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Lignin distribution in wood cell walls determined by TEM and backscattered SEM techniques

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Abstract

The lignin distribution in cell walls of spruce and beech wood was determined by high-voltage transmission-electron-microscopy (TEM) in sections stained with potassium permanganate as well as by field-emission-scanning-electron-microscopy (FE-SEM) combined with a back-scattered electron detector on mercurized specimens. The latter is a new technique based on the mercurization of lignin and the concomitant visualization of mercury by back-scattered electron microscopy (BSE). Due to this combination it was possible to obtain a visualized overview of the lignin distribution across the different layers of the cell wall. To our knowledge, this combined method was used the first time to analyse the lignin distribution in cell walls. In agreement with previous work the highest lignin levels were found in the compound middle lamella and the cell corners. Back-scattered FE-SEM allows the lignin distribution in the pit membrane of bordered pits as well as in the various cell wall layers to be shown. In addition, by using TEM as well as SEM we observed that lignin closely follows the cellulose microfibril orientation in the secondary cell wall. From these observations, we conclude that the polymerisation of monolignols is affected by the arrangement of the polysaccharides which constitute the cell wall.

Keywords: Back-scattered electron microscopy (BSE); Cell wall ultrastructure; FE-SEM; Lignin; Mercurization; TEM; Wood anatomy

1. Introduction

The structural organization of the wood cell wall is highly orchestrated and complex. It consists of cellulose which is a linear polymer containing glucose units organized in crystalline as well as amorphous regions, polyoses which are branched polymers composed of various polysaccharides, and lignin, which is an aromatic amorphous molecule containing phenylpropane monomers. Until now, considerable effort has been applied to the investigation of the process of lignin formation and its distribution in the cell wall. While the presence of lignin in high concentrations in the cell wall is regarded as a positive benefit for example in fibreboard industries, it is generally regarded as undesirable by the pulp and paper industry. Therefore, various processes to delignify wood have been developed (e.g., Weinstock et al., 1998). On the other hand, various electron-microscopic methods have been developed over the years to analyse the lignin distribution within different wood elements. For example, the distribution of lignin can be determined by interference microscopy and confocal laser scanning microscopy (Donaldson et al., 2001). Furthermore, the ultraviolet (UV) technique was first introduced by Lange (1954) and later greatly improved by the use of thin sections (Fergus et al., 1969; Scott et al., 1969). The quality of the results depends on the absorptivity of the lignin molecule which is quite sensitive to different structural factors. Especially the presence of double bonds or carbonyl groups can lead to a considerable increase in absorptivity. For correct determination the absorptivity of the different morphological tissues has to be exactly the same; otherwise it would have to be determined in separate experiments. UV microspectrophotometry was also applied to the deterioration of lignin during fungal wood decay (Bauch et al., 1976) and to localize and analyse cell wall wood

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extractives (Kleist and Bauch, 2001; Koch and Kleist, 2001).

The technique of bromination and subsequent detection by energy-dispersive X-ray analysis (EDXA) was first developed by Saka et al. (1982) and Saka and Thomas (1982). This method also depends on the structure of the wood: In uncondensed guaiacyl units and in biphenyl units a monobromination of the aromatic part occurs; in contrast to dibromination. It is evident that structural variations in lignin can affect both, the UV as well as the bromination method; therefore, a technique which proves less sensitive to structural variations would be of great advantage.

On the other hand, potassium permanganate ($KMnO_4$) was established as a general electron-dense staining agent for lignin (Bland et al., 1971; Hepler et al., 1970). In principle, e.g., a coniferylalcohol molecule is oxidised by $KMnO_4$. In such a reaction the permanganate anion is reduced to manganese dioxide which then precipitates, indicating the site of reaction. The staining of ultrathin sections with $KMnO_4$ in order to determine the lignin distribution in woody cell walls experienced something of a renaissance in the 1980s and 1990s. Maurer and Fengel (1991), in particular, used the staining intensity with subsequent densitometric evaluation as an indicator for lignin distribution within woody cell walls. Donaldson (1994) studied the process of lignification in the secondary wall of *Pinus radiata* D. Don and found that it occurred by deposition of protolignin monomers onto the ends of expanding lignin lamellae between cellulose microfibrils. This pattern of deposition leads to greatly elongated patches of lignin due to the greater rate of deposition along the microfibril axis compared to that across it.

