J Wood Sci (2002) 48:95–98


ORIGINAL ARTICLE

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# Ultrastructure of the $S_2$ layer in relation to lignin distribution in *Pinus radiata* tracheids

Received: January 5, 2001 / Accepted: May 18, 2001

**Abstract** The ultrastructure of the $S_2$ layer in relation to its lignin distribution was examined using transmission electron microscopy in the tracheids of *Pinus radiata*. The $S_2$ layer had a striated appearance at low magnification. Observations at higher magnifications showed lignin to be distributed inhomogeneously in this layer, appearing as a mosaic of electron-dense and electron-lucent regions. These regions are scattered, showing a pattern of often interconnecting sinuous features in a predominantly radial profile. The significance of these features of the $S_2$ layer is discussed, particularly in relation to the available information from recent ultrastructural observations on the appearance of cellulose microfibrils and the pattern of their distribution in the $S_2$ layer using rapid freeze-deep etching in conjunction with transmission electron microscopy. Predictions are made as to the likely distribution and arrangement of cellulose microfibrils in the $S_2$ layer based on the pattern of lignin distribution observed in this layer.

**Key words** $S_2$ layer · Lignin distribution · Ultrastructure · Transmission electron microscopy · *Pinus radiata*

## Introduction

Wood scientists and technologists have been interested in understanding the ultrastructure and composition of the $S_2$ layer of wood cell walls for a long time because this layer occupies a large proportion of wood cell walls, making important contributions to strength properties of both solid wood and fiber products. Certain aspects of the composition and ultrastructure of the $S_2$ layer are better known than others. The lignin distribution of this and other wall layers has been characterized for both soft- and hardwood species using a range of microscopic, cytochemical, and microanalytical techniques, including ultraviolet (UV) microscopy, scanning electron microscopy (SEM), energy dispersive X-ray microanalysis (EDXA), transmission electron microscopy (TEM), interference microscopy, immunocytochemistry, and confocal laser scanning microscopy (CLSM).[1–10] The ultrastructural studies have mainly been concerned with examining the orientation of cellulose microfibrils and in some cases their distribution in relation to lignin and hemicelluloses, the noncellulosic components of wood cell walls. Several models of the macromolecular organization of wood cell walls have been developed based primarily on the information available from ultrastructural studies of the $S_2$ layer, of which Kerr and Goring's model[11] is most widely accepted. These models have contributed much to our understanding of the spatial relation of cellulose microfibrils to hemicellulose and lignin, but more recent studies on wood cell walls in general and the $S_2$ layer in particular suggest that the macromolecular organization may be considerably more complex and there may be several levels of microstructural organization. TEM, SEM, and field emission SEM studies[10,12–16] have shown the $S_2$ layer to have radial features, in addition to the well-known concentric lamellations[12] or tangential features.

The TEM observations presented in this paper describe the ultrastructure of the $S_2$ layer in *Pinus radiata* tracheids. They extend our understanding of the microdistribution of lignin in this layer.

## Materials and methods

Small pieces of *P. radiata* earlywood were treated with 1% aqueous potassium permanganate ($KMnO_4$) for 1 h at room temperature and subsequently washed with several changes

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of distilled water. The samples were then dehydrated in graded series of acetone and embedded in Spurr's low-viscosity resin.[17] Ultrathin sections from the embedded samples were cut with a diamond knife, poststained with 1% $KMnO_4$ (prepared in 0.1% sodium citrate buffer), and examined with Philips 201 and 300 TEM. This stain was used to contrast lignin to examine its microdistribution in the $S_2$ layer of tracheid walls.

## Results

The pattern of lignin distribution in the $S_2$ layer of *P. radiata* tracheids was not clearly observable at low magnifications, but there was some indication that this layer may be striated in the radial direction (Fig. 1A). Observations at higher magnifications revealed that lignin in the $S_2$ layer is not homogeneously distributed. The $S_2$ layer in Fig. 1B, which shows part of a pit border at a magnification approximately 2.5 times greater than that of Fig. 1A, appears to be a mosaic of irregular electron-dense and electron-lucent regions, although this feature is more readily distinguishable in some parts than in others.

