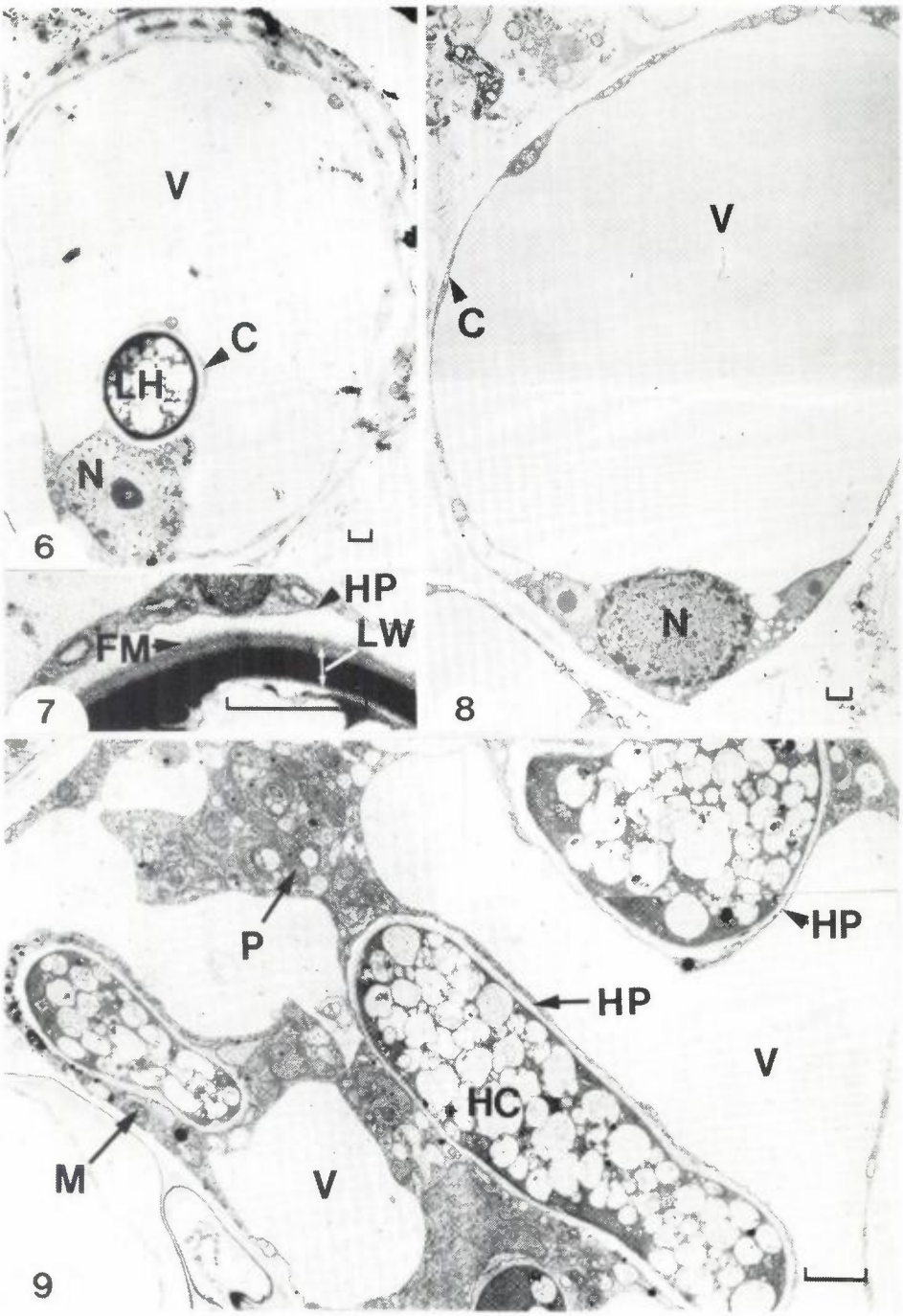
Figures 1–5. (1) Light micrograph of a *G. lutea* root infected by *G. intraradices*, in cross section; a linear hypha (LH) is in an outer cell and coils (C) or arbuscules (A) in parenchymal cells. No hyphae are in the intercellular spaces (IS) or stele (ST). (2) An external hypha (EH) of *G. mosseae* surrounded by soil particles (SP) in the mucilage layer. (3) An external hypha (EH) of *G. intraradices* covered by mucilage and in contact with the walls of two cells (CW) where they meet. Lacunae (L) in the hyphal wall contain a microorganism (M). (4) Idem 3; external hypha entering the root. A clear zone (CZ) forms in the host wall between the symbionts. (5) Detail of 4. Bar represents 25 μm in Fig. 1 and 0.5 μm in Figs. 2 to 5.