Another technique to analyse lignin in the cell wall is based on mercurization; it was first used in the early history of lignin chemistry to characterize its structure (Freudenberg et al., 1931). Mercurization is a reaction occurring under mild conditions, it seems to be less sensitive to the substitution pattern of the aromatic ring. Mercuric acetate reacts under mildly acidic conditions via an electrophilic substitution reaction with the aromatic moiety of the lignin, producing a covalent bond between the aromatic ring and the acetoxymercuric group. The scanning-electron-microscopy–energy-dispersive-X-ray-technique (SEM–EDX) was successfully used to quantify the lignin distribution in the middle lamella and cell wall of mercurized and brominated wood (Eriksson et al., 1988; Saka et al., 1978; Westermark et al., 1988). In the present paper we studied the lignin distribution in cell walls from spruce and beech wood by using $KMnO_4$ in high voltage TEM technique. The results are compared with those we obtained by a new technique which combines the mercurization of wood with analysis by field-emission-scanning-electron-microscopy (FE-SEM) using a back-scattered electron detector.

2. Materials and methods

2.1. Plant material

Wood from an 80-years-old spruce tree (*Picea abies* (L.) Karst.) and an 80-years-old beech tree (*Fagus sylvatica* L.), grown at the research plot close to Munich, was used for the analysis. The Munich site is a fresh, sandy loam, and the climate is characterized by high total annual precipitation (1009 mm) and a mean annual temperature of about 9.2 °C. Specimens were taken from the sapwood at a tree height of 1.3 m in winter and dissected into small blocks.

2.2. Specimen preparation for transmission electron microscopy

Approximately 1–2 mm^2 segments of dry wood were extracted with cyclohexane–ethanol and embedded in Spurr's epoxy resin (Spurr, 1969). Sixty–ninety nanometers thick sections were cut with a diamond knife on a Reichert ultramicrotome and stained for 10 min with 1% $KMnO_4$. Photographs were taken using a Zeiss 10C TEM operated at 80 kV and a Philips CM20 with FEG operating at 160 kV (resolution 0.1 nm).

2.3. Mercurization

Small sticks (2mm × 2mm × 5 mm) were taken from the sapwood of both trees and extracted with acetone. Then they were treated with a solution of mercuric acetate and acetic acid at 95 °C with methanol as solvent (according to Westermark et al., 1988). After mercurization, they were transferred to boiling methanol and washed twice by refluxing in methanol for 8 hours.

2.4. Scanning and transmission electron microscopy of mercurized wood tissue

For SEM and TEM, 1–2 mm^2 segments of mercurized wood tissue were dehydrated in a graded ethanol series and embedded in Spurr's low viscosity epoxy resin. Sections approximately 100 nm thick were cut with a diamond knife on a Reichert Ultracut E ultramicrotome and coated with 2–3 nm carbon by evaporation. A field emission scanning electron microscope (Hitachi S-4100) equipped with a back-scattered electron detector (YAG-type; Autrata) and an EDX microanalysis system (Vantage; Noran Instruments) was operated at 15 kV, with an approximate resolution of 5 nm. Specifity of the back-scattered electron microscopy (BSE) signal for mercury was controlled by EDX-analysis before taking

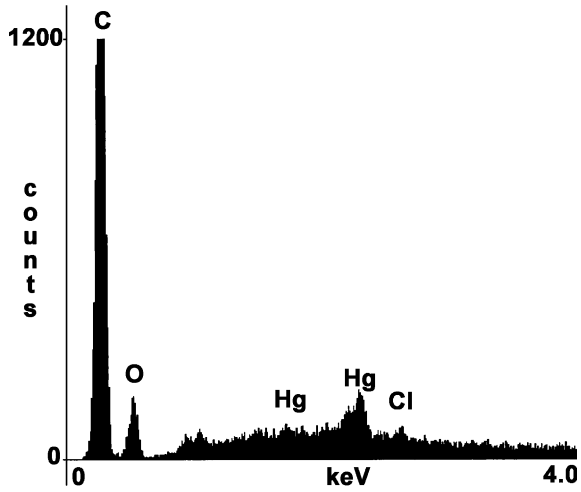

Fig. 1. Typical EDX-spectrum of a mercurized wood cell wall indicating high amounts of C, O, and Hg. Mercury is responsible for the BSE-signal.