The distribution of lignin in the $S_2$ layer appeared distinctly irregular when observed at much greater magnifications. As seen in Fig. 2A, the $S_2$ layer is well differentiated into dense and lucent regions (less electron-dense). Although a gradation in the electron density between the dense and lucent regions can also be seen, here the terms dense and lucent regions are used for convenience when describing the $S_2$ wall microstructure. These regions are scattered and also have a pattern of often interconnecting sinuous features in a predominantly radial profile. The micromorphological appearance of the $S_2$ layer as a mosaic of dense and lucent regions is highly variable with respect to the spatial relation of these regions relative to each other. As seen in Fig. 2B, it is significant that the microchecks present in the mechanically damaged part of the tracheid wall are also sinuous, displaying a radial profile closely corresponding to the distribution profile of the dense and lucent features present in the $S_2$ layer.

The width of 20 electron-lucent regions of the $S_2$ layer were randomly measured with the assumption that the electron-lucent regions are more likely to contain a larger proportion of cellulose than electron-dense regions. Thus we obtained an indication of the distribution of cellulose in cellulose-rich regions. The measurements showed a range of 20–100 nm, with most of the electron-lucent regions having a width of 20–40 nm.

## Discussion

Information available from microscopic studies of wood cell walls suggests that there may be several levels of microstructural complexities within individual wall layers. The presence of concentric/tangential lamellation in the $S_2$ layer of conifer tracheids has been demonstrated at both light and electron microscopic levels.[12] This feature is best revealed after specific staining of wood cell walls or selective removal of cell wall constituents.[12] Use of TEM has also provided information on the spatial relations between cell wall macromolecules, particularly between cellulose microfibrils (the basic cell wall structural units that can be resolved by microscopy) and lignin.[11,18] The pattern of lignin distribution in the $S_2$ layer of *P. radiata* wood cell walls shown here provides an example of the microstructural complexity at an intermediate level, which may be widely present in wood cell walls but about which information is virtually nonexistent, partly because of limited resolution of the techniques, such as UV microscopy, which have been commonly used to study lignin distribution.

It is well known that the concentration of lignin varies across wood cell walls, and the compound middle lamella generally is more highly lignified than other wall regions. The concentration of lignin also varies among the layers of the secondary wall, with the extent greater in some wood

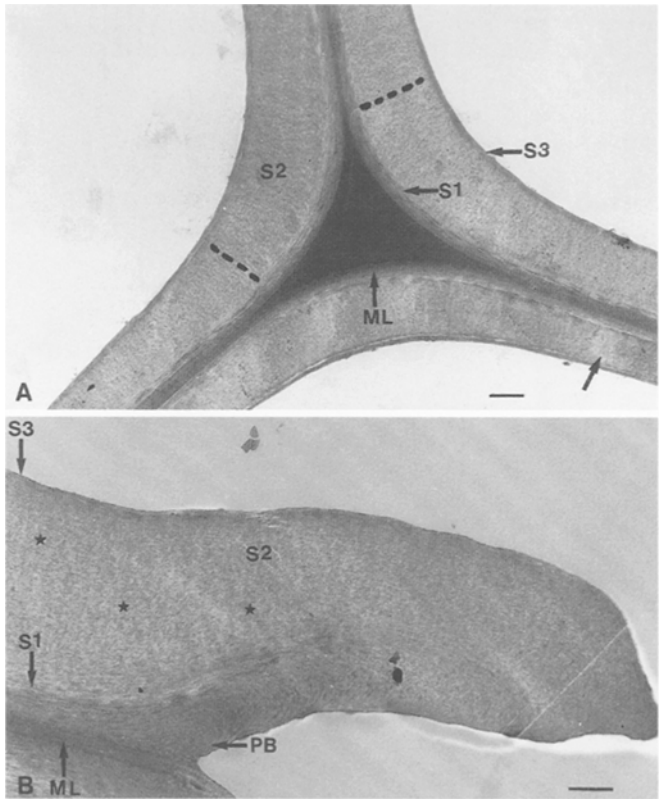


**Fig. 1. A** Transverse section through a corner region between adjoining tracheids. At this low magnification there is no clear indication that lignin distribution is irregular in the $S_2$ layer. However, this layer appears to be radially striated (*stippled lines*). The *unlabeled arrow* indicates a region where the tracheid wall appears to have been stretched during sectioning and is therefore less dense than other wall regions. *ML*, middle lamella; *S1*, *S2*, *S3*, secondary wall layers. *Bar* 1 μm. **B** Transverse section through part of a tracheid pit border (*PB*). The lignin distribution in the $S_2$ layer does not appear to be homogeneous at this magnification, which is approximately three times greater than the magnification in **A**. The $S_2$ layer is differentiated into irregular electron-dense and -lucent regions (*stars*) and consequently appears mottled. *Bar* 500 nm

