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Characterisation of Wound Reaction Compounds in the Xylem of *Tilia americana* L. by Electron Microscopy and Cellular UV-Microspectrophotometry [1,2]

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Abstract

Wound reactions in cells of the boundary layer in the xylem of *Tilia americana* L. were investigated by transmission electron microscopy and cellular ultraviolet (UV)-microspectrophotometry. Transmission electron microscopy revealed that in vessels and fibres wound reaction compounds are deposited in the lumen as balloon-like structures and/or as wall attachments. Parenchyma cells frequently show atypical innermost wall layers, either similar to regular wall layers or as a nearly electron transparent structure resembling suberinised cell wall layers. UV-microspectrophotometry allowed for a closer chemical characterisation of the wound reaction compounds. According to their absorbance behaviour with a distinct maximum beyond 300 nm, these were identified as flavonoid compounds. Precursor substances with an additional maximum at 278 nm were found in parenchyma cells. The combination of transmission electron microscopy and UV-microspectrophotometry also revealed that wound reaction compounds are attached to but do not impregnate the vessel walls.

Keywords: wound reactions, xylem, vessels, parenchyma cells, cell wall, electron microscopy, UV-microspectrophotometry, *Tilia americana* L.

[1] Based on a paper presented at the 3rd Joint New Zealand-German Symposium on Plant Cell Walls, 13-15 February 2008, Auckland, New Zealand

[2] Dedicated to our dear colleague Dr. H.G. "Jorgo" Richter on the occasion of his 70th birthday.

Introduction

Wounding of tree xylem causes responses in living cells endeavouring to compartmentalization of affected tissue portions (Shigo, 1984; Shigo & Marx, 1977; Liese & Dujesiefken, 1996). To effectively protect the inner xylem against air embolism, desiccation and invasion of microorganisms, the wound-associated metabolic processes in parenchyma cells result in the formation of a thin, distinctly visible zone along the interface between affected and unaffected xylem portions. This reaction zone or boundary layer separates the discoloured and decayed xylem tissue from the healthy and functional xylem without discolouration (e.g. Pearce, 2000). Within the boundary layer, vessels and fibres in hardwoods contain granular or fibrillar deposits mostly filling the entire lumen. It is variously described that xylem parenchyma cells are responsible for these blockages by synthesising wound reaction compounds which are then secreted through the pits into adjacent vessels and fibres (Hillis, 1987; Schmitt & Liese, 1990; Bonsen, 1991; Fink, 1999). In addition, wound reaction compounds are frequently involved in the modification of fibre and vessel walls by aggregating along wall surfaces and/or by incrusting inner wall layers (Pearce, 2000; Melcher et al., 2003). Such

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mechanisms successfully enhance the resistance of the boundary layer mainly against decaying microorganisms as reported by Pearce (1991), Schwarze & Fink (1997) and Stobbe et al. (1999).

There is some information on the chemical nature of these wound reaction compounds. Hillis (1987) described numerous accessory compounds in wound xylem of soft- and hard-woods. Pearce (1996) gave an overview on the chemical nature of antimicrobial compounds produced in trees during defence against fungal attacks, such as phenylpropanoids, flavonoids and terpenoids. In wound wood of *Tilia* spp., Melcher et al. (2003) identified the sesquiterpene 5-hydroxy-calamenene.

Although we know much about the fine structural processes during wound reactions and the chemical nature of some wound response compounds, there is little known about their subcellular localisation. To obtain more detailed information on the topochemistry of aromatic compounds synthesised and deposited in response to wounding and on a possible impregnation of cell walls, we used a combination of transmission electron microscopy and cellular UV-microspectrophotometry for analyses of wound xylem in *Tilia americana* L.. This investigation focused on phenolic extractives deposited in vessels, fibres and parenchyma cells of the boundary layer.

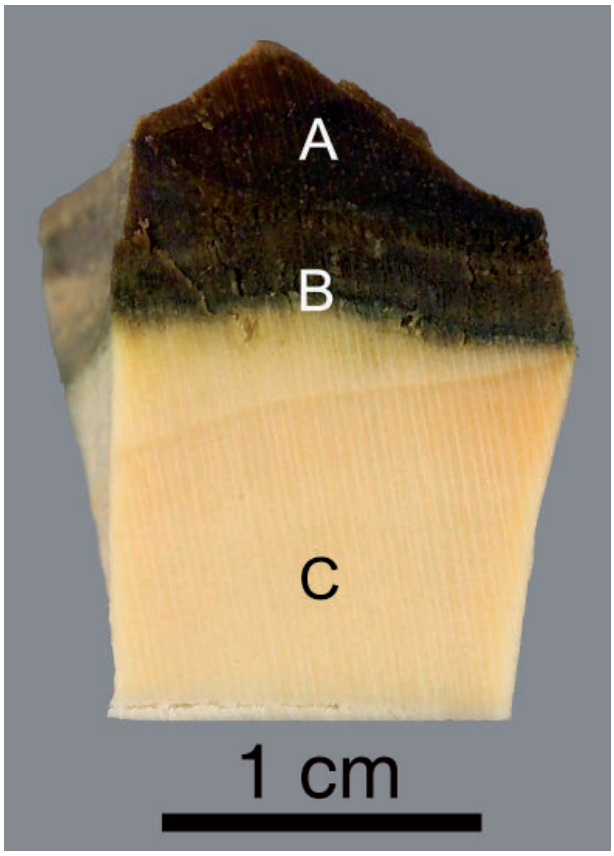

FIGURE 1: Macroscopic view of discoloured xylem close to the wound (A), greenish boundary layer (B), and inner unaffected xylem (C).

Materials and Methods

The stem of a T. *americana* tree (about 45 years old with a diameter at breast height of 55 cm) was wounded mechanically during early May 2007. Four wounds of approximately 50 x 50 mm^2 were induced with a chisel by removing bark, cambium and the outermost 2-4 mm of the xylem. After a response period of two months, the wound surface appeared distinctly dark brown discoloured. There were no qualitative differences between the wounds. Sampling of wound reaction tissue at that time was performed with a chisel so that the entire discoloured outer and some unaffected inner xylem was obtained. Discolourations extended from the wound surface about 20 mm into the xylem with a narrow but clearly visible, greenish boundary layer at the transition from discoloured to unaffected xylem (Figure 1). For transmission electron microscopy (TEM) and cellular UV-microspectrophotometry (UMSP), the size of each sample was reduced with a razor blade to 2 x 2 x 5 mm^3 containing the greenish boundary layer and some cell rows of the dark brown outer xylem as well as the inner unaffected xylem. Samples for TEM analysis were fixed for one day in a mixture of a 2% paraformaldehyde and a 2.5% glutaraldehyde solution (buffered in 0.1M sodium cacodylate buffer adjusted with hydrochloric acid to a pH value of 7.2 after Karnovsky, 1965), washed in the same buffer, osmicated with a 1% aqueous osmium tetroxide solution, again washed in buffer, dehydrated in a graded series of acetone, and embedded in Spurr's epoxy resin (Spurr, 1969). Samples for UMSP analysis were air-dried and then directly infiltrated with Spurr's epoxy resin under mild vacuum with several cycles of evacuation and ventilation as described by Kleist and Schmitt (1999). The resin was polymerised at 70 °C for 24 hours. For TEM, the ultrathin sections were post-stained with 1% aqueous uranyl acetate and 6% aqueous lead citrate (Reynolds, 1963) and examined with a Philips CM12 TEM at an accelerating voltage of 80 or 100kV. UV-microspectrophotometry was carried out with a ZEISS UMSP 80 microspectrophotometer equipped with a scanning stage for the determination of image profiles at a constant wavelength of 278 nm using the software APAMOS® (Zeiss). All UV analyses were carried out with 1 µm semi-thin sections mounted on quartz slides, embedded in glycerine and covered with quartz coverslips. Image profiles were recorded with a local geometrical resolution of 0.25 µm x 0.25 µm and a photometric resolution of 4096 greyscale levels and converted into 14 basic colours representing the measured absorbance intensities (see Koch & Kleist, 2001; Koch & Grünwald, 2004 for details). Additional sections were analysed by photometric point measurements with a spot size of 1 μm^2. Spectra were taken in 1nm-steps in the wavelength range between

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240 and 700 nm using the software LAMWIN® (Zeiss). This technique is well established for the determination of lignin in the various layers of woody cell walls (e.g. Fergus et al., 1969; Saka et al., 1982; Takabe, 2002), where lignin displays a characteristic UV-absorbance mainly around 212 nm and additionally at 280 nm due to the presence of associated phenylpropane groups with several chromophoric structural elements (e.g. Sarkanen & Hergert, 1971; Hesse et al., 1991). No other component in the mature cell wall shows these UV-absorbance characteristics. Phenolic compounds have maximum absorbances slightly above 280 nm (bathochromic shift) and simultaneously distinctly above 300 nm forming a shoulder peak (e.g. Goldschmid, 1971; Koch et al., 2003).

Results and Discussion

Wounding of trees causes distinct discolorations in affected tissues. In the xylem, wound reactions are restricted to certain regions around the wound causing a usually brownish discoloration. With time, a sharp transition to the unaffected xylem develops. Such a transition zone is characterised by intense wound reactions as indicated by the fact that nearly all vessels and fibres contain deposits of accessory compounds produced in parenchyma cells (see also Stobbe et al., 1999). In *Tilia americana*, this narrow reaction zone or boundary layer (Pearce, 2000) displays a greenish colour (Figure 1) and its contours do not follow growth ring boundaries. Regarding functional aspects, the boundary layer represents a static wound boundary retaining its function for an extended time to restrict embolism and spread of invading microorganisms into inner xylem portions (Pearce, 1996). However, if microorganisms penetrate this layer, new xylem becomes colonised with the subsequent establishment of a new boundary layer (Pearce, 1987, 1991; Boddy, 1992). Wood-degrading fungi are present in the brownish xylem adjacent to the wound and usually cause intense decay as variously described (e.g. Hillis, 1987; Blanchette & Biggs 1992; Pearce, 2000). They are absent from cells forming the boundary layer.

Vessels and fibres of a boundary layer frequently show occlusions of varying structure, easily visible with both the light and electron microscopes. Tyloses are a type of vessel occlusion representing cytoplasmic outgrowths from a parenchyma cell through a pit in a vessel. In 1949, Chattaway concluded that the diameter of the aperture of pit pairs between parenchyma cells and vessels plays an important role in the formation of tyloses, whereby species with small pit apertures are unable to produce tyloses. Bonsen and Kučera (1990) confirmed this hypothesis and stated that a minimum pit-diameter of about 3 µm is necessary. The minimum diameter of pit-apertures in *Tilia americana* is distinctly below that value (e.g. Schmitt & Liese, 1992). This might explain why *T. americana* does not form tyloses unlike species in other hardwood genera such as *Populus*, *Quercus* or *Robinia*. Figure 2A shows the lumen of a *T. americana* vessel shortly after wounding. The balloon-like structures do not contain a cytoplasm which is further evidence that they are not tyloses. Instead, they consisted of an electron-dense envelope with more or less loosely packed fibrillar or granular fillings or uniformly dark staining material (Figure 2A). Similar results have been observed in *Betula pendula* Roth (Schmitt & Liese, 1992). It has been frequently reported that these wound response compounds are synthesised in neighbouring parenchyma cells from which they are extruded through the pits into vessel and fibre lumina (Bauch et al., 1980; Dujesiefken et al., 1989; Bonsen & Kučera, 1990; Schmitt & Liese, 1990, 1992; Bonsen, 1991). Our subcellular studies of *T. americana* boundary layer cells also show electron dense compounds attached to the vessel wall, which often appear irregular towards the lumen. Figure 2B shows the secondary wall of a vessel composed of three sub-layers of similar thickness. At the interface between the innermost wall layer and the attached substances is an abrupt transition without any evidence that the attached compounds impregnate the vessel wall. The substances attached to the wall resembled the envelope structures already described for lumen deposits. The structural differences between envelopes of the larger balloons and their contents indicate that these structures undergo modifications in the lumina, such as polymerisation and oxidation (Koch et al., 2006).

Results of our TEM studies indicated that parenchyma cells of the boundary layer frequently showed an additional inner layer when compared with cells of the control tissue so that the secondary wall consisted of two to four broad layers (Figures 2C & 2D). In contrast to such an electron dense layer (shown by arrows in Figure 2C), which structurally resembles a cellulose and lignin containing secondary wall layer, parenchyma cells sometimes also showed a nearly electron transparent innermost wall layer (Figure 2D). This could be interpreted as shrinkage of cell content but, in fact, this consisted of fibrillar/granular material at early stages of development and in late developmental stages of a few sub-layers, often described as similar to “a suberinised cell wall layer” (see Figure 2D insert) (Biggs, 1987; Pearce, 1990), because it stains positive with Sudan IV (Schmitt & Liese, 1993). Such a wall also resembles suberinised parenchyma cells identified in wound-associated bark tissue (Biggs, 1984, 1985; Biggs et al., 1984; Trockenbrodt, 1994). Sudan dyes are widely used for suberin staining because of their lipophilic properties; suberin staining is probably due to a physical association rather than a chemical reaction (Krishnamurthy, 1999). In addition, we found that parenchyma cells of the boundary layer also contained large electron dense bodies and fibrillar aggregations, both filling a large part of the protoplast (Figures 2C & 2D).

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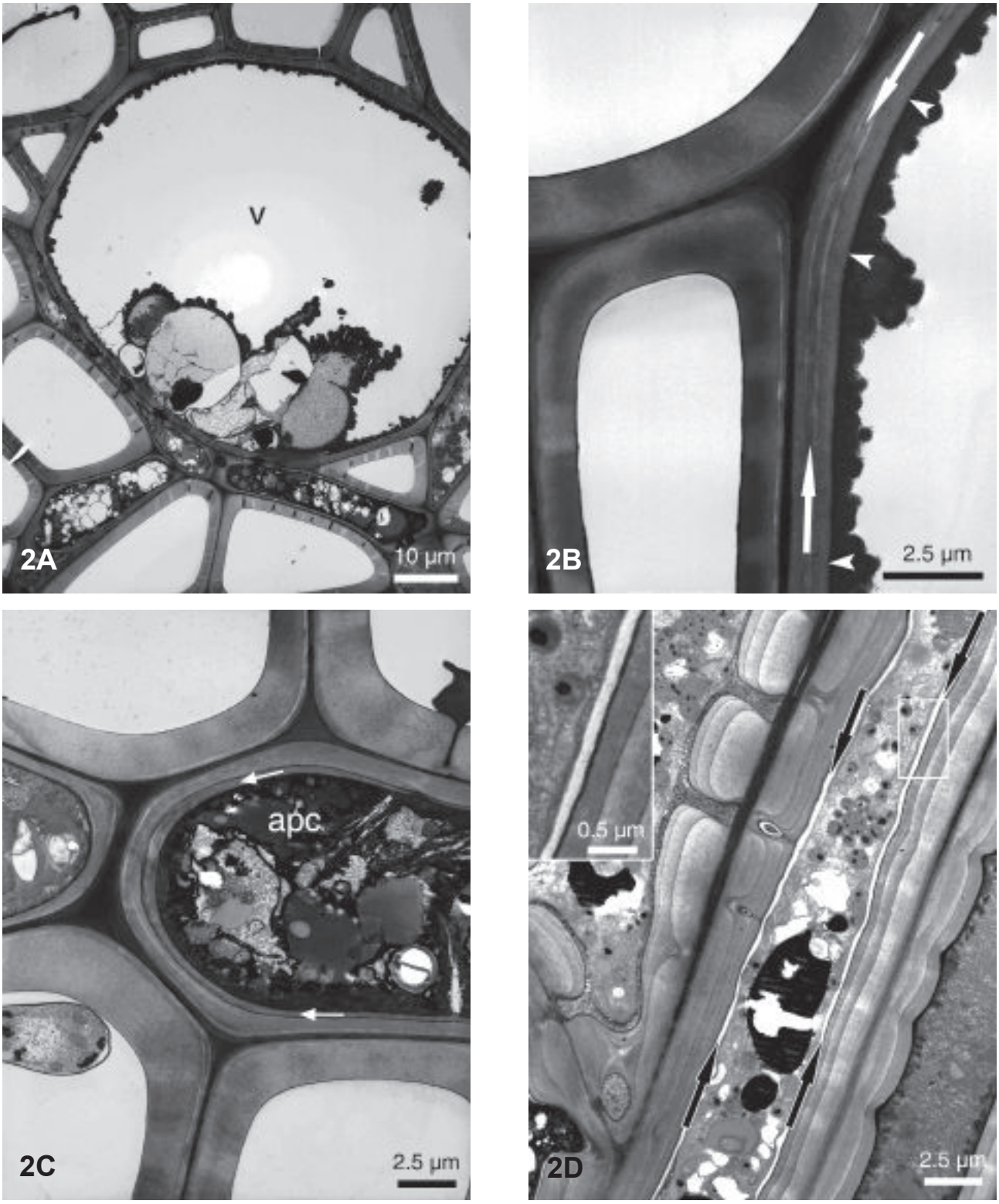

FIGURE 2: TEM micrographs of different cell types in the boundary layer: 2A - vessel (v) with balloon-like and wall-attached wound reaction compounds in its lumen. 2B - vessel wall with typical three-layered structure of its secondary wall (arrows) and dark staining wound reaction compounds directly attached to the wall; note that these compounds are not penetrating the wall (arrowheads). 2C – axial parenchyma cell (apc) with additional inner secondary wall layer (arrows) and large amounts of fibrillar wound reaction compounds. 2D – ray parenchyma cells with multilayered secondary wall and electron transparent early stage suberin-like layer (arrows); large electron dense bodies and fibrillar material in vacuoles of the protoplast. Inset: Detail of the suberin-like layer consisting of fibrillar/granular material.

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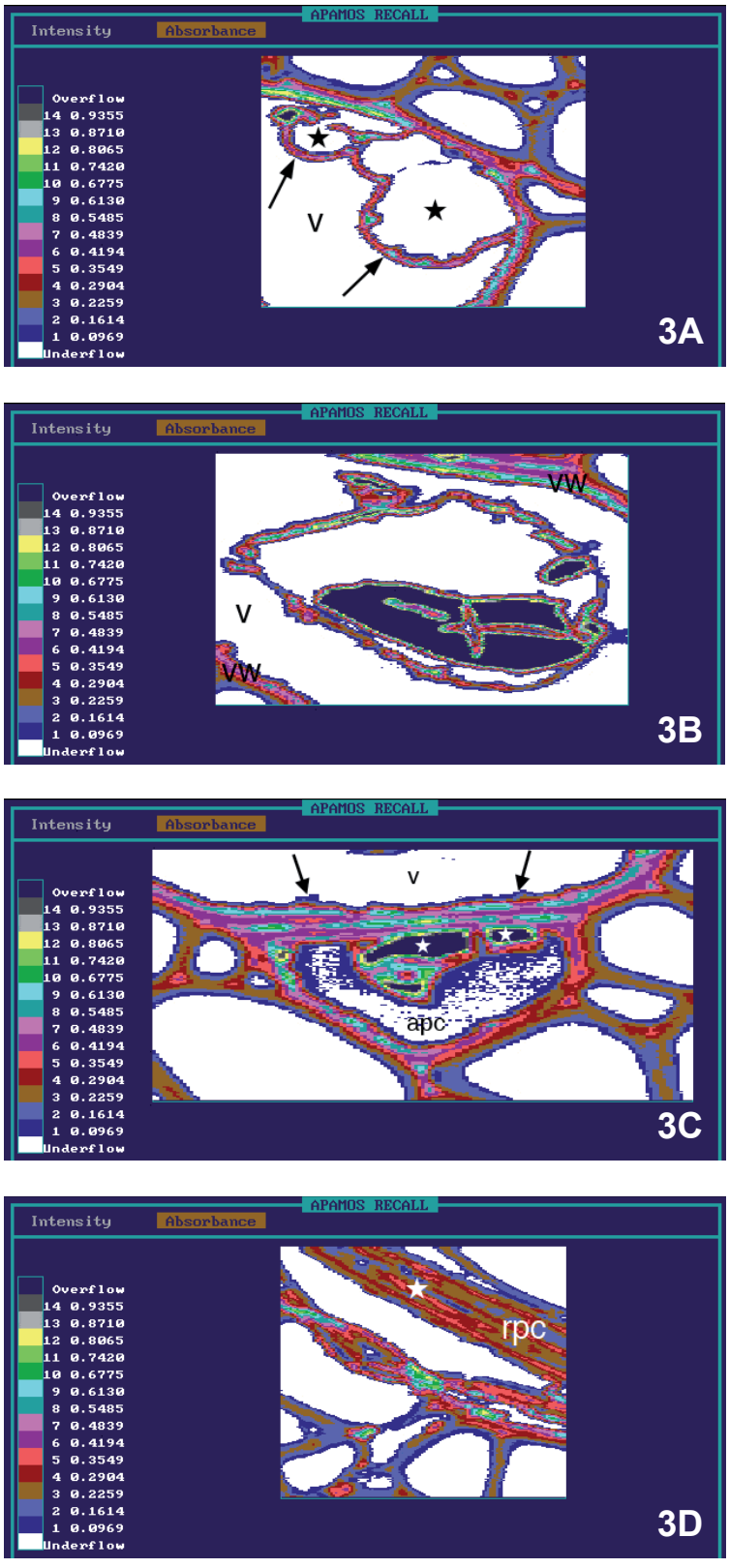

FIGURE 3: UV-microscopic scanning profiles of different cell types in the boundary layer; the colour pixels represent different UV absorbance values measured at a wavelength of 278 nm (geometric resolution of 0.25 µm x 0.25 µm): (A) – vessel (v) with balloon-like wound reaction compounds (★); envelopes of the balloons (arrows) with absorbance values between 0.1 and 0.3. (B) – vessel with balloon-like wound reaction compounds containing strongly absorbing fillings; vessel wall (vw). (C) – axial parenchyma cell (apc) with strongly absorbing wound reaction compounds (★); vessel (v) with wall-attached wound reaction compounds (arrows). (D) – ray parenchyma cell (rpