

Differentiation of Terminal Latewood Tracheids in Silver Fir Trees During Autumn

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• **Background and Aims** The differentiation of terminal latewood tracheids of silver fir (*Abies alba*) trees grown in Slovenia was investigated in autumn/winter 2001/2002.

• **Methods** The experimental trees were divided into three groups: one with narrow annual rings, width less than 1 mm; one with annual ring widths between 1 and 4 mm; and one group with broad rings larger than 4 mm. The differentiation of terminal latewood tracheids was investigated by light-, electron- and UV-microscopy in tissues sampled in October and November 2001 and March 2002.

• **Key Results** In the middle of October, cambial divisions did not occur any more in any of the trees. In trees with narrow annual rings, cell wall deposition as well as lignification were completed in terminal latewood tracheids at this date, whereas in trees with annual ring widths of more than 1 mm these processes still continued. Electron microscopy as well as UV microscopy revealed an unligified inner S₂ layer and the absence of S₃ and warty layers. With increasing distance from the cambium, wall formation and lignification gradually appeared to be completed. Samples of all trees taken in the middle of November only contained differentiated terminal latewood tracheids. At the structural and lignin topochemical level, November and March samples showed completed differentiation of walls of terminal latewood tracheids.

• **Conclusions** In trees with broader annual rings, the final steps of differentiation of the youngest latewood tracheids near the cambium still continued during autumn, but were finished prior to winter. It was concluded from structural observations that duration of cambial activity is longer in trees with broad annual rings than in trees with narrow rings.

Key words: Silver fir (*Abies alba*), latewood tracheids, cell wall structure, autumn differentiation, lignification, light microscopy, transmission electron microscopy, UV-microspectrophotometry.

INTRODUCTION

Xylem formation is to a great degree regulated by genetic and environmental factors. The process of differentiation of tracheary elements and fibres can be divided into four successive stages: (1) postcambial enlargement, which determines cell dimensions and shape, (2) formation of multilayered cell walls, (3) deposition of lignin within the cell wall polysaccharide matrix, and (4) cell death and autolysis of the protoplasm (Wardrop, 1965; Denne and Dodd, 1981; Fujita and Harada, 1991; Savidge, 1996, 2000; Plomion *et al.*, 2001; Wodzicki, 2001). In gymnosperms, more than 90 % of the newly formed cells differentiate into axially orientated tracheids responsible for both water conduction and mechanical support. At the end of the growing season the cambium becomes dormant, although its most recently produced derivatives continue differentiating for some time (Murmanis and Sachs, 1969; Nix and Villiers, 1985; Donaldson, 1991, 1992, 2001; Gindl *et al.*, 2001; Gričar *et al.*, 2003; Schmitt *et al.*, 2003).

The aim of this study with fir trees (*Abies alba*) of the Dinaric region in Slovenia was to add information at the ultrastructural level on the final steps of cell wall differentiation. Special attention was paid to the question as to how

long wall formation continues after cessation of cambial activity. Samples taken in the autumn and winter of 2001/2002 were examined by light- and electron-microscopy and for topochemical aspects of lignin distribution within cell walls by UV-microspectrophotometry.

MATERIAL AND METHODS

This study was conducted in a permanently monitored forest stand located at Ravnik/Slovenia (elevation 500–700 m), about 50 km south-west of Ljubljana (approx. 46°N, 14°E). The site is in the Dinaric region and is typical of silver fir/beech forests in Slovenia. Samples were taken from ten apparently healthy silver fir (*Abies alba* Mill.) trees, 150–180 years old with a mean stem diameter at breast height of approx. 50 cm. Samples containing inner phloem, cambium and outer xylem (30 × 10 × 10 mm³) were taken from all trees at breast height on 17 October 2001 (early autumn) and 14 November 2001 (late autumn), and on 8 March 2002 (late winter). They were immediately immersed in FEA (formaldehyde–ethanol–acetic acid) and stored for 1 week in a refrigerator. For light microscopy, sample blocks were then dehydrated using a graded ethanol series. Transverse sections (20 µm thick) were prepared without further processing using a Leica SM 2000R sliding microtome and double-stained with safranin and astra blue.