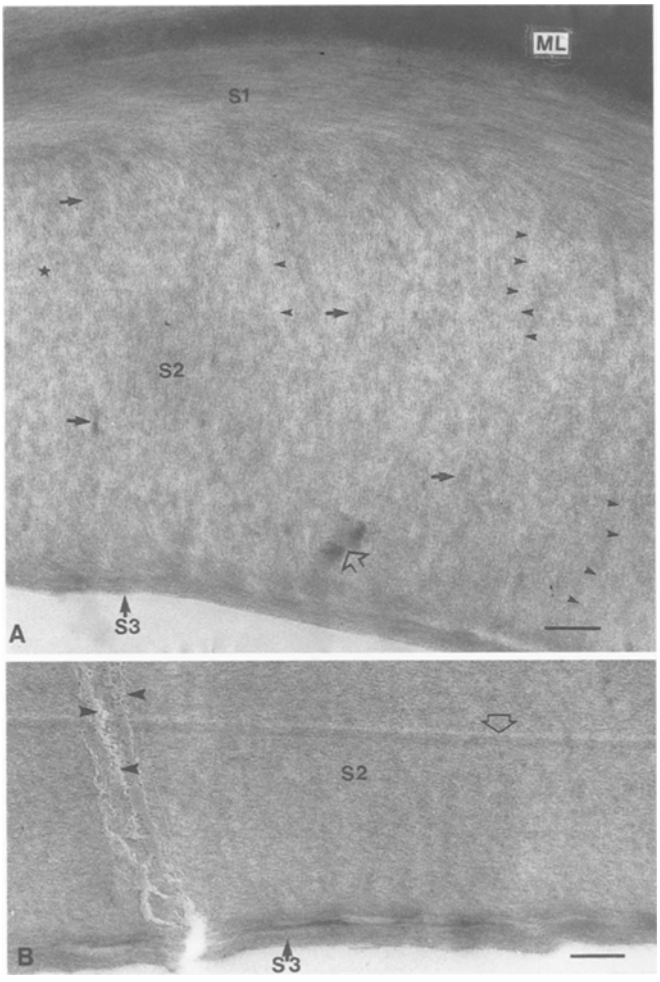


**Fig. 2.** **A** Transverse section through part of a tracheid wall. This high-magnification micrograph shows the lignin distribution in the $S_2$ layer to be distinctly inhomogeneous, with the wall appearing to be a mosaic of electron-dense (*arrows*) and electron-lucent (*arrowheads*) regions. The lucent regions have a pattern of interconnecting sinuous features with a predominantly radial alignment (*arrowheads*). **B** Transverse section through part of a tracheid wall. The microchecks present in the $S_2$ layer (*arrowheads*) have a radial profile and are sinuous, corresponding to the form and orientation of the dense and lucent sinuous wall regions. *Open arrow* indicates a knife mark. *Bars* 250 nm

species than in others. Recent observations suggest that lignin may not be evenly distributed even within individual wall layers or regions. For example, the distribution of lignin has been shown to be uneven in the cell corner middle lamella of both soft- and hardwoods.[7,10,19,20]

$KMnO_4$ has been widely used to contrast lignin in the electron microscopic studies of plant and wood cells.[10,21,22] This stain appears to be specific for lignin,[23] although there are suggestions that it may also stain some hemicelluloses,[24] but not cellulose. The observations of $KMnO_4$-stained ultrathin sections in this study show the distribution of lignin to be irregular within the $S_2$ layer, as is evident from the presence of numerous electron-dense and electron-lucent regions in this layer, giving this part of the wall a striated appearance at low magnification and a mottled appearance at high magnification. The pattern of lignin distribution and micromorphological features of the $S_2$ layer shown here in the tracheids of normal wood is similar, although somewhat less striking, to that widely present in the inner $S_2$ wall of mild compression wood tracheids of *P. radiata*[10,13,14] and appears to be also present in the $S_2$ layer of *Pinus sylvestris* wood from which polysaccharides had been selectively removed, leaving the lignin skeleton behind.[12]