Root penetration and hypha development in the outer root layers of G. lutea

Hyphae growing over the root surface of *G. lutea* developed in the mucilage layer, which could be distinguished due to the electron-dense soil particles it contained (Fig. 2), then came into contact with the external cells of the roots (Fig. 3). External root cells were characterised by a thick wall consisting of an inner layer (primary wall), a thin electron-dense layer (pectin layer ?) and an external wall layer which thinned out at the junction of two cells (Figs. 3 and 4). With wall to wall contact between the two partners, hyphae became enclosed by the mucilage; this stage can be considered as the first step in the morphological integration between the mycorrhizal fungus and the host root. Subsequently, hyphal penetration into the root tissues occurred, most often at the junction between two epidermal cells where the external wall was thinnest (Fig. 4).

The walls of external hyphae were very thick (0.4–0.6 μm) (Table 1) and pluristratified (Figs. 2, 3, 4 and 5). The outer wall layer frequently consisted of large electron-translucent lacunae, similar to those described for *G. fasciculatum* by Bonfante-Fasolo and Grippiolo (1982). Bacteria-like organisms were present in the lacunae (Figs. 3 and 4); if these had a lytic activity, they could contribute to the disappearance of the external wall layer. Hyphal penetration of the root tissues was characterised by deformation and a slight thinning out of the fungal wall (Fig. 4). An irregular electron-translucent zone could be observed in the host cell wall at the zone of contact with the fungal hypha (Fig. 5); this is suggestive of localised enzymic degradation of the host wall.

Cells in the outer root tissues showed little reaction to fungal penetration (Fig. 6); hyphae were surrounded by host plasmalemma and a thin layer of protoplasm, with the host nucleus frequently situated close to the fungus. Host plasmalemma and fungal wall were always separated by a fibrillar layer of material (Fig. 7). The host cell otherwise retained the aspect of an un-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Figures 6–9. (6) Linear hypha (LH) of *G. intraradices* in an outermost root cell, surrounded by host cytoplasm (C). The host cell has a large vacuole (V) and a nucleus (N) close to the hypha. (7) Detail of 6, showing the two layered hyphal wall (LW) surrounded by host plasmalemma (HP) and fibrillar material (FM). (8) An uninfected parenchymal cell with a large central vacuole (V), a peripheral nucleus (N) and cytoplasm (C). (9) Sections of a coiled hypha (HC) of *G. intraradices* in a parenchymal cell and surrounded by host plasmalemma (HP), vacuole (V) and cytoplasm rich in mitochondria (M) and plastids (P). Bar represents 1 μm in Figs. 6 to 9.

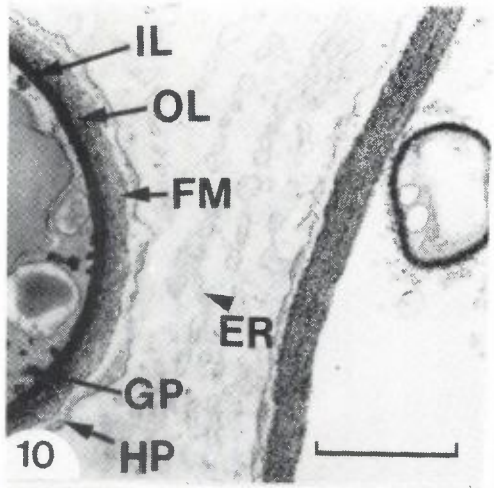
infected cell. Hyphae in these cells had a rather thick wall (0.2–0.3 μm) (Table 1) with an electron-dense inner and a lighter outer layer, and contained cytoplasm with numerous small vacuoles (Figs. 6 and 7).