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Sections were mounted on glass slides using Euparal and examined with a Nikon Eclipse 800 light microscope. Two October samples containing cambium and outer xylem were chosen for detailed ultrastructural analyses by means of transmission electron microscopy (TEM) and UV-microscopy. These samples were dehydrated in a graded series of ethanol and water-free acetone and embedded in Spurr's epoxy resin (Spurr, 1969). For TEM, ultrathin transverse sections (80–100 nm thick) were stained with potassium permanganate according to Donaldson (1992). Examination was carried out with a Philips CM 12 TEM at accelerating voltages of 40 or 60 kV to enhance contrast. For UV spectroscopic measurements, unstained transverse sections (1 μm thick) were prepared from the epoxy resin-embedded samples, transferred to quartz slides, immersed in a drop of non-UV absorbing glycerine and covered with quartz cover-slips. UV-absorbance spectra were recorded using a Zeiss UMSP 80 micro-spectrophotometer. The specimens were investigated by point measurements with a spot size of 1 μm^2 using the programme LAMWIN[®] (Zeiss International). The spectra were taken at wavelengths from 240 to 400 nm in 2 nm steps. For each point analysis, the measurements were automatically repeated 50 times. Spectra from October samples were taken from the first, second, fourth, sixth, eighth and tenth cell row from the cambium. For the November and March samples, spectra were taken from the first and second cell row from the cambium. UV absorbance was determined for the outer (near compound middle lamella), middle and inner (near cell lumen) regions of tangential secondary cell walls.

RESULTS AND DISCUSSION

Light microscopy revealed that the cambium in all samples taken on 17 October 2001 consisted of only three to six cells with a very small radial diameter, indicating dormancy. In addition, no developing xylem cells in early stages of post-cambial growth were present. Staining with safranin and astra blue revealed that in trees with an outermost annual ring comprising more than 100 cells radially (typically giving a ring width of more than 4 mm), more than five cells were still actively differentiating. Staining also indicated that the inner regions of their secondary walls were incompletely lignified and that cytoplasm was still present in the cell lumina (Fig. 1). Trees with an outermost annual ring composed of 20–100 cells per radial row (corresponding ring width of 1–4 mm) also contained differentiating tracheids adjacent to the cambium, although in most cases this was limited to one or two cells per radial row. However, in trees with a narrow outermost annual ring, i.e. fewer than 20 cells per radial row and a ring width less than 1 mm, differentiation of terminal latewood tracheids was complete. Generally, the number of incompletely differentiated tracheids per radial row was related to the width of the outermost annual ring (Table 1). Four weeks later, on 14 November 2001, the fact that the entire wall of the terminal latewood tracheids stained red indicated that the cells were completely differentiated and fully lignified in all trees (Fig. 1). Samples collected in March were similar to

those collected in November in that they contained completely differentiated latewood tracheids with thickened and lignified secondary walls. From this it may be concluded that the differentiation of the terminal latewood tracheids was completed by the middle of November.

In recent years, potassium permanganate staining has been widely used in electron microscopy as an indicator for lignin distribution in different morphological regions of a woody cell wall (e.g. Maurer and Fengel, 1991; Donaldson, 1992; Singh and Donaldson, 1999; Schmitt and Melcher, 2004). In this study, sections stained with potassium permanganate revealed that the youngest latewood tracheids adjacent to the cambium of trees with broad annual rings were still in late stages of differentiation in the middle of October. Deposition of the polysaccharides of the S_1 and S_2 layers was evident in the cell walls, while the S_3 and warty layer were not yet present. The compound middle lamella, S_1 layer and outer S_2 layer were stained with potassium permanganate, but staining decreased from the outer to the inner S_2 layer (towards the lumen). From this it may be concluded that lignification had not yet taken place in the inner part of the S_2 layer (Fig. 2). These results on the distribution of lignin within developing cell walls are in good agreement with the frequently described delay between polysaccharide deposition and lignin incorporation (Wardrop, 1965; Kutscha and Schwarzmann, 1975; Saka and Thomas, 1982; Terashima and Fukushima, 1988; Donaldson, 1991, 1992, 2001; Fukushima and Terashima, 1991; Terashima, 2000). In October samples, secondary wall formation appeared complete at a distance of about eight cells from the cambium. The S_3 layer and the warty layer were clearly lignified, as demonstrated by their intense staining with potassium permanganate. By the middle of November, in all samples both secondary wall formation and lignification were completed also in the last-formed latewood tracheids. These observations confirm the conclusions drawn from light microscopy.

