Ultrastructural aspects of rubber tree root rot diseases

By M. Nicole, J. P. Geiger and D. Nandris

Abstract

The aggression of Hevea brasiliensis roots by two root-rot fungi, Rigidoporus lignosus and Phellinus noxius is studied at cellular level. Ultrastructural observations reveal the root penetration and tissues colonization by the fungal hyphae, showing clearly alteration in the host cell wall, leading to the root decay. This paper describes the different mechanisms used by these two fungi in degrading lignified and non lignified tissues.

1 Introduction

Rigidoporus lignosus and Phellinus noxius are two of the most severe decay-causing fungi of living trees in West Africa (Piché 1956) especially in the Ivory Coast (Nandris et al. 1985). The root rot they cause is a lethal disease of Hevea brasiliensis (rubber tree). The fungi, living in the soil, contaminate the trees by developing rhizomorphs (R. lignosus) or mycelial sleeves (P. noxius) respectively. Light microscopical observations have shown that these organisms penetrate the tap root of rubber trees through lenticels or wounds and then destroy a large part of root tissues (Nicole et al. 1982 a). Biochemical studies confirm that the major cell wall components are degraded by extracellular fungal enzymes (Geiger et al. 1986a, c, d). But little information is available on the ultrastructure of white rots caused by R. lignosus and P. noxius.

This paper reports some accurate observations on H. brasiliensis infected with these fungi in an attempt to clarify the infective process of both parasites which was investigated separately in two parallel experimental protocols, to examine whether differences exist.

2 Material and methods

2.1 Pathogen material and inoculum

Isolates of R. lignosus (strain 1) and of P. noxius (strain 4) were collected in 1978 on rubber trees naturally infected in plantations. Sticks of rubber tree wood (8 cm long by 2 cm diam.) were sterilized in Roux flasks and seeded with 8 mycelial implants collected from a 2 % malt-agar culture of each fungus. Because of the different degradative abilities of each parasite, the incubation before plant inoculation lasted 5 months for P. noxius and 11 months for R. lignosus. Such infected segments constitute the inoculum.

2.2 Artificial infection of rubber trees

The following methods were applied under controlled conditions (Nandris et al. 1983). Seed of H. brasiliensis (clone GT1) was collected in plantations of the rubber research
institute of the Ivory Coast (IRCA). After germination in sandy tubs, the young seedlings were pricked in tubs (1×1×1 m) filled with forest soil in which a high humidity level was monitored by watering to saturation. The control of this level was realized with a neutronic moisture gauge (Solo 20). For each one-month-old plant, five inoculum segments were fixed to the tap root at 20 cm depth in the soil. Infected rubber trees were then collected at different stages of infection (Nicole et al. 1983) for electron microscopy preparation.

2.3 Transmission electron microscopy

Classical techniques of plant preparation for electron microscopy were modified and adapted to rubber root tissues as follows:

The infected and non-infected plants were immediately fixed “in toto”, after sampling, with 3 % glutaraldehyde (2 h, 4°C) buffered with 0.1 M sodium cacodylate (pH 7.2). The roots were then cut in small segments and fixed again (2 h, 4°C). After several washings in buffer (4 × 15 min. and one night, 4°C), the samples were postfixed (2 h, 4°C) in 1 % osmium tetroxid. Following a further rinse in sodium cacodylate, the segments were dehydrated in a gradual ethanol series from 5 to 100 % and embedded in Spurr’s resin (Spurr 1969) or in Epon 812 (Luft 1961) preceded of an epoxy propane change (2 × 1 h duration). Because of the woody nature of the roots, the pieces were infiltrated in resin for at least 5 days. Polymerisation was carried out for 3 days at 70°C. Sections of 60–50 nm were cut with glass or diamond knives on a LKB ultramicrotom, and mounted on 200 mesh grids. After staining with a saturated alcohol solution of uranyl acetate (5 min.), followed by lead citrate (Reynolds 1963), the sections were examined on a Siemens Elmiskop 102 electron microscope, operating at 80 kv.

2.4 Scanning electron microscopy

After the double fixation (glutaraldehyde-osmium tetroxid), samples of infected roots were dehydrated in a gradual aceton series and then subsequently dried in a critical point drying apparatus. Blocks were coated with gold and examined under a Stereoscan 180 microscope.

3 Results

3.1 Root penetration

Microscopical observations of R. lignosus rhizomorphs spreading along the root show differences in the hyphal wall thickness which ranges between 0.2 and 0.7 μm (Fig. 1, 2). The medium layer, which constitutes 60 to 70 % of the wall, is reticulated. When hyphae invade the root, the fungal wall thickness decreases to less than 0.2 μm (Fig. 3). Only the inner side of the fungal wall is affected by this modification (Fig. 4).

During active penetration, intercellular spaces between two peridermic cells (Fig. 5) can be highly degraded making the decay resemble a lace (Fig. 6). The dissolution of the middle lamella appears to facilitate the penetration of hyphae. Such observations were not conducted on roots infected with P. noxius because a crusty mycelial sleeve along the root, consisting of stones, sand and hyphae, prevents accurate observations.

For both fungi, penetration occurs through lenticels or wounds. R. lignosus-rhizomorphs exert a pressure on the cortical tissues until they tear (Fig. 7). This mechanical action is relayed by a more active mechanism causing the loss of the cortical cells rigidity. Examination of Figure 8 reveals a granular zone in front of the apex of some hyphae, suggesting a cell wall degradation by fungal secretions.
Abbreviations used for the figures: c: host cell; cw: cellulosic cell wall; h: hypha; mh: microhypha; mi: mitochondria; ml: middle lamella; pw: primary wall; r: rhizomorph; s: suber; st: sieve tube; sw: suberized wall; S1, S2, S3: secondary wall layers; v: vacuole – Fig. 1. Extracellular hypha in a rhizomorph of *R. lignosus*, note the thickness of the fungal wall. Its central fraction is reticulated (arrow); (x 15000) – Fig. 2. Scanning electron-micrograph of a *R. lignosus*-rhizomorph in contact with the host root showing both hyphae with thick and thin wall; (x 500) – Fig. 3. Intracellular position in an host cell; the wall thickness of *R. lignosus* hyphae does not reach more than 0.2 μm (arrow); (x 10000) – Fig. 4. Decrease of fungal wall thickness during penetration, hyphae with special structures (*) for perforation the wall of peridermic cells; only the inner side of the fungal wall seems to be affected by this transformation (arrows); (x 12500) – Fig. 5. Degradation of the intercellular space between two peridermic cells during active penetration by *R. lignosus* hyphae; intercellular material is eroded in contact with the hyphae (arrows); (x 5000)
Fig. 6. The highly degraded intercellular space shows the aspect of a lace; (× 10000) – Fig. 7. Mechanic penetration of a rhizomorph of *R. lignosus* in a host root. The pressure exerts by the hyphae on the cortical tissue causes the degradation of the suberized cells in contact with the rhizomorph (arrows); (× 400) – Fig. 8. Host cell wall degradation around *R. lignosus* during root penetration; note the granular zone limited to the mycelium, which may indicate an enzymatic alteration of the wall (arrows); (× 10000) – Fig. 9. Intercellular colonization of cortical tissues; adjacent cells are separated by a fungal ramification differentiated during the fungal growth (arrow); (× 5000)

3.2 Colonization and degradation of non lignified tissues

Non lignified tissues of a young rubber tree are among other constituted of primary parenchyma (until 5 weeks old), suber and phloem. Phloem is highly differentiated and contains parenchymatous rays, companion cells, sieve tubes, tannin cells and laticifers. Thus, before wood invasion, *R. lignosus* and *P. noxius* have to degrade the major cell wall components of these tissues: cellulose, hemi-cellulose, pectin and suberin.
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Fig. 10. The intracellular position of hyphae in cortical tissues result in wall-perforation; the digestion of the cellulosic framework is first characterized by granular zone near the fungal filament (arrow); (× 8000) – Fig. 11. Cellulosic wall penetration; the enzymatic degradation of the wall (double arrows) is completed by mechanical processes (arrows); (× 15000) – Fig. 12. Aveolar aspects (arrows) of a cellulosic wall of a phloem cell degraded by *R. lignosus*, the fibrillar structure of the wall is profoundly pertubated (× 8000) – Fig. 13. Middle lamella and cellulose fibers of a sieve tube cell wall in a healthy phloem; (× 20000) – Fig. 14. The degradation of the middle lamella causes the increase of its osmiophilic properties (arrows) before its perforation; (× 10000)