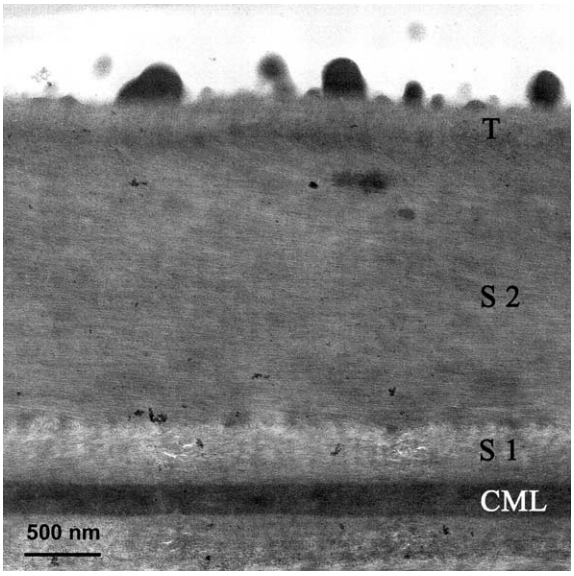

Fig. 2. TEM micrograph of an ultrathin radial section of the cell wall of a beech libriform fibre, taken at 80 kV. The specimen was stained with 1% $KMnO_4$. The dark staining of the CML indicates that it is strongly lignified. CML, compound middle lamella; S1, secondary wall 1; S2, secondary wall 2; T, tertiary wall.

micrographs (Fig. 1). For better comparison with the TEM results, the BSE-signal was inverted.

3. Results and discussion

3.1. Potassium permanganate staining in TEM

Since the early 1950s conventional transmission electron microscopy has been used for studying various aspects of the wood cell wall structure (Liese, 1951; Liese and Fahnenbrook, 1952). From the 1990's on the $KMnO_4$ technique was variously used in order to find out more details on both the course of lignification during cell wall differentiation (Donaldson, 1992) as well as delignification during pulping processes and, also, during fungal decay. The capability of $KMnO_4$ to stain lignin represents a very suitable technique for TEM. In the present study we used transmission electron microscopy at 80 and 160 kV in order to obtain detailed information on the ultrastructural aspects of lignin distribution. The various wall layers clearly appear in thin radial sections of libriform fibres from beech wood (Fig. 2). The middle lamella which glues the cells together shows no clear transition to the adjacent wall layers. Therefore, the middle lamella and both adjacent primary walls are termed compound middle lamella ((CML), approx. 300 nm width). The dark staining of the CML indicates that it is strongly lignified.

In contrast, the subsequent secondary wall 1 (S1) is the brightest layer of the wall for a much lower lignin concentration. This observation agrees with other determinations of lignin concentration by ultraviolet micrography of black spruce tracheids where the highest levels occur in the CML (50–100%) while the secondary wall has only a concentration of 20–25% (Fergus et al., 1969). However, by using the SEM–EDX technique Westermark and coworkers (1988) measured a relatively low lignin content of 55–58% in the true middle lamella. The S1 is approximately 300–400 nm in width (Fig. 2) and composed of three to four lamellae of cellulose microfibrils which spin right or left in opposite direction to the cell length with an inclination of 60–80 ° (Higuchi, 1997).

The thickest wall layer is the secondary wall 2 (S2); its width is 1 μm in earlywood and approximately 5 μm in latewood. It is composed of up to 150 lamellae with an inclination in the direction of the cell length of 5–30 ° (Higuchi, 1997). The last fibrillar layer at the luminal border is the tertiary wall (T) which is 0.1–0.2 μm in width and composed of several lamellae. Microfibrils spin in the right or left direction with an inclination of 70–90 ° (Côté, 1967). Additionally, the warty layer as part of T forms the innermost wall layer.