UV-microspectrophotometry was used for a semi-quantitative determination of lignin within individual cell wall layers. Softwood lignin is mainly composed of guaiacylpropane units and gives an absorption maximum at a wavelength of 280 nm, whereas hardwood lignin is a mixture of guaiacylpropane and syringylpropane units in varying ratios resulting in a shifted peak maximum towards 278–270 nm (Fergus and Goring, 1970; Fukazawa and Imagawa, 1981; Fengel and Wegener, 1989; Takabe *et al.*, 1992; Koch and Kleist, 2001; Koch and Grünwald, 2004). In this study, UV microscopy of tangential secondary walls of differentiating latewood tracheids in trees with broad annual rings sampled in October showed an increasing absorbance at 280 nm with increasing distance from the cambium. This indicated lower lignin content in immature cell walls. Figure 3 shows representative spectra of developing secondary walls adjacent to the cambium and up to a distance of ten cell rows from it. In each tracheid, spectra were taken from the outer, middle and inner region of secondary walls. Absorbance values between $\log \text{abs}_{280\text{nm}}$ 0.2 and 0.3 were recorded in the outer secondary walls of tracheids adjacent to the cambium. Structurally, such an outer secondary wall area represents S_1 and outer S_2 . These values were lower

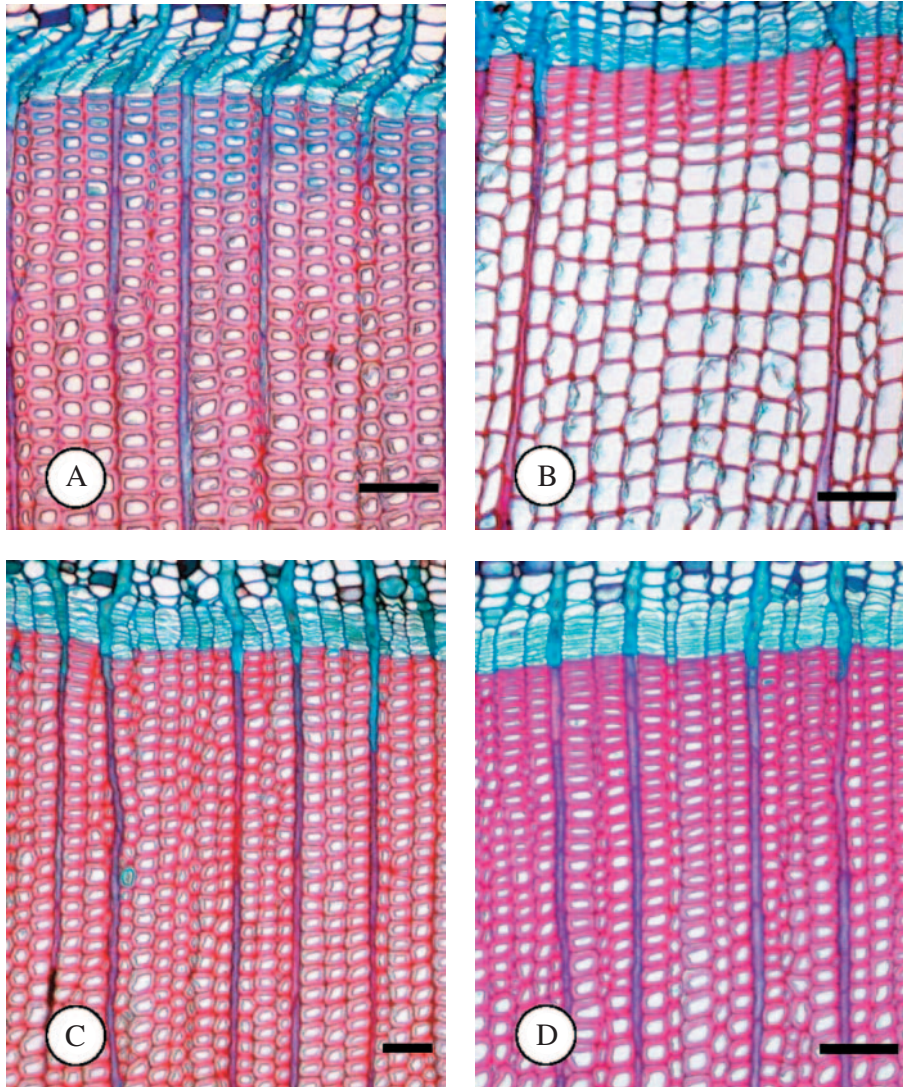