Cellular modifications during fungal colonisation of parenchymal cortical cells

Uninfected parenchymal cells of *G. lutea* roots were characterised by a large central vacuole, delimited by the tonoplast, and filling practically the entire volume of the cell (Fig. 8). The thin layer of peripheral cytoplasm contained all the other organelles (nucleus, mitochondria, plastids, ribosomes, endoplasmic reticulum).

With infection of these cortical cells, the mycorrhizal fungus first developed large hyphal coils, represented by several large (about 4 μm) sections per cell, and the host vacuole became divided into several interconnected compartments of different sizes (Fig. 9). Continuous host plasmalemma extended around the hyphae and irregular amounts of host cytoplasm surrounded each hyphal section. Numerous organelles could be observed in the host protoplast: plastids, mitochondria, endoplasmic reticulum (Figs. 9 and 10) and dictyosomes. This aspect of the host protoplast indicated an enhanced metabolic activity in the host cell with fungal invasion, and contrasted with the weak host reaction observed in the outer cell layers of the root. Host plasmalemma was positively stained by the PATAg reaction (Fig. 10) but less by the Swift reaction (Fig. 11). The interfacial matrix between the host plasmalemma and the fungal wall formed an electron-translucent area after contrasting with uranyl acetate and lead citrate (Fig. 9), but the PATAg test revealed the presence of polysaccharide material (Fig. 10), perhaps of host origin.

Fungal walls became a little thinner (0.1–0.2 μm) (Table 1) as hyphae formed coils; the PATAg reaction revealed a two-layered wall structure with an electron dense inner and a lighter outer zone (Fig. 10), while only one wall layer was stained by the Swift test (Fig. 11). The coiled hyphae con-



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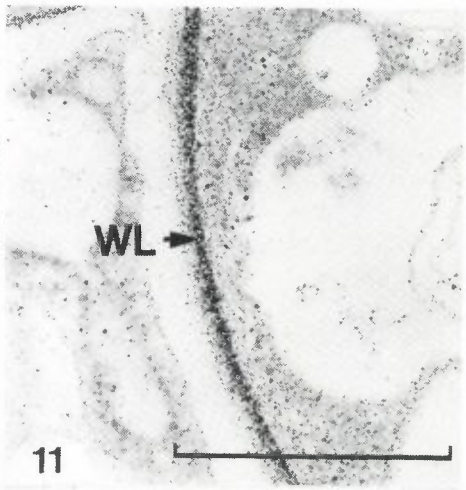

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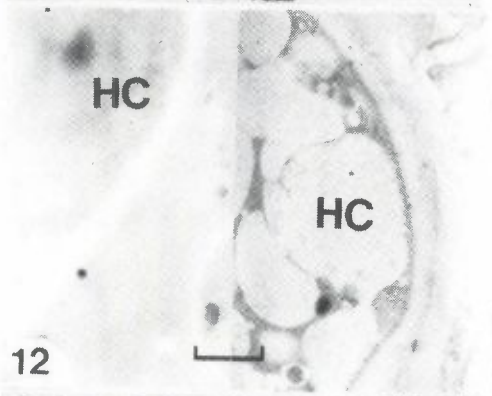

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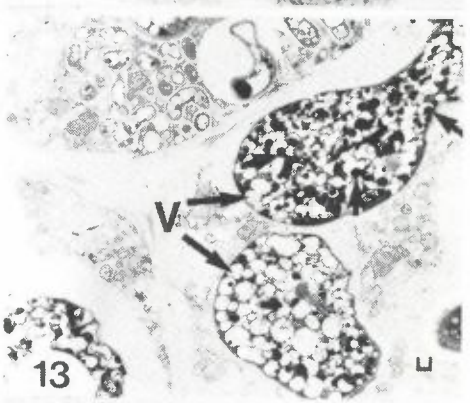

