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Forestry Canada, Quebec Region, Sainte-Foy, Quebec, Canada

Cellulose is degraded during phloem necrosis of Hevea brasiliensis

By M. NICOLE, H. CHAMBERLAND, D. NANDRIS and G. B. OUELLETTE

Abstract

A cytochemical investigation was performed for studying cellulose alteration during phloem necrosis of the rubber tree. An exoglucanase-gold complex was used for localizing β-(1,4)-D-glucan linkages of non reducing ends of the polymer. Variation in cell wall labelling indicates that cellulose is degraded during this disease and suggests that cellulases are involved in this alteration. Wall appositions and periplasmic vesicles were also observed.

1 Introduction

In West African plantations, a disease affecting *Hevea brasiliensis* since several years, causes severe disturbances of the phloem, the latice vessel-containing tissue. This disease, of which the etiology remains unknown, was called "phloem necrosis" when discovered in the Ivory Coast a few years ago (NANDRIS et al. 1984). Biochemical studies of latex characteristics demonstrated that phloem necrosis differs from other phloem disorders such as tapping panel dryness (NANDRIS et al. 1991a). Phloem necrosis is mainly characterized by the occurrence of small gray circular patches and thin cracks on the surface of the bark where woodborer holes also cause latex to flow. The necrosis first appears as brown sheets located within the feeder phloem, close to the vascular cambium, and then leads to a disorganization of the tissue. Eventually, affected trunks become deformed through cracking of the bark between the collar and the tapping area (NANDRIS et al. 1984). Despite a wide range of experiments dealing with identification of possible causal microorganisms (NANDRIS et al. 1991b), no pathogen has been identified so far, although several data suggested a biological origin of phloem necrosis (NANDRIS et al. 1991b; NICOLE et al. 1991).

A recent cytological investigation has greatly improved our knowledge of cellular pertubations occurring during phloem necrosis (NICOLE et al. 1991). It demonstrated major alterations of cytoplasm, and walls of phloem cells such as tylosoid formation in latice vessels, and vesiculation of endomembranes of sieve tubes and parenchymatous cells. Ultrastructural observations also revealed strong modification of cellulosic walls of all cell types, mainly in proximity of highly disorganized plasmodesmata. In addition, vesicles and broken membrane pieces were seen in association with fibrils close to cell walls. These ultrastructural studies strongly suggest that degradative processes are involved in phloem cell wall breakdown.

Enzymes conjugated to colloidal gold have been widely used in plant biology to localize specific substrates (BENDAYAN 1985). The exoglucanase-gold complex presents a high binding affinity for β-(1–4)-D glucans widely distributed in non reducing ends of cellulose chains (BERG et al. 1988; BENHAMOU 1989; BENHAMOU et al. 1990). It was successfully used for studying the cellulose localization in various pathosystems (BENHAMOU et al. 1987; BLANCHETTE et al. 1989; CHAMBERLAND et al. 1989; BONFANTE-FASOLO et al.

1990; NICOLE and BENHAMOU 1991). The present paper reports a cytochemical investigation of phloem necrosis in rubber trees by means of an exoglucanase-gold complex with the aim to gain a better understanding of cellulose alteration during this disease.

2 Materials and methods

2.1 Tissue processing

Samples from healthy and diseased rubber trees were processed as described previously (NICOLE et al. 1986, 1991). Briefly, glutaraldehyde (3 % aqueous solution) was injected into the phloem of selected rubber trees near the area sampling. After a few minutes, the prefixed phloem, including the cambium and some cells of the xylem, was removed from the tree, cut into small pieces in 3 % cold glutaraldehyde buffered to pH 7.2 with 0.1 M sodium cacodylate, and fixed for 2 h in the same solution. After several washings in the buffer, fragments of the xylem were discarded and the samples were then postfixed with 1 % osmium tetroxide in cacodylate buffer for 2 h at 4 °C. Following a further rinse in the buffer, samples were dehydrated in a graded series of ethanol and embedded in Epon 812.

2.2 Enzyme

The exoglucanase used in this study was a gift from Dr. C. Breuil (Forintek, Canada). It was purified in a five-step procedure from a cellulase produced by the fungus *Trichoderma harzianum* Rifai. (SADLER et al. 1982). This enzyme presents a high affinity for β-(1–4)-D glucans widely distributed in non reducing ends of cellulose chains. The isoelectric point of the enzyme was found to be 4.5–4.8 and its optimal activity pH around 6.5.

2.3 Cytochemical experiment

Colloidal gold with particles averaging 15 nm in diameter was prepared according to FRENS (1973). Ten millilitres of the gold suspension, adjusted to pH 6.0 using 0.2 M K_2CO_3 was added to 0.5 mg of exoglucanase in 0.1 ml phosphate-buffered saline (PBS), pH 6.5; 0.5 ml of 1 % polyethylene glycol (PEG 20000) was further added and the mixture was centrifugated at 28000 × g for 60 min at 4 °C. The red mobile pellet was recovered in 0.5 mL of PBS, pH 6.5, containing 0.02 % PEG. This constituted the stock solution.