FIG. 1. Light micrographs of transverse sections through cambium adjacent xylem. (A) Sampling on 17 October (early autumn): trees with wide annual growth rings show incompletely differentiated terminal latewood tracheids, as indicated by blue stained inner wall parts. (B) Sampling on 17 October: trees with narrow annual growth rings show completed differentiation of terminal latewood tracheids, as indicated by red stained cell walls. (C) Sampling on 14 November (late autumn): trees with wide annual growth rings show completed differentiation of last-formed tracheids, as indicated by red wall staining. (D) Sampling in March (late winter): no differences to November samples in trees with broader annual growth rings. Scale bars = 100 μm .

TABLE 1. Relationship between total number of cells per radial row (total width) of the annual ring and number of incompletely differentiated latewood tracheids per radial row (width of incompletely differentiated tracheids) in silver fir trees in October

Tree number	Number of xylem cells per radial row in outer growth ring	Number of incompletely differentiated xylem cells per radial row	Width of annual growth ring (mm)	Width of zone of incompletely differentiated xylem cells (μm)
1	11.2	0.0	0.3	0.0
2	15.6	0.0	0.5	0.0
3	36.3	0.2	1.3	1.9
4	40.6	0.4	1.6	4.4
5	48.8	0.4	2.0	4.9
6	59.0	2.2	2.2	7.9
7	69.2	1.0	2.4	26.0
8	108.0	7.0	4.0	90.6
9	117.3	5.6	4.3	96.1
10	124.8	5.7	4.7	140.7

In all cases, number of measurements = 5.