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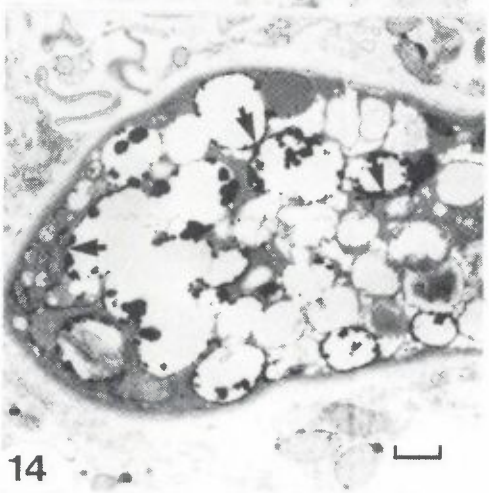

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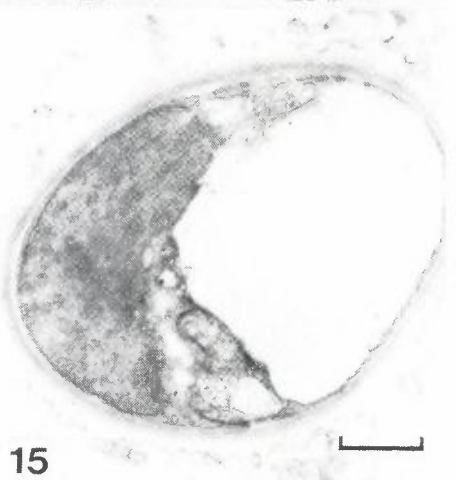

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Figures 10–15. (10) Hyphal coil (HC) of *G. intraradices* after the PATAg reaction revealing a dark inner (IL) and lighter outer wall layer (OL), covered by fibrillar material (FM). Glycogen particles (GP) and host plasmalemma (HP) are positively stained; endoplasmic reticulum (ER) is in the host cytoplasm. (11) Idem 10 after the Swift test which stains one wall layer (WL). (12) Controls of PATAg (a) and Swift (b) reactions. (13) Hyphal coil of *G. intraradices* with numerous, small vacuoles (V) enclosing osmiophilic granules (arrows). (14) Alkaline phosphatase activity (arrows) along the tonoplast of *G. mosseae*. (15) KCN control of 14. Bar represents 0.6 μm in Figs. 10 to 15.