Assuming that $KMnO_4$ can contrast all or most of the lignin present in the $S_2$ layer of *P. radiata* tracheids, we interpret the striated/mottled appearance of the $S_2$ layer to indicate that the electron-lucent regions of the wall contain more cellulosic polysaccharides than the electron-dense regions, and conversely the electron-dense regions contain more lignin than the electron-lucent regions. If $KMnO_4$ also stains hemicelluloses containing acidic groups,[24] dense regions are likely to contain more such hemicelluloses. However, their amount is likely to be fairly small relative to the amount of lignin present in these regions. Support for higher lignin content of the dense regions also comes from observations of the pattern of copper-chromium-arsenate (CCA) distribution in the $S_2$ layer of *Pinus abies* tracheids that had been delignified and subsequently treated with CCA. The CCA particles appeared to be preferentially concentrated in those regions that had become more porous after delignification because of their higher initial lignin content (Singh and Daniel, unpublished observations).

Recent TEM studies[25] using the technique of rapid freeze-deep etching (RFDE) of secondary walls prior to lignification show that cellulose microfibrils and their bundles in the $S_2$ layer are slightly sinuous in places rather than being straight rods, as the well known models of wood ultrastructure depict. During lignification, the lenticular spaces widely present among microfibril bundles apparently become filled with lignin and are no longer visible at the conclusion of the lignification process. Hafrén[26] has also observed microfibril bundles to vary in their size. Although this information is based on observations in the plane of individual prepared replicas, the pattern described is likely to be present throughout the thickness of the $S_2$ wall.

The micromorphological appearance of the $S_2$ layer in this study suggests that the microfibrils/microfibril bundles are more tightly grouped in the lucent regions than in the dense regions. The densest regions are likely to include regions corresponding to spaces[25] among microfibril bundles present in unlignified secondary walls. Although there were indications that there may be a gradation in the density of the $S_2$ wall, serial sectioning and computer-assisted image enhancement would be necessary to determine whether the variation in the density is related to the location of these regions within the plane of ultrathin sections or reflects the pattern of distribution of microfibrils. However, in the absence of this information and with the assumption that lucent regions are rich in cellulose and poor in lignin, the measurements made of the width of the lucent regions enable us to predict the size of the population of cellulose microfibrils in these cellulose-rich regions. The 20–40 nm width for most lucent regions would roughly correspond to the presence of 5–10 microfibrils based on the recent estimate of an average diameter of 3.6 nm for the

microfibrils in *P. radiata* holocellulose.[27] Confirmation of this predicted pattern of microfibril distribution must wait until it becomes possible to make a more precise assessment.

The sinuous pattern of the lucent regions (and correspondingly of the dense regions) across the thickness of the $S_2$ layer can be explained in part by the observations of Hafrén et al.,[25] which showed the microfibrils and their bundles to be sinuous in places in secondary walls, and by the observations of Kataoka et al.[28] showing microfibril angles to change in successive waves of deposition, leaving spaces between stripes (bundles). The sinuous appearance of the lucent regions suggests that microfibril bundles are not likely to be linear in their distribution across the width of the $S_2$ layer but may be more randomly arranged. The presence of microchecks in the mechanically damaged parts of the $S_2$ layer in *P. radiata* tracheid showing a sinuous profile supports this interpretation. The microchecks are likely to have formed in the interface regions between cellulose-rich (electron-lucent) and lignin-rich (electron-dense) areas, which also have sinuous profiles radially across the $S_2$ layer.

## Conclusions

It is apparent from the observations presented in this study that the macromolecular organization of wood cell walls is more complex than previously considered, and that the complexity of organization may be at different levels. The complexity shown in this work is at a level above the organization of individual microfibrils.[11] Future wood cell wall models will also have to take into consideration the forms and the pattern of distribution of microfibril bundles as observed in RFDE preparations as well as the complex pattern of lignin distribution shown in this work. The information thus presented can enhance our understanding of the effect of physical and chemical treatments on wood cell walls during wood processing.

**Acknowledgments** This work was carried out within the framework of the Wood Ultrastructure Research Centre (WURC), a competence center at the Swedish University of Agricultural Sciences (http://www-wurc.slu.se). WURC was established in cooperation with the Swedish National Board for Industrial and Technical Development, seven companies within or connected to the Swedish forest industry, and four Swedish universities/research institutions.

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