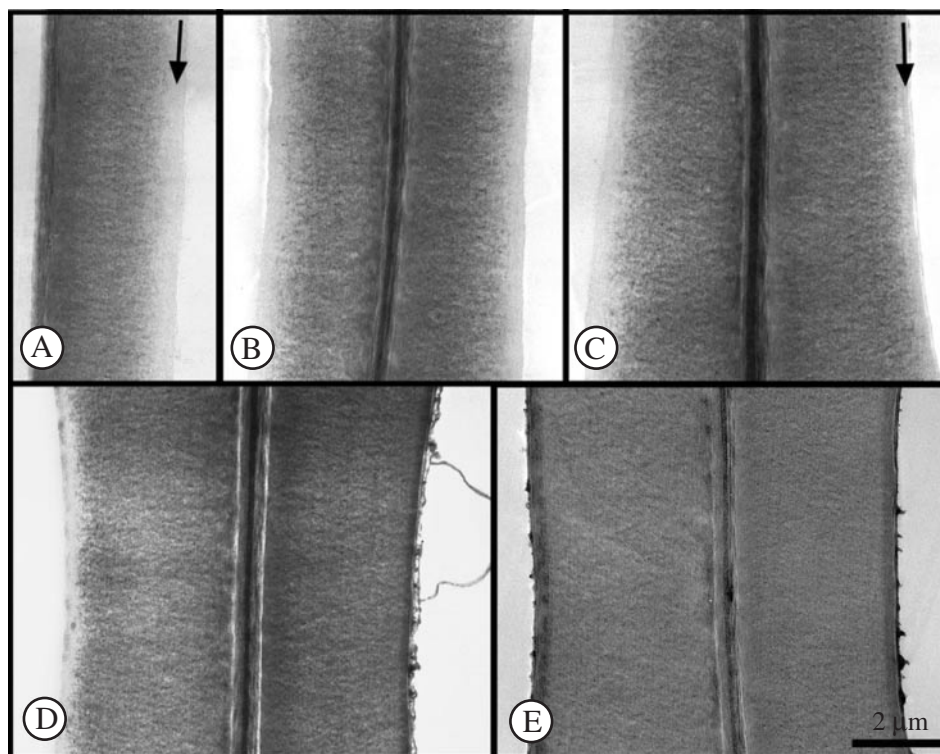


FIG. 2. Electron micrographs of tangential tracheid walls of the outermost growth ring of a tree with broad rings; samples taken on 17 October. (A) Cambium-adjacent tracheid; inner S_2 layer unlignified (arrow), S_3 and warty layer not deposited. (B) Second (left) and third (right) tracheid row, inner S_2 less lignified, S_3 and warty layer not yet deposited. (C) Fourth (left) and fifth (right) tracheid row, fifth with S_3 layer (arrow), S_2 layer still less lignified. (D) Sixth (left) and seventh (right) tracheid row; sixth without warty layer, inner S_2 and S_3 less lignified; seventh with warty layer under formation, inner S_2 and S_3 lignified. (E) Tenth (left) and eleventh (right) tracheid row, both with differentiated walls and distinctly visible warty layer.

than typically measured for the S_2 layers of spruce tracheids (Koch and Kleist, 2001), where absorption is around $\log \text{abs}_{280\text{nm}}$ 0.35 and 0.54. Extremely low absorption values between $\log \text{abs}_{280\text{nm}}$ 0 and 0.2 were found in the middle region of secondary walls of the last-formed tracheids (mainly the central S_2 layer). Spectra from the innermost secondary wall, mainly representing the inner S_2 layer, showed an absorbance of less than $\log \text{abs}_{280\text{nm}}$ 0.1. At about four cell rows from the cambium, the outer secondary wall reached an absorbance maximum of $\log \text{abs}_{280\text{nm}}$ 0.35 to 0.45, and at about the sixth cell row the middle region showed a $\log \text{abs}_{280\text{nm}}$ 0.3 to 0.4. The UV spectra taken from the inner secondary wall had their maximum in the eighth cell row with $\log \text{abs}_{280\text{nm}}$ 0.3 to 0.4. It can be concluded from these measurements that in trees with broad annual rings, the differentiation of the latewood tracheids was not completed within eight cell rows from the cambium, although cambial activity had ceased. Corresponding absorbance values of spectra taken from November samples already showed maximum values for all three secondary wall regions in last-formed tracheids. This was also true for March samples (Fig. 4), clearly indicating that lignification was completed in the period between the middle of October and middle of November.

It has been shown by several authors that differentiation of secondary xylem produced late in the year is not completed simultaneously with the onset of cambium

dormancy. Murmanis and Sachs (1969) investigated seasonal development of secondary xylem in *Pinus strobus*, and found cytoplasm still present in one-to-three tangential rows of latewood tracheids in samples taken in September, October and even in January. Nix and Villiers (1985) observed differentiation of the last-formed tracheids of loblolly (*Pinus taeda*) and slash (*Pinus elliotti*) pine during winter dormancy in South Carolina. Increasing wall thicknesses measured between November and March led them to conclude that the last-formed latewood cells of the current annual ring continued to deposit cell wall material until the following spring. Donaldson (1991) also reported incomplete lignification of secondary walls of latewood tracheids in *Pinus radiata* sampled in late autumn in New Zealand. In another study, Donaldson (1992) found that latewood was only partially lignified in most *Pinus radiata* trees sampled in late winter. Donaldson (2001) suggested from various observations that lignification is sensitive to environmental conditions, especially temperature, which seems to play an important role in slowing down this process. In support of our observations, Gindl *et al.* (2001) found the time course of cell wall thickening and lignification of tracheids in Norway spruce continued throughout the autumn. In the case of a valley-grown tree (elevation 580 m), cell wall thickening of latewood tracheids was completed on 20 October, but lignification was still taking place. However, at the end of the year, by 28 December, lignification of