For labelling, sections of diseased phloem were first floated on a drop of PBS-PEG, pH 6.5 for 5 min, then incubated on a drop of the diluted enzyme-gold (1:2 or 1:4 in PBS-PEG, pH 6.5) complex for 30 min at room temperature, in a moist chamber. They were then thoroughly washed with PBS, pH 7.2, rinsed with distilled water, and contrasted with uranyl acetate and lead citrate, before examination under a JEOL 1200 EX electron microscope operating at 80 Kv.

Specificity of labelling was assessed by means of the following control tests: (i) incubation with the exoglucanase-gold complex to which 5 mg/ml of D-glucans from barley was previously added, (ii) incubation with a bovine serum albumin (BSA)-gold complex, a nonenzymatic protein-gold complex, and (iii) incubation with stabilized gold suspension.

2.4 Quantitative analysis

The intensity of labelling obtained with the enzyme-gold complex was quantitatively evaluated. Micrographs were taken at a primary magnification of ×10000 and enlarged to a final magnification of ×26000. Areas of walls in the necrosed phloem were visually selected on micrographs according to their apparent degradation (see Figs. 4 and 5)

[A: wall regions with no visible degradation, Fig. 4 (arrows); B: wall regions of moderate degradation, Fig. 4 (double arrows); C: highly degraded areas, Fig. 4 (arrowheads)]. Manual counting was then performed and the surface of each area was processed on a Leitz Tas Plus image analyser. The density of gold particles per square micron was calculated by dividing the number of granules by the surface area.

3 Results

The secondary phloem of *Hevea brasiliensis* is a complex tissue composed of several cell types; these include articulated and anastomosed laticiferous vessels arranged in concentric rings, tannin cells and parenchymatous cells closely associated with laticifers, sieve tubes, stone cells with sclerified walls, cells specialized for crystal production, and parenchymatous rays connected with wood rays. The organization of the phloem reveals two distinct zones:

– the soft inner phloem which lies close to the cambium, containing active sieve tubes and functional laticifers which are tapped for latex;

– the hard outer phloem which contains clustered stone cells and the older mantles of laticifers (for more details see De Fay and Jacob 1989).

Incubation of sections from healthy phloem trees with the exoglucanase-gold complex showed a regular distribution of gold particles over the walls of all cell types, including the middle lamella (Fig. 1). Walls of plasmodesmata areas were regularly labelled even in regions close to plasmodesmata channels (Fig. 2). In contrast, the cytoplasm and cell organelles including the plasma membrane and rubber particles of latice vessels were not labelled.

Observations of sections from necrosed phloem revealed an extensive cell wall degradation. This degradation, characterized by the occurrence of more or less large light areas, was predominant in the middle lamella (Fig. 3) and appeared to extend toward the neighbouring wall regions. Few gold particles were present over these light areas (Fig. 3) while more numerous gold particles occurred over the adjacent unaltered walls. Such wall degradation also occurred in areas close to intercellular spaces. In these wall regions of varying electron opacity, the distribution of gold particles was irregular (Figs. 4 and 5). Some electron lucent areas of the walls appeared labelled (Fig. 5, areas B), while other light translucent areas were weakly labelled (Fig. 5, areas C). A quantitative analysis demonstrated that gold particle density was similar over cell walls of the healthy phloem, and over moderately degraded (Table 1, region B) and unaltered cell wall portions

Table 1. **Quantitative evaluation of labelling over healthy and necrosed phloem cell walls**

Phloem		n	Gold particles/μm^2
necrosed	cell wall : region A	15	508,85 ± 119,21
	cell wall : region B	13	507,55 ± 113,71
	cell wall : region C	10	285,92 ± 46,78
	background	7	0,73 ± 0,37
	control	7	0,80 ± 0,25
healthy	cell walls	8	483,11 ± 27,71
	background	8	0,89 ± 0,22
	control	7	0,76 ± 0,35

A : wall regions with no visible degradation; B: wall regions of moderate degradation; C : highly degraded wall areas; n: number of microphotographs used for gold particle quantitation.