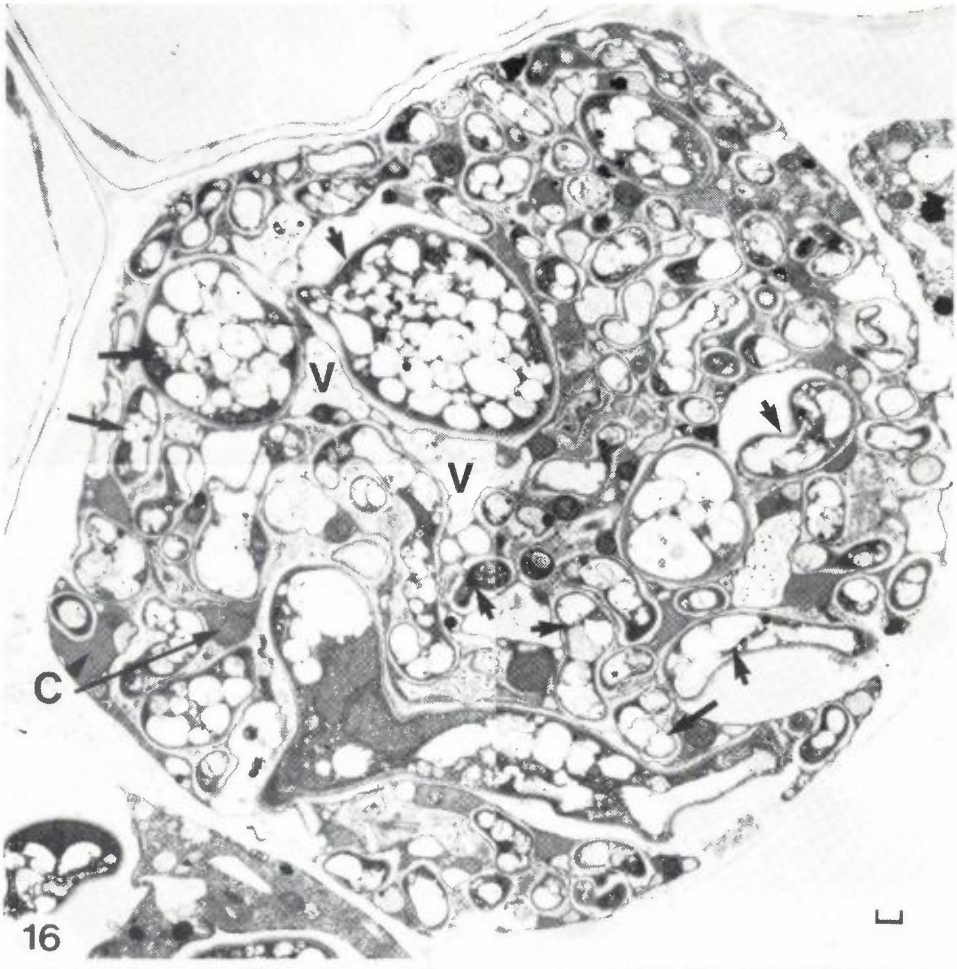
tained cytoplasm, nuclei, mitochondria, PATAg positive glycogen particles (Fig. 10) and numerous, small vacuoles which often enclosed alkaline phosphatase activities (Fig. 14) and osmiophilic granules (Fig. 13) described as polyphosphate by other authors (Cox et al., 1975; Strullu et al., 1981).

As the fungus continued to develop in these host cells, numerous small hyphal sections could be observed dispersed around the large sections (Fig. 16). These represented fine hyphal branches arising by repeated division from the hyphal coil; the diameter of the smallest was about 0.4 μm, the size of a host mitochondrion. Fungal hyphae filled the host cell which completely lost its initial aspect: very little remained of the vacuole, and cytoplasm scattered among the fungal branches was rich in organelles, especially mitochondria and plastids (Fig. 16). Each section of fungal hypha was continuously surrounded by PATAg positive host plasmalemma (Fig. 17).

As this highly divided fungal structure formed, the fungal wall became extremely thin and in the smallest branches it was only 0.02 μm thick. Here, it reacted strongly with the PATAg and Swift tests to give one uniformly stained layer (Figs. 17 and 18). The fungal cytoplasm resembled that in the hyphal coils, except that plasmalemmasomes, stained by the PATAg and Swift reactions, were very frequent (Figs. 17 and 18).

Senescence of intracellular fungal structures

The first stages of fungal deterioration, both in outer root cells and in parenchymal cortical cells, were characterised by a deformation of the fungal wall and alteration in the hyphal contents, with the accumulation of moderately electron-dense lipidic material (Fig. 19). Deterioration of arbuscules began in the smallest branches and progressed to the arbuscule trunk. The metabolic activity of the host cell still appeared greatly enhanced during the early stages of fungal senescence; the host cytoplasm contained numerous mitochondria, plastids, dictyosomes and an extensive endoplasmic reticulum



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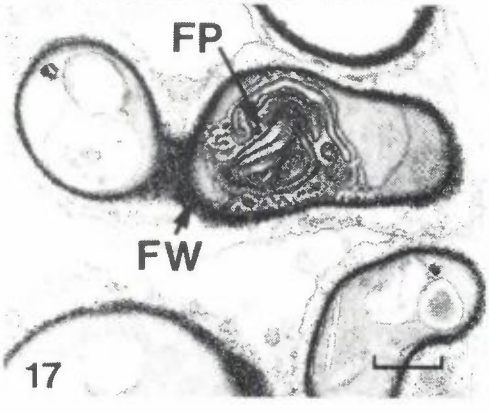