Examination of radial spruce sections at high voltage (160 kV) reveals that the secondary wall has a fibrillar texture due to the presence of alternating dark and light lines (Fig. 3a). The dark lines are believed to be deposits of lignin in the spaces between cellulose microfibrils which are oriented in different directions in the CML, S1, and S2 (Fengel and Wegener, 1989). When microfibrils are cut transversely as shown in the CML, the electron-opaque material has a very fine granular appearance in contrast to the fibrillar texture of the S1 and S2 (Figs. 3a and b). The electron-opaque particles of the CML are interpreted to be deposits of lignin due to the electron opacity of MnO_2 which complexes with lignin

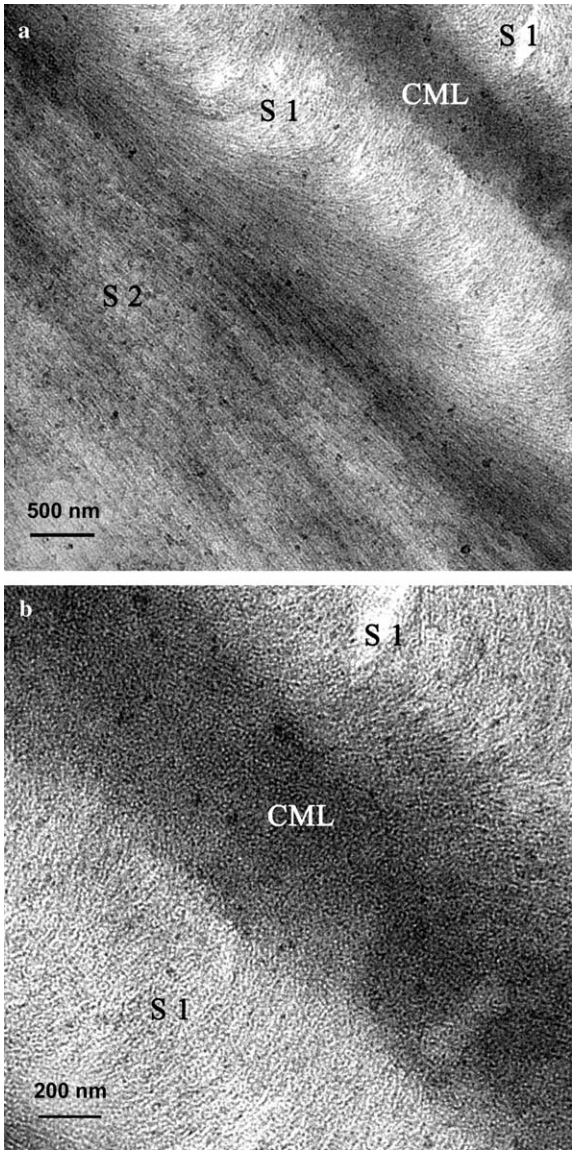

Fig. 3. TEM micrographs of ultrathin radial sections of spruce tracheids, taken at 160 kV. The specimens were stained for 10 min with 1% $KMnO_4$. (a) The secondary wall has a fibrillar texture due to the presence of alternating dark and light lines. In the S2 the cellulose microfibrils are oriented at a steep angle while the lignin fills the spaces in-between. (b) In the CML microfibrils are cut transversely showing a fine granular appearance of the dark lignin matrix. CML, compound middle lamella; S1, secondary wall 1; S2, secondary wall 2.

(Bland et al., 1971; Maurer and Fengel, 1991). The lignin particles occur in high concentrations throughout the CML and intermingle with the microfibrils of the S1 which appears brighter indicating a low lignin concentration. The transition between S1 and S2 is characterized by a change in microfibril direction and also by an increase in lignin concentration. In addition, there are significant differences in the physical and chemical properties between the protolignins in the CML and the lignin of the secondary wall (Hardell et al., 1980; Whiting and Goring, 1982). In the S2 all cellulose microfibrils are oriented at a steep angle while the lignin fills the spaces in-between (Fig. 3a). Thus, the use of $KMnO_4$ in high-voltage TEM permits the detailed analysis of the lignification pattern in the woody cell wall where lignification occurs most strongly in the CML and, to a much lesser degree, in the secondary wall.

3.2. Visualization of mercurized wood by TEM

Incubation with mercuric acetate caused electron-dense deposits basically in all layers of the cell wall at different strengths as shown by TEM in tangential sections of spruce wood (Fig. 4b). In all sections, the precipitates occurred primarily in the CML indicating the strong lignification of this layer. Also the CML of the cell corners is heavily labeled by mercuric complexes (Fig. 4a, right). By contrast, the S1 often lacked reaction products or showed less label. In the S2 a homogeneous distribution of the mercuric staining was visible in almost all sections, but in a much lower density than in the CML (Figs. 4a and b). This shows that in tangential sections, it becomes evident that there is no preference for direction in the lignification of the S2.