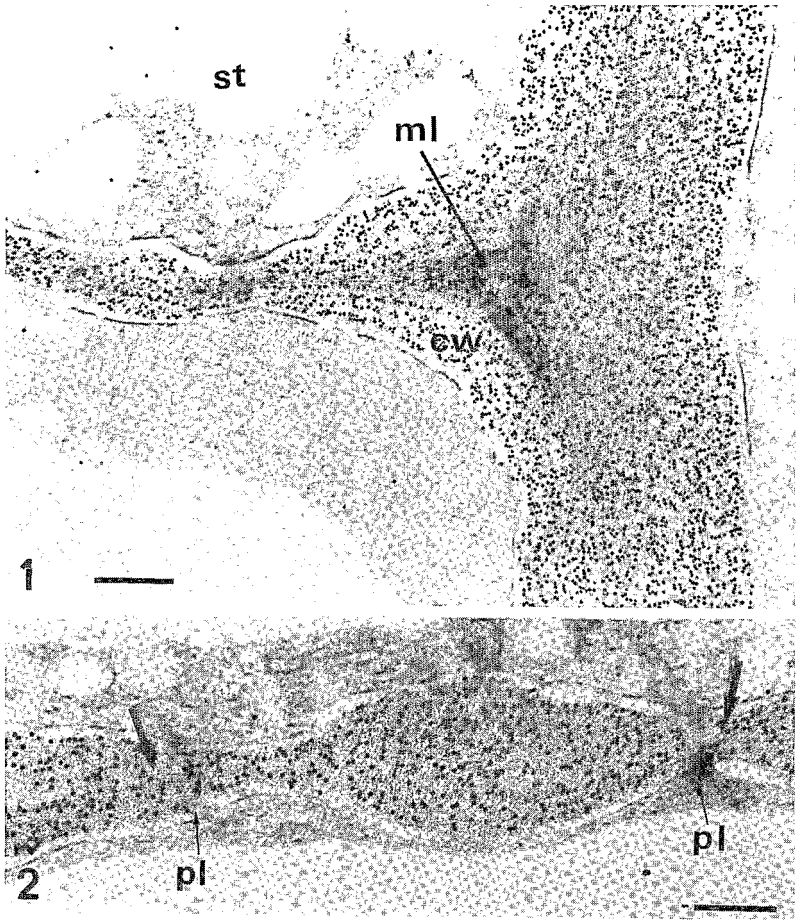


Fig. 1. Healthy trees: gold particles are evenly distributed over phloem cell walls (cw) and the middle lamella (ml) of sieve tubes from healthy trees. Few gold particles are seen in cell cytoplasm, (st: sieve tube), (bar = 0.55 μm). – *Fig. 2.* Healthy trees: an almost regular labelling is noted over cell walls (arrows) even in areas close to plasmodesmata (pl), (bar = 0.5 μm)

of the necrosed phloem (Table 1, region A). A reduction in the labelling of about 50% occurred over highly degraded cell wall areas of the necrosed phloem (Table 1, areas C) (Fig. 5).

The degraded phloem also showed modification in the plasmodesmata areas varying from light alteration to nearly complete digestion of neighbouring wall material. In sieve tubes and parenchymatous cells with no visible wall degradation, the labelling was less intense over walls close to the plasmodesmata than over adjacent walls (Fig. 6 and 7). In other plasmodesmata areas, only a few gold particles were noted over loose wall material (Fig. 6) in contrast to the unaltered highly labelled middle lamella and neighbouring walls. Labelling was also found to be weak over abnormal electron-lucent areas close to plasmodesmata channels (Fig. 7). Examination of transverse sections from the necrosed feeder phloem showing severe symptoms revealed large areas devoid of gold particles including plasmodesmata areas (Fig. 8). Unlabelled fibrillar material, possibly corresponding to wall remnants, was frequently noted in the proximity of plasmodesmata (Figs. 8 and 9). These necrosed cells exhibited an almost completely disrupted plasma membrane (Figs. 8 and 9).

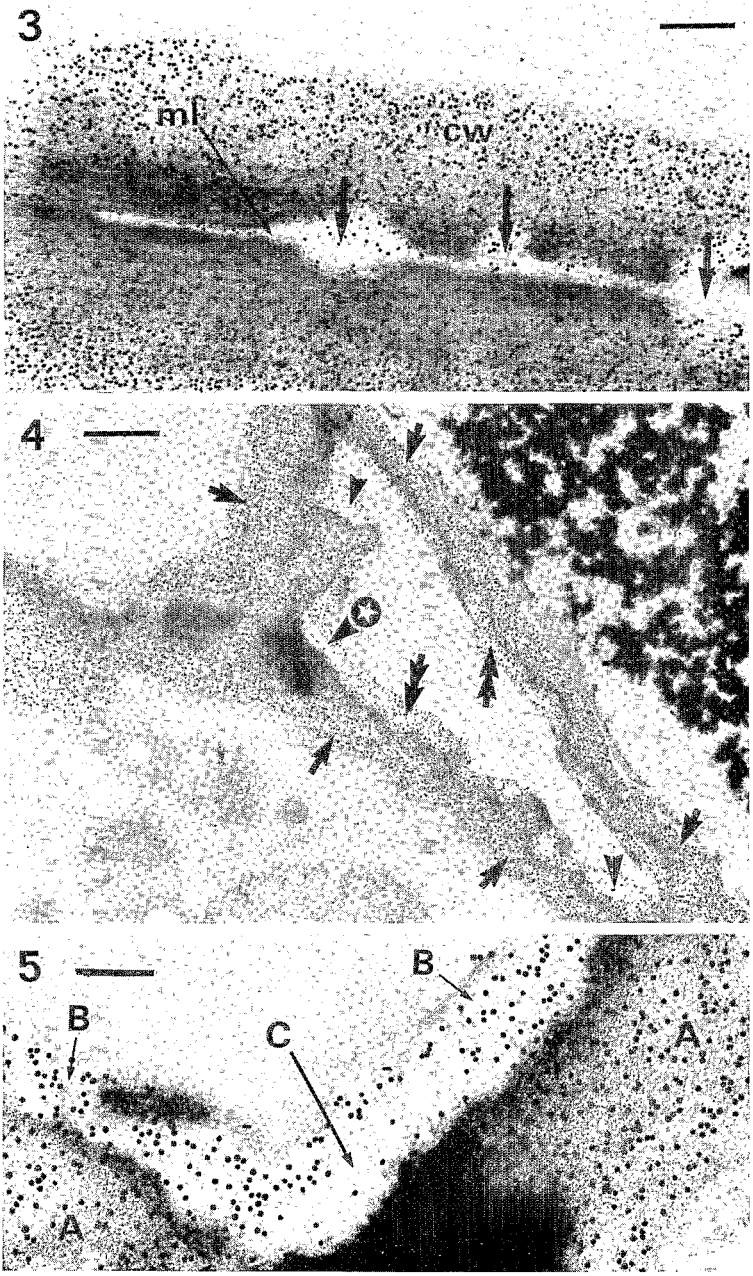


Fig. 3. Necrosed trees: a few gold particles occur over these light areas of the middle lamella (ml) (arrows), (bar = 0.4 μm). – *Fig. 4.* Necrosed trees: intact regions of walls (arrows) are regularly labelled as well as some light areas (double arrows). Few gold particles are observed over other electronlucent regions (arrowheads), (✪ : enlarged area in Fig. 5), (bar = 1 μm). – *Fig. 5.* Necrosed trees: enlargement of Fig. 4. Unaltered regions of walls (A) as well as some degraded areas (B) are regularly labelled while only few gold particles occur over other altered zones (C), (bar = 0.33 μm)

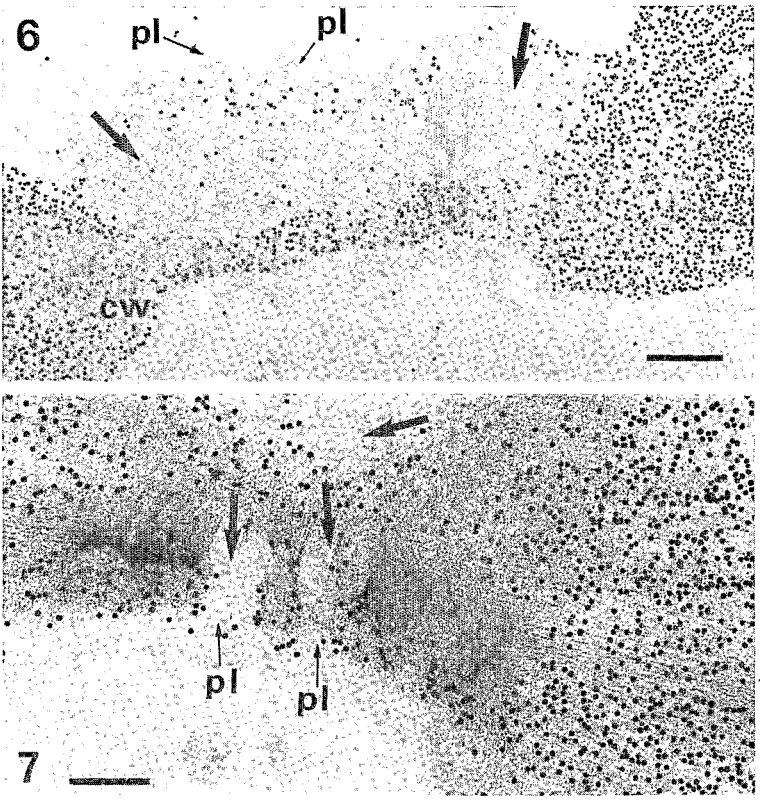
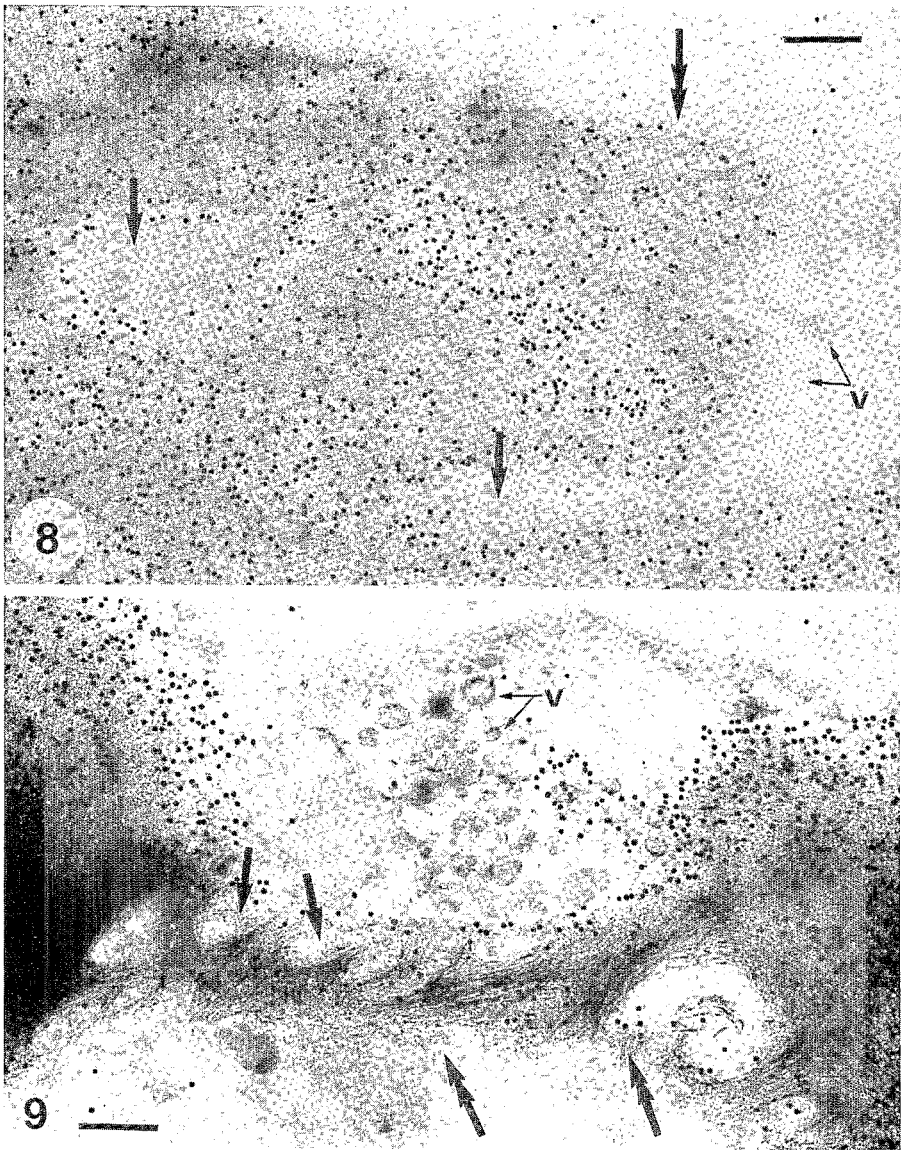


Fig. 6. Necrosed trees: loose wall material close to plasmodesmata (pl) are weakly labelled (arrows) as compared to adjacent wall areas (cw), (bar = 0.5 μm). – *Fig. 7.* Necrosed trees: wall regions of degraded tissue close to plasmodesmata (pl) are devoid of gold particles (arrows), (bar = 0.33 μm)

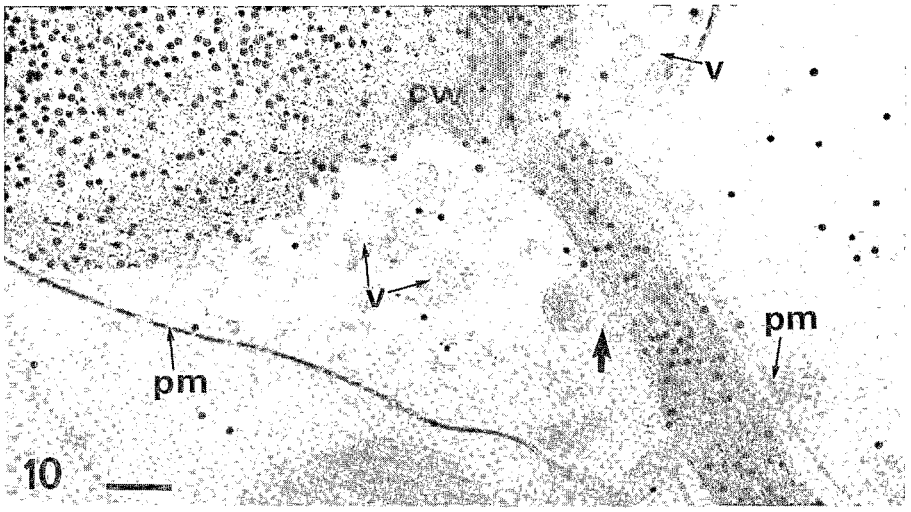
Another morphological change frequently noted in phloem necrosis was the accumulation of paramural vesicles (Fig. 10). They were occasionally found close to degraded walls of disorganized plasmodesmata or in the periplasmic space of unaltered cells (Fig. 10). Nearly no labelling was observed over these vesicles while only a few gold particles occurred over small fibrils.

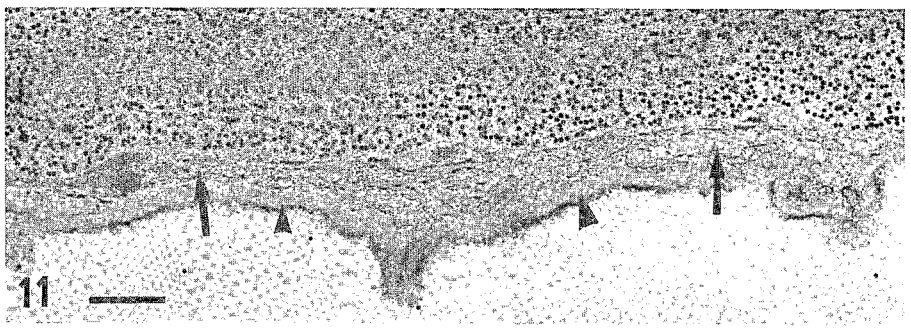
Unaltered sieve tubes and parenchyma cells of damaged phloem often exhibited additional wall depositions of more amorphous (Fig. 11) or fibrillar-like material (Figs. 11 and 12). Vesicular structures, containing fibrils that appear to be linked with the plasmalemma, were sometimes noted close to these wall appositions (Fig. 12). No labelling was observed over both type of appositions or fibrils in contrast to highly labelled adjacent walls (Figs. 11 and 12).

All control tests including incubation of sections with (i) the exoglucanase-gold complex to which was previously added an excess of β-(1,4)-D-glucan, (ii) BSA-gold, and (iii) a stabilized gold solution, did not yield any significative labelling over cell walls of both healthy and diseased phloem of rubber trees (Figs. 13 and 14; Table 1).



Figs. 8 and 9. Necrosed trees: transverse sections of degraded walls showing a weak labelling (arrows) over plasmodesmata areas. No gold particles are observed over fibrillar material (double arrows) as well as over vesicle-like structures (v) close to the walls. Plasma membranes are absent in these cells (Fig. 8: bar = 0.33 μm; Fig. 9: bar = 0.4 μm)





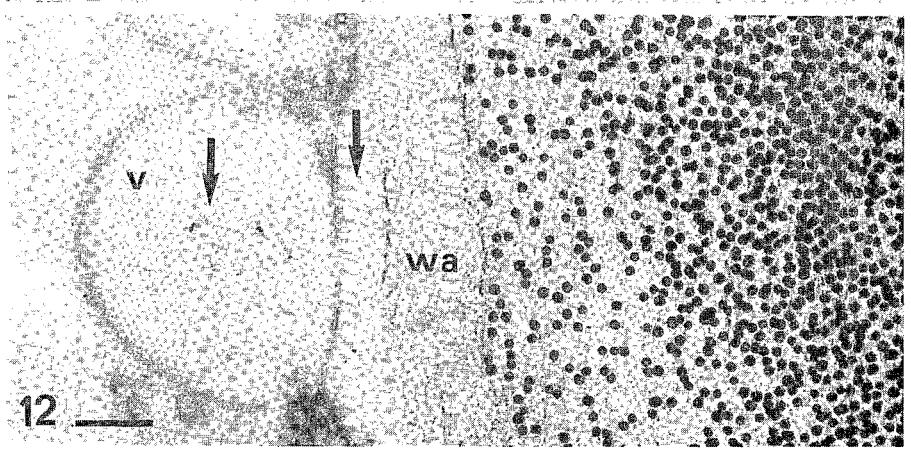
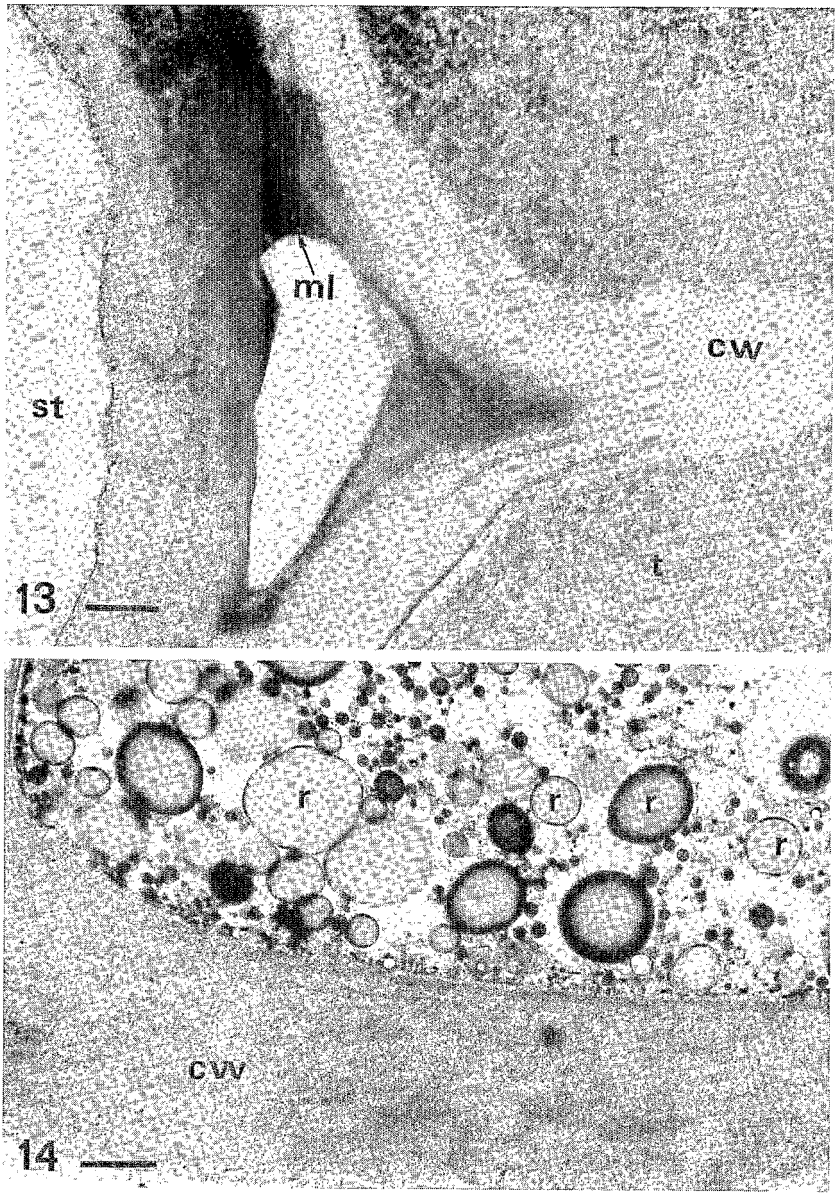


Fig. 10. Necrosed trees: paramural vesicles (v) are noted between the plasma membranes (pm) and the cell walls (cw). No gold particles occur over these vesicles. An unlabelled amorphous material (arrow) appears to link one of these vesicles to the cell wall, (bar = 0.25 μm). – *Fig. 11.* Necrosed trees: fibrillar (arrows) and amorphous (arrowheads) wall appositions occur close to non altered phloem cell walls in a necrosed tree. No labelling is seen over these depositions while the adjacent cell wall is evenly labelled, (bar = 0.5 μm). – *Fig. 12.* Necrosed trees: unlabelled fribrils (arrows) are observed in a vesicle-like structure (v) associated with the plasma membrane (pm). An adjacent wall apposition is devoid of labelling (wa), (bar = 0.2 μm)



Figs. 13. and 14. Control tests. Nearly no gold particles are seen over the cell walls of necrosed phloem (Fig. 13) and healthy phloem (Fig. 14) after incubation of sections with the exoglucanase-gold complex which has been previously incubated with an excess of glucans. (cw: cell wall; ml: middle lamella; r: rubber particles; st: sieve tubes; t: tannin cell), (Figs. 13 and 14 bar = 0.83 μm)

4 Discussion

From a previous ultrastructural study on phloem necrosis of the rubber tree, it was shown that walls of cell types were highly affected thus suggesting that hydrolytic enzymes were involved in cell wall degradation (NICOLE et al. 1991). In the present study, the use of a highly specific and sensitive probe for β-(1–4)-D-glucans provided new information concerning wall digestion during this disease. The significant decrease of labelling over altered cell walls confirms the occurrence of cellulose degradation. Moreover, variation in labelling was observed over wall areas close to intercellular spaces (Table 1). This observation suggests that hydrolysis of non-reducing ends of cellulose is probably preceded by other enzymatic events. Enzymes such as endo-1,4-β-glucanases could first hydrolyze β-(1–4)-glucosidic linkages producing shorter units that would be attacked later by exo-1,4-β-glucanases (CBH). In contrast, in wall regions close to plasmodesmata labelling decreases significantly without an apparent degradation of walls. It appeared that in the plasmodesmata regions, CBH are involved in a very early stage of the digestion process. This could be explained by the high accessibility of these regions to cytoplasmic enzymes.

These data raise the question of the origin of the cellulase complex capable of altering cell walls during phloem necrosis: (1) cellulases have been demonstrated to occur in rubber trees (SCHELDRAKE 1969; SCHELDRAKE and MOIR 1970). Extremely intense activity of such enzymes was shown to be located mainly in the latex cytosol. Moreover, in healthy rubber trees, the function of cellulase is related to the formation of the articulated latice vessels (SCHELDRAKE 1969) which leads to anastomosis of latex cells. Cellulase activity has also been found to be strong in both young and older laticiferous cells. During phloem necrosis, the cellulase complex of the rubber tree may be triggered by the necrotic process, thus favoring cell wall degradation. It is likely that synergisms between the cellulase complex and other enzymes (i. e. pectinases) might be associated with the phloem autolysis. (2) Cellulase may also be of pathogen origin. In this respect, previous works revealed that symptomatology and epidemic spread in plantations were in accordance with a pathological cause of the disease. However, microscopic observations, attempts at causal agent transmission, biochemical studies and chemical or antibiotic treatments did not result in detection of any pathogen (NANDRIS et al. 1991b).

Our observations also revealed the accumulation of vesicles in periplasmic areas of both affected and apparently normal cells. This change may be related to the disease, since it was not observed in healthy trees. Such membranous structures, often reported to be associated with the plasmalemma (MARCHANT and ROBARDS 1968), may be involved in cell wall degradation by releasing hydrolytic enzymes. Such so-called paramural bodies also have been suggested to be a primitive but effective defense reaction to various pathogens (HEATH 1980). They are thought to contain sugar residues possibly associated with glycoproteins or phenolic compounds (CHAMBERLAND et al. 1989). Wall appositions are other morphological changes known to occur in many host-pathogen interactions (AIST 1976) as in abiotic disorders. The absence of labelling over both types of appositions observed in diseased phloem cells indicates the lack of β-(1–4)-D-glucans in this material. It is likely, however, that the nature of the fibrillar deposition could be related to cellulose in an intermediate form. In contrast, the amorphous apposition may be of suberin origin as already described for other pathosystems (RIOUX and OUELLETTE 1991). Thus, the presence of periplasmic vesicles and wall appositions suggests that microorganisms may be directly or indirectly involved in the development of rubber tree phloem necrosis.

In conclusion, the present cytochemical study suggested that non-reducing ends of cellulose are hydrolyzed, indicating that cellulose is degraded during phloem necrosis. Whether cellulases supposed to be involved in wall alteration are from rubber tree and/or from pathogen origin is still a matter of speculation.

Acknowledgement

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Summary

Phloem necrosis is a severe disease affecting rubber trees in West African plantations. Among disturbances recorded in the degraded tissue, wall alteration of all cell types is one of the most significant cellular modification. In this study, cytochemical investigation was performed in the aim to gain further information on the cellulose breakdown during phloem necrosis. An exoglucanase-gold complex was used for localizing β-(1,4)-D-glucan linkages of non reducing ends of the cellulose polymer. The use of this probe showed a variation of labelling over walls of altered cells. In plasmodesmata areas, labelling decreased over walls which did have an apparent unaltered aspect. In regions adjacent to intercellular spaces, labelling did not quantitatively decrease although walls appeared to be highly digested. These data indicate that cellulose is degraded during phloem necrosis and that exoglucanases are involved in an early stage of cell wall degradation in plasmodesmata areas. Cellulases of laticifers or exogenous enzymes may be responsible for cellulose degradation.

The presence of periplasmic vesicles and wall appositions in the necrosed phloem have also been observed, suggesting that microorganisms may be directly or indirectly involved in the development of phloem necrosis in rubber trees.

Résumé

La cellulose des parois cellulaires est dégradée au cours de la nécrose du phloème d'Hevea brasiliensis

La nécrose du phloème d'Hévéa est une grave maladie répandue dans les plantations ouest-africaines. A l'échelle ultrastructurale, l'altération des parois cellulaires est la modification la plus fréquemment observée. Une étude cytochimique a été entreprise dans le but de mieux comprendre les mécanismes de la dégradation de la cellulose des parois. A ce titre, une exoglucanase conjuguée à l'or colloïdal a été utilisée pour localiser les liaisons β-(1,4)-D-glucanes des extrémités non réductrices du polymère. Une variation du marquage des parois altérées a été observée à tous les stades de nécrose du phloème. Dans les régions riches en plasmodesmes, le marquage des parois est modifié alors que celles-ci paraissent intactes. A l'inverse, au voisinage des espaces intercellulaires, le marquage des parois dégradées est quantitativement identique à celui des régions pariétales non altérées. Ces résultats montrent d'une part que la cellulose des parois du phloème est digérée au cours du processus de nécrose tissulaire et, d'autre part, que des exoglucanases sont impliquées dans les stades précoces de la dégradation des parois au voisinage des plasmodesmes. Des cellulases endogènes ou exogènes à l'Hévéa pourraient être responsables des altérations de la cellulose.

Les observations microscopiques ont également montré la présence de vésicules paramurales et de dépots le long des parois dans les espaces périplasmiques, suggérant la participation directe, ou indirecte, de mircroorganismes à la nécrose du phloème d'Hévéa.

Zusammenfassung

Zelluloseabbau im Verlauf der Phloemnekrose von Hevea brasiliensis

Die Phloemnekrose ist eine gefährliche Krankheit des Kautschukbaumes in Pflanzungen Westafrikas. Zellwandveränderungen gehören zu den folgenschwersten Funktionsstörungen bei dieser Krankheit. In der nun vorliegenden Arbeit wurden zytochemische Untersuchungen mit dem Ziel durchgeführt, Genaueres über den Zelluloseabbau während der Phloemnekrose zu erfahren. Ein Exoglucanase-Gold Komplex wurde dazu verwendet, die β-(1,4)-D-Glucan Bindungen der nicht reduzierenden Enden der Zellulose zu markieren. Dieser Komplex wurde von den Zellwänden geschädigter Zellen unterschiedlich stark gebunden. In scheinbar intakten Zellwänden in der Nähe von Plasmodesmata war die Häufigkeit gebundener Komplexe meist gering. In der Nähe von Interzellularen konnte keine Abnahme in der Häufigkeit gebundener Komplexe festgestellt werden, obschon die Wände den Eindruck erweckten, bereits stark abgebaut zu sein. Diese Daten zeigen, daß Zellulose während der Phloemnekrose abgebaut wird, und daß Exoglucanasen bereits in einem frühen Stadium am Zellwandabbau in der Nähe von Plasmodesmata beteiligt sind. Zellulasen aus den Milchröhren oder exogene Enzyme dürften für diesen Zelluloseabbau verantwortlich sein.

Es wurden des weiteren periplasmatische Vesikel und Wandanlagerungen in nekrotischem Phloem beobachtet, welche darauf hindeuten, daß Mikroorganismen direkt oder indirekt an der Phloemnekrose des Kautschukbaumes beteiligt sein könnten.

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Authors' address: M. NICOLE, H. CHAMBERLAND, D. NANDRIS, G. B. OUELLETTE, Forestry Canada, Quebec Region, 1055 Rue du PEPS, Sainte-Foy, Quebec, G1V4C7, Canada (*Corresponding address:* M. NICOLE, ORSTOM, Laboratoire de Phytopathologie, B. P. 5045, 34000 Montpellier, France)

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