3.2.1 Colonization

Intercellular and intracellular positions are adopted by both fungi. The location in the middle lamella causes pectin dissolution, favoured by hyphal ramifications (Fig. 9). Moving through the wall results in a digestion of this wall. The perforation begins with a digestive action (Fig. 10) which is often completed by a mechanical process (Fig. 11), as it is revealed by the compression of wall cellulose fibers during fungal growth.
3.2.2 Degradation

The degradation of cell walls occurs in contact with the hyphae or at some distance in front of them. In the parenchymatous cells, the cellulosic wall turns into an alveolar structure and loses its fibrillar structure (Figs. 12 and 13). The degradation is often achieved by the loss of the wall. Separation of cells is accompanied, especially in the secondary phloem, by a conspicuous change in the intercellular space. The osmiophilic properties of the middle lamella increase (Fig. 14) and after its perforation, the erosion extends to the cellulosic wall (Fig. 15) before the complete digestion.

The progression of *R. lignosus* and *P. noxius* in a young bark of rubber tree roots also affects suberized cell walls. The degradation is characterized by the increase of the wall electron opacity or by a direct perforation of walls (Fig. 16).
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Fig. 19. After meatus formation resulting in an alteration of middle lamella, the digestive action extends to the primary wall (arrows); (x 20000) – Fig. 20. Organization of walls of a xylem vessel of a healthy young root; (x 12500) – Figs. 21 and 22. Wood degradation by R. lignosus: a cross section through a vessel wall shows the dissolution of the S1/S2 border layer of the secondary wall characterized by small cavities (arrows) (21); the alteration then extends to the S2 layer (arrows) (22). (Fig. 21: x 25000; Fig. 22: x 15000) – Fig. 23. Major wood degradation and disorganization by P. norius affect mainly the secondary wall which is highly eroded (arrows); (x 40000) – Fig. 24. Digestion of the middle lamella and the primary wall of a lignified cell by P. norius; note the dislocation of the S1 and S2 layers (arrows); (x 15000)

3.3 Colonization and degradation of lignified tissues

3.3.1 Wood colonization

In vivo, xylem invasion by R. lignosus is independent of cortical tissue-colonization and occurs through the parenchymatous rays (Nicole et al. 1982a). The hyphae can be found both within ray cells and the lumen of vessels. Their propagation from cell to cell frequently proceeds through pit membranes which are progressively eroded. The differentiation of microhyphae is rare (Fig. 17). For P. norius, wood invasion starts after the colonization of all cortical tissues. The development of hyphae is similar to R. lignosus.
3.3.2 Wood degradation by R. lignosus

The cell wall degradation is progressive and proceeds in contact with or in front of hyphae. The alteration occurs inwards, from the middle lamella towards the lumen. Two stages characterize the middle lamella and the primary wall degradation: a) an apparition of a granular matrix, after the disorganization of the native structure (Fig. 18); b) a progressive perforation of this matrix, causing meatus formation (Fig. 19). Then, the alteration reaches the less lignified secondary wall. The junction of S1 and S2 layer is particularly susceptible to the fungal action. Indeed, small cavities appear at this level (Figs. 20 and 21), amalgamate and then extend inwards until causing the disparition of the half S2 layer (Fig. 22), showing a strong digestion of the wall framework. S3 layer is saved up by this mechanism. When the progression of decay occurs outwards, the S3 layer is degraded; on this case only S2 and S1 layers are less altered.

3.3.3 Wood degradation by P. noxius

Unlike R. lignosus, wall degradation by P. noxius occurs as well inwards as outwards, in contact or not with the mycelium. However, the secondary wall is more susceptible to the enzymatic action (Fig. 23). This degradation often begins by the apparition of a strong osmiophilic reaction of the wall. The middle lamella and the primary wall generally show less alteration and complete digestion was rare (Fig. 24).