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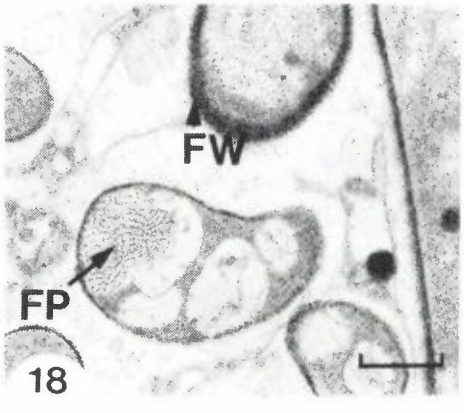

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Figures 16–18. (16) Cortical cell containing an arbuscule of *G. intraradices*. Host cytoplasm (C) rich in organelles surrounds hyphal sections (arrows) which fill the cell lumen; little host vacuole (V) remains. (17 and 18) Arbuscular hyphae of *G. intraradices* after the PATAg and Swift reaction respectively. The fungal wall (FW) and fungal plasmalemmasome (FP) react positively. Bar represents 0.3 μm in Figs. 16 to 18.

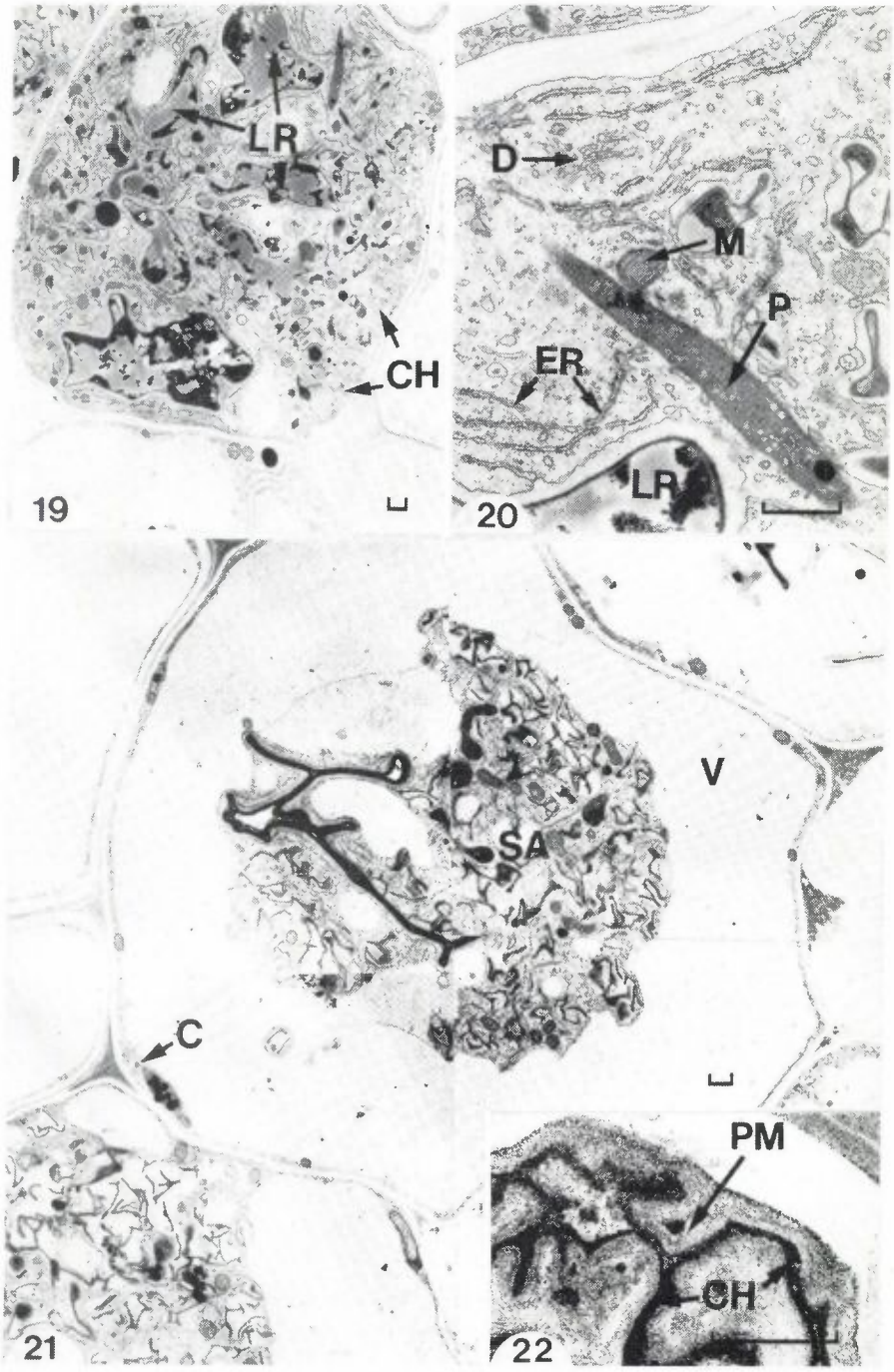
(Fig. 20). As fungal senescence advanced in a cell, all the hyphae emptied and the collapsed fungal walls clumped together (Figs. 19 and 21). The clumps of hyphal walls were still surrounded by a small amount of host cytoplasm, but at this stage of arbuscule senescence the host cell no longer had the aspect of an infected cell and it resembled an uninfected cell with a large vacuole and a thin layer of peripheral cytoplasm (Fig. 21). The collapsed fungal hyphae were encased by PATAg positive material (Fig. 22).