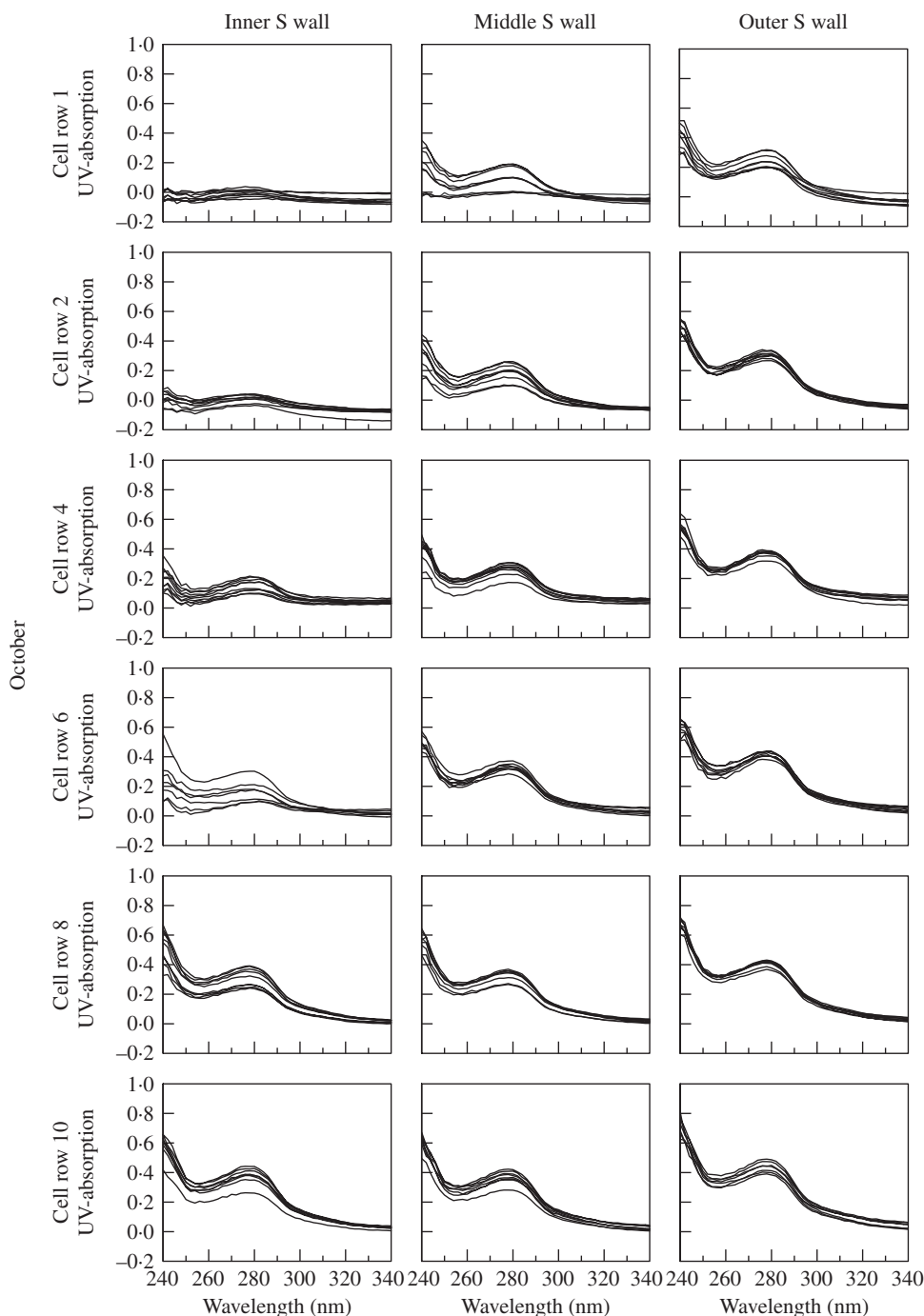


FIG. 3. UV-absorption spectra measured in outer (near compound middle lamella), mid- and inner (near lumen) secondary wall of latewood tracheids. Absorption at wavelength 280 nm increases with increasing distance from the cambium in October.

latewood tracheids was completed. All these observations confirm that lignification of latewood tracheids may be considerably delayed when compared with deposition of secondary wall polysaccharides.