4 Discussion

The decrease of the wall thickness of R. lignosus hyphae is a major fact occurring in root penetration; it corresponds an important stage of the biological cycle of this fungus. Indeed, two types of mycelium have been identified with fundamental differences in morphological characteristics (BOISSON 1973) and in enzymatic secretions (GEIGER 1975). One type of mycelium (called B) is specified in propagation by rhizomorph formation, while the other (A) possesses the infectious potential.

So, the decrease of wall thickness during penetration can be related to the transformation of mycelium B into mycelium A. Cytological tests have shown that polysaccharid components of the wall are involved in this modification (unpublished data). The chitin level being constant in the wall of both A and B mycelium (NICOLE 1982), other polymers may probably be hydrolysed during the wall transformation, for example α and β D-glucan, major polysaccharides of Basidiomycetae (BURNETT and TRINCI 1978; CHET and HÜTTERMANN 1980). This coincides with important modifications in the glucose pathway during B to A transformation, mainly under anaerobic environment (BAREYRE and BOISSON 1969). Such anoxic conditions are fundamental for the initiation or R. lignosus pathogenesis (NANDRIS et al. 1983).

Root penetration and colonization of non-lignified tissues by both fungi result in wall perforation caused by hyphal growth or (and) a chemical digestion (PERIES and IRUGAL-BANDARA 1973; NICOLE et al. 1982 a). Nevertheless, several features indicate that wall degradation is predominantly enzymatic: a) irrespective of the contact with the hyphae the cell wall is more electron-opaque than the rest of the wall; b) the fibrillar structure of cellulose and the structure of suberin are disorganized; c) the disintegration of tissues. Cellulases, xylanases, glucosidases, glycosidases and galactosidases were identified as causing cellulose and hemi-cellulose degradation, and pectic enzymes – polygalacturonase, pectin methyl-esterase, pectate lyase – as causing middle lamella alteration (GEIGER et al. 1986a, d). However, their activities are higher in P. noxius, especially for the pectic enzymes thus explaining the faster alteration of these tissues by this fungus. As
reported by other authors (FERNANDO et al. 1984; ZIMMERMANN and SEEMÜLLER 1984) the significance of suberin-degrading enzymes in root penetration and suberin alteration can also be strongly suggested (NICOLE et al. 1986). Evidences given by microscopic observations are completed by the characterization of such enzymes in culture filtrates of both fungi (GEIGER et al. 1986a).

Wood invasion by *R. lignosus* and *P. noxius* is based upon the active penetration of cells, as described in other wood rotting fungi (CHOU and LEVI 1971; PECK et al. 1972). Differences appear mainly during xylem degradation. Wood alteration of young rubber tree seedlings by *R. lignosus* is selective, and occurs inwards from the middle lamella to the secondary wall, removing first the lignin-rich fraction and then polysaccharids. Except for Eastern Hemlock infected with *Ganoderma tsugae* (BLANCHETTE 1984), such a degradation has rarely been reported infecting living trees, but mainly in vitro on sterilized wood-blocks (SANTRA and NANDI 1975; NOGUCHI et al. 1980). These observations suggest that *R. lignosus* possesses effective lignolytic potentialities. The laccases (E.C. 1.30.3.2.), fungal extracellular enzymes probably involved in lignin decomposition (ANDER and ERIKSSON 1976 and 1977; NOGUCHI et al. 1980), have been characterized in rubber tree tissues infected with *R. lignosus* (GEIGER 1976, 1986a). One of these laccases, the L1 laccase, induces a modification of the polymerization degree of a rubber tree thioglycolic lignin (TGL), leading to both condensation and depolymerization of this macromolecule (GEIGER et al. 1986c). The partial resistance of the S3 layer to the enzymatic attack, also suggested by other authors (COWLING 1961; LIESE 1970; DIROL and RAVILLY 1979), may result from the low porosity of this layer, limiting the enzyme-diffusibility. The presence of some enzyme inhibitors of phenolic origin has been envisaged (RUEL et al. 1981).