4. Discussion

The present ultrastructural observations of mycorrhizal roots of *G. lutea* (naturally or control-infected) complete and confirm previous light microscope descriptions (Jacquelinet-Jeanmougin and Gianinazzi-Pearson, 1983). The mycorrhizal infection only develops intensely in the parenchymal cortical cells and fungal spread from cell to cell in the host roots is entirely intracellular. Although intercellular spaces develop in the root cortex of *G. lutea*, intercellular hyphae have never been observed.

When a VA mycorrhizal fungus develops in roots of *G. lutea*, the morphology of the hyphae and the ultrastructural modifications occurring in the host cell are determined by the type of host cell that is colonised. A cell in the outer root layers shows very little reaction to the simple fungal hypha crossing it, while deep in the root cortex the fungal hyphae divide intensely and the host protoplast shows signs of an enhanced metabolic activity. The formation of highly branched structures in host cells confirms that arbuscules develop in this host plant; the reasons for arbuscule formation specifically occurring in host parenchymal cells and the mechanisms controlling it are not known. It is, however, interesting that host cell type has also been reported to influence plant-fungus interactions in pathogen infections (Hinch et al., 1985; Wetherbee et al., 1985).

The pattern of arbuscule development in *G. lutea* differs from that in most VA mycorrhizal associations, and this is probably responsible for the particular morphological features of endomycorrhiza in this plant. In most VA mycorrhizae, infection spreads in root tissues by intercellular hyphae and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Figures 19–22. A senescing arbuscule of *G. mosseae* with lipid-rich (LR) and collapsed hyphae (CH). (20) Detail of 19 showing a plastid (P), mitochondrion (M), dictyosome (D) and endoplasmic reticulum (ER) in host cytoplasm around lipid-rich (LR) hyphae. (21) Parenchymal cell containing a completely senescent arbuscule (SA) of *G. mosseae*, a large vacuole (V) and peripheral cytoplasm (C). (22) Collapsed hyphae (CH) of *G. intraradices* enclosed by polysaccharide material (PM) (PATAg reaction). Bar represents 0.8 μm in Figs. 19 to 22.

branches from these enter cortical cells to give rise to arbuscules, which in this case represent terminal haustoria in each cell (see Bonfante-Fasolo, 1984 and Gianinazzi-Pearson, 1984 for references). In contrast, hyphae that propagate the infection in *G. lutea* are uniquely intracellular and arbuscules arise directly from the hyphal coils that are formed as the fungus passes from one parenchymal cell to another. This pattern of arbuscule formation is quite similar to that observed in certain woody species like *Liriodendron tulipifera* and *Ginkgo biloba* (Kinden and Brown, 1975a,b; Bonfante-Fasolo and Fontana, 1985).

Hyphal penetration of *G. lutea* roots occurs preferentially at the junction of two host cells, suggesting that fungal passage may somehow be promoted here. It is certain that the thinner host walls at this site would greatly reduce the physical barrier for the fungus and so facilitate its entry into the root. This pattern of hyphal penetration at cell junctions is reminiscent of that described for certain pathogens (Hinch et al., 1985). The mechanisms by which VA mycorrhizal fungi penetrate host tissues are still unknown, but the present observations of fungal penetration into *G. lutea* roots indicate that these fungi probably possess cell wall degrading enzyme activities. Indeed, the electron-translucent zone observed in the host cell wall where it comes into contact with the fungal hypha suggests the existence of an enzymic destruction of the former. Kinden and Brown (1975a) and Gianinazzi-Pearson et al. (1981) have already suggested that enzymic processes may be involved in the spread of hyphae through the intercellular middle lamella material in other hosts.

The wall of VA mycorrhizal fungi thins out considerably as hyphae penetrate deep into the host root tissues; the wall of the arbuscule in the parenchymal cells of *G. lutea* was up to 30 times thinner than the wall of external hyphae (Table 1). This modification in the fungal wall seems to be characteristic of vesicular-arbuscular mycorrhiza formation; it has previously been described in associations with *Ornithogalum umbellatum* (Bonfante-Fasolo

Table 1. Wall thickness (μm) of hyphae of *G. intraradices* in association with *G. lutea* roots

• External hyphae	0.4–0.6
• Intracellular hyphae in the external root layers	0.2–0.03
• Intracellular hyphae in the parenchymal cortical cells forming:	
– coils	0.1–0.2
– arbuscules	0.02–0.04

and Scannerini, 1977), *Phaseolus vulgaris* (Holley and Peterson, 1979), *Rubus ideaus* (Gianinazzi-Pearson et al., 1981), *Vitis vinifera* (Bonfante-Fasolo and Grippiolo, 1982), *Trifolium repens* (Pons, 1984) and *Gingko biloba* (Bonfante-Fasolo and Fontana, 1985). The decrease in wall thickness is accompanied by an important simplification in its texture, composition and molecular organisation (Bonfante-Fasolo and Gianinazzi-Pearson, 1986). In *G. lutea* roots, the formation of a simplified fungal wall where there is hyperramification of the arbuscule creates an exceptionally large surface of contact and a greatly reduced apoplast between the VA mycorrhizal fungus and the host protoplast. This structure must be particularly favourable to solute movement between the symbionts and may therefore explain, at least partly, how a relatively low degree of mycorrhiza infection can be highly effective in the phosphate nutrition of the host plant (Jacquelinet-Jeanmougin et al., 1986). The present study did not give any clues,however, as to why *G. lutea* roots, which are receptive to infection by VA mycorrhizal fungi, limit the spread of these symbionts once inside the root tissues (McGee, 1985; Jacquelinet-Jeanmougin et al., 1986). There was no obvious impairment of fungal physiology; the ultrastructural features of hyphae and their contents (vacuolar alkaline phosphatase, polyphosphate granules, glycogen and lipid accumulation) were identical to those observed in VA mycorrhizal fungi in association with other hosts (see Bonfante-Fasolo, 1984 and Gianinazzi-Pearson, 1984). Other aspects of the physiological relationship between fungus and root in gentianaceous species could differ from that in 'typical' VA mycorrhizas (McGee, 1985). The possibility, for example, that certain unusual carbohydrates, or their derivatives, found uniquely in the Gentianaceae may somehow be implicated in this particular host control over the fungal symbiont (Lewis, 1986) deserves further investigation.

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