3.3. Analysis of the lignin distribution by the FE-SEM–BSE technique

Field-emission-scanning-electron-microscopy (FE-SEM) has been a commonly used method for some 15 years but it is only in the last few years that this technique has also been used for research on wood structure.

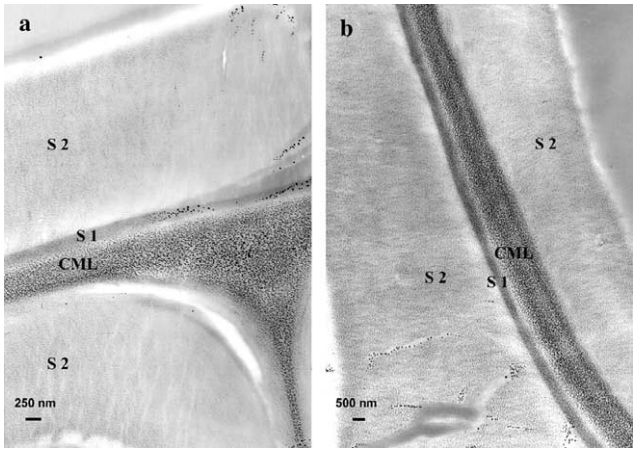

Fig. 4. TEM micrographs of ultrathin tangential sections of spruce tracheids, taken at 80 kV. The specimens were incubated with mercuric acetate which forms black precipitates in almost all layers of the cell wall at different strengths. (a) Label accumulated mainly in the CML and the cell corner. (b) In the S2 there is the typical homogeneous distribution of mercury grains, but in a much lower density than in the CML. CML, compound middle lamella, S1, secondary wall 1; S2, secondary wall 2; T, tertiary wall.

FE-SEM has the benefit of higher resolution than conventional SEM. Therefore, this technique has been successfully used to observe the microfibrillar orientation of the developing secondary wall of japanese fir earlywood tracheids (Abe et al., 1991). In this study the authors describe that the boundaries between layers (S1, S2, and S3) could not be exactly determined because the angle of microfibrils shifted progressively from the outermost to the innermost lamellae in the secondary wall.

Studies on mercurized wood showed that it is well suited for lignin determination by EDX technique (Westermark et al., 1988). With this method it was possible to measure the lignin distribution between the secondary wall and the middle lamella in spruce wood. Based on the chemical advantages of lignin mercurization the visualization of mercury by BSE became possible. A BSE-image of a cross-section of mercurized spruce wood is shown in Fig. 5a. The lignin content is highest in the black areas of the cell corners and in the CML of the radial walls. In radial sections, too, the lignin distribution can be observed in the various wall layers (Fig. 5b), indicating that the space between the bright cellulose microfibrils of the S1 and S2 is completely filled with dark aggregates of globular lignin molecules. Fig. 6 shows a transverse section of the CML and the S1 at higher magnification. Bright microfibrils and the dark lignin matrix form slightly wavy, interrupted lamellae which have also been proposed for the ultrastructure of the S2 layer (Kerr and Goring, 1975; Ruel et al., 1978, 1979; Scallan and Green, 1975; Stone et al., 1971). The gap between cellulose microfibrils is filled with a thin layer of the lignin-hemicellulose complex (Terashima, 2000).

FE-SEM observations of thin cross sections of beech latewood clearly revealed the various cell wall layers of libriform fibres (Fig. 7a), again indicating high amounts of lignin in the CML and the cell corners. Wood cells are anatomically characterized by the presence of simple, half bordered and bordered pits (Fig. 7b). In growing trees, their function is to ensure the interchange of water and nutritive liquids between cells. Based on this physiological function the pathway for chemicals during impregnation is derived. Because of pits being natural irregularities in the wood cell wall and vary greatly in dimension (Sirviö and Kärenlampi, 1998), they are considered as weak points which reduce the strength of wood pulp fibres (Page et al., 1972). Pit regions are also preferred areas of enzymatic hydrolysis which causes a loosened texture. The ultrastructure of pits was examined by Imamura and Harada (1973) and the width of cellulose microfibrils in the pit membrane of spruce was determined as being larger than the width of those found in cell walls (Saka et al., 1976). In the present study we show that bordered pits of beech latewood have heavily lignin encrusted pit membranes (Fig. 7b, thin arrow). Also, the pit chamber appears to be covered with lignin (thick arrows).

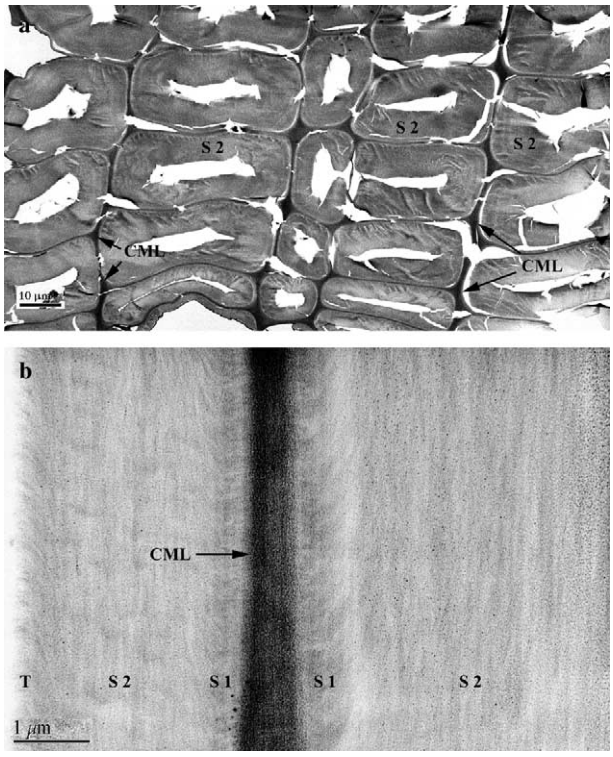

Fig. 5. FE-SEM micrographs taken with a back-scattered electron detector on thin sections of mercurized spruce tracheids. (a) Transverse section, showing the homogeneous lignin distribution in the S2 and high lignin contents in the CML of radial walls (arrows). (b) Radial section, indicating high amounts of lignin in the CML. In the S1 and S2 the dark lignin closely follows the changing microfibril orientation. CML, compound middle lamella; S1, secondary wall 1; S2 secondary wall 2; T, tertiary wall.

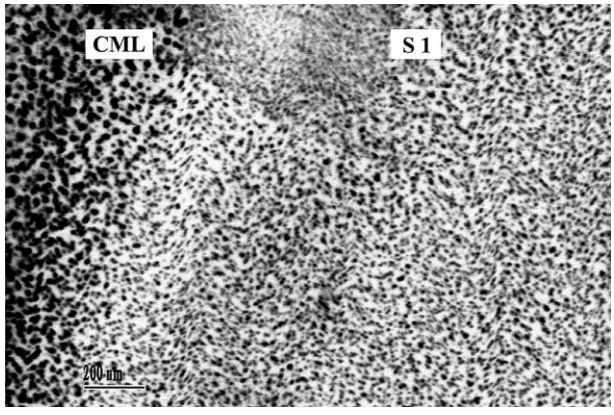

Fig. 6. FE-SEM micrograph taken with a BSE detector at higher magnification on a mercurized spruce tracheid. The transverse section shows lamellae of bright cellulose microfibrils and dark lignin aggregates. In the S1 there is no preferred orientation of the lamella in any direction of the cell wall. CML, compound middle lamella; S1, secondary wall 1.

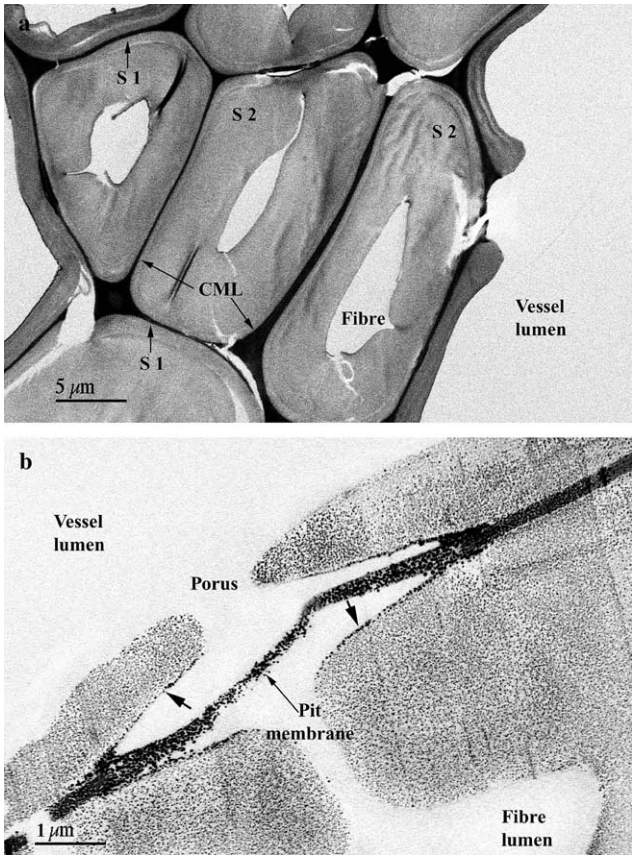

Fig. 7. BSE-image (FE-SEM, 15 kV) of mercurized cross sections of beech latewood. BSE-images were inverted for better comparison with the TEM micrographs. (a) In the S1 and S2 of the fibres lignin is distributed homogeneously while its level increases in the CML and the cell corners. (b) Lignin distribution of a bordered pit between vessel and fibre. The pit membrane is highly encrusted with lignin; also, the pit chamber is covered with lignin (thick arrows). CML, compound middle lamella; S1, secondary wall 1; S2, secondary wall 2.

3.4. Conclusion and perspectives

The biology and chemistry of the wood cell wall plays a key role in the determination of properties of solid wood and products derived from wood. For example, both lignin content and composition are known to have an impact on pulp and paper. Recently a wide variety of detailed techniques of the cell and molecular biology of wood formation was published, including immunolocalisation of enzymes of lignification (Chaffey, 2002). With regard to microscopical techniques conventional electron microscopy has been successfully employed for the characterization of cell wall ultrastructure. Since the late 1980s, new types of microscopes have been commercially available. These include, for instance, atomic force microscopy which can profile wood specimens and, as shown in this study, high voltage TEM and FE-SEM in combination with back-scattered electron detectors. Both methods are able to visualize the lignin distribution in the wood cell wall and provide invaluable information on cell wall ultrastructure. Back-scattered SEM on mercurized wood is a novel combination of techniques which enabled us to confirm previous results on cell wall ultrastructure. As regards the lamellar structure of the tracheid cell wall different orientations, concentric as well as radial ones, have been observed by various groups over the past decade. The theory of a radial rather than concentric structural configuration of the S2 wall layer was proposed by Sell and Zimmermann (1993). However, the concentric lamellar structure of the S2 is the generally accepted model (Kerr and Goring, 1975; Stone et al., 1971), recently confirmed by Fahlen and Salmen (2002). In our study we succeeded in showing, by TEM and FE-SEM, that the lignin distribution of the secondary wall layers of spruce tracheids and beech fibres closely follows the direction of the cellulose microfibrils which are therefore responsible for the lamellar structure. Thus, the polymerisation of monolignols seems to be affected by the arrangement of the polysaccharides that constitute the cell wall.

The EM techniques described in the present study could also be of interest for the investigation of wood from transgenic trees. Because of the great economic importance of the secondary wood cell wall many genes in the biosynthesis pathway of cellulose, hemicellulose and lignin have already been identified (Mellerowicz et al., 2001). Currently, biotechnological efforts are underway to improve the quality of wood for paper production by genetic modification of the pathway of lignification (Chen et al., 2001). For this purpose, poplars (*Populus* spp.) have been widely chosen as models suitable for genetic manipulation (Bradshaw et al., 2001). Poplars with diminished lignin concentrations or with a modified wood composition have been obtained by suppression of enzymes involved in the production of lignin precursors (Chen et al., 2001; Hu et al., 1999). Recently, Ruel et al. (2001) used tobacco plants genetically modified in their monolignols biosynthesis pathway to study the topochemical distribution of various lignin structures by immunocytochemistry. The results show that the plants exhibiting a marked loosening of their secondary wall were lacking non-condensed lignin in the loosened areas, suggesting that non-condensed lignin contributes to the cohesion of the cell wall. In recognition of the developments in the field of molecular biology of wood research, application of the techniques described in the present study would also be suitable to determine the lignin distribution of transgenic plants and therefore indicate the effect of a genetic modification on the ultrastructure of the cell wall.

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