Wood alteration of roots of rubber trees by *P. noxius* is unspecific. Nevertheless, the middle lamella and the primary wall decomposition seem to be lower than the secondary wall degradation, unlike *P. pini* (BLANCHETTE 1979) and *P. isabellinus* (ANDER and ERIKSSON 1976 and 1977) which selectively attack the lignified structures. A laccase has also been identified and isolated from *P. noxius* infected tissues (GEIGER et al. 1986b). Meanwhile, this enzyme differs from *R. lignosus* laccases by its molecular weight and its lower activity, explaining the lower lignin degradation observed in roots.

This ultrastructural study, performed in vivo, describes some aspects of *H. brasiliensis* root colonization and decay, while the main works on wood degradation by white root rot fungi were generally realized in vitro (SANTRA and NANDI 1975; NOGUCHI et al. 1980; MURMANIS et al. 1984; RUEL et al. 1984; HIGHLEY and MURMANIS 1984). This work completes biochemical investigations described on this host-parasite system (GEIGER et al. 1986a). Although the role of extracellular enzymes of *R. lignosus* and *P. noxius* in wall alteration is partly understood, their role in pathogenesis remains nevertheless undetermined. Involvement of other fungal secretions in the infection is possible, too (NICOLE et al. 1982). Indeed, PERIES (1959) has characterized a thermostable toxin produced in vitro by different strains of *R. lignosus* in Sri Lanka; but no correlations have been established with its pathogenicity.

Summary

In order to describe root colonization and degradation an ultrastructural study was performed on young rubber trees infected with two root rot fungi, *Rigidoporus lignosus* and *Phellinus noxius*. Inoculation of roots took place by passive mechanisms or after cell wall perforation. In root tissues, both fungi have been observed in intracellular, intercellular and intraparietal positions. Components of different root tissues—suberin, pectin, cellulose, hemi-cellulose and lignin—are digested and cell walls disorganized by these parasites. Colonization and degradation of non-lignified tissues are faster with *P. noxius*. Xylem degradation by *R. lignosus* begins in strongly lignified regions—middle lamella and primary wall—and extends towards the secondary wall. The S3 layer is rarely altered, however. On the other hand, wood degradation by *P. noxius* is not selective; the layers, enriched with lignin and the middle lamella seem to be less eroded.
The role of degrading-enzymes for the infection process is discussed in comparison to biochemical data recorded elsewhere.

Résumé

Aspects ultrastructuraux des pourridiés d’Hevea brasiliensis

Une étude ultrastructurale a été réalisée sur de jeunes Hévéa infectés artificiellement par deux champignons agents de pourridié: Rigidoporus lignosus et Phellinus noxius. Les différentes étapes de la colonisation et de la dégradation du système racinaire sont décrites dans cet article. La pénétration de la racine par les parasites s’effectue par les voies naturelles ou après perforation de la paroi. Dans les tissus, les hyphes ont été observées tant dans les cellules qu’en positions intercellulaire et intrapariétale. Les composants des parois cellulaires hôtes sont par conséquent soit désorganisés soit dégradés par chacun des champignons. L’altération des tissus non lignifiés par P. noxius est cependant plus rapide que celle réalisée par R. lignosus. À l’inverse, ce dernier est plus actif dans les tissus lignifiés.

Le rôle des enzymes dégradantes dans le processus infectieux de chaque parasite est discuté en comparaison avec les résultats biochimiques acquis par ailleurs.

Zusammenfassung

Feinstrukturen im Zusammenhang mit Wurzelfäule an Hevea brasiliensis


Abschließend wird die Bedeutung von Enzymen für den Infektionsvorgang unter Berücksichtigung der einschlägigen biochemischen Literatur diskutiert.

Literature


ULTRASTRUCTURAL ASPECTS OF RUBBER TREE ROOT ROT DISEASES


Authors’ address: Dr. M. NICOLE; Dr. J. P. GEIGER, Dr. D. NANDRIS, Laboratoire de Phytopathologie, ORSTOM, BPV 51, Abidjan, Ivory Coast

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