In conclusion, the results obtained by light-, electron- and UV-microscopy for silver fir trees growing in mountainous regions of Slovenia clearly demonstrate that in early autumn (October) lignin deposition was still progressing in

trees with broad annual rings. Similarly, we observed incompletely differentiated terminal latewood tracheids in trees with wide growth rings in the middle of October 1999 (Schmitt *et al.*, 2003) and in the middle of October 2002 as well (J. Gričar, unpubl. res.). It is suggested that the date of completion of differentiation in the last-formed latewood tracheids is partly related to the duration of cambial activity. The cambium of more productive trees remains active

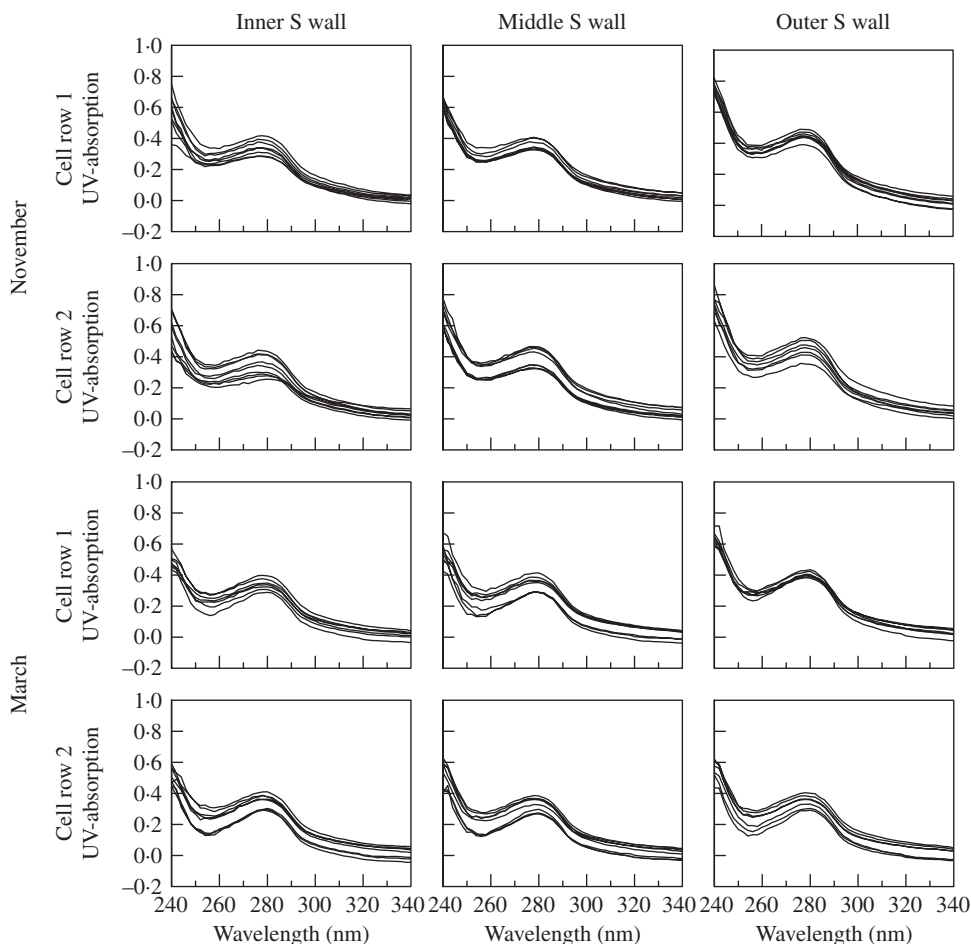


FIG. 4. UV-absorption spectra measured in outer (near compound middle lamella), mid- and inner (near lumen) secondary wall of latewood tracheids at the cambium in November and March. Absorption values at wavelength 280 nm are similar.

longer and therefore produces more cells. On the other hand, the cambium of less productive trees stops earlier with its cell division activity leading to narrower annual rings. As a consequence of these differences, differentiation of tracheids laid down late in the season continues in October and perhaps even in the first days of November. Differentiation of the latewood tracheids was completed in all investigated trees before the middle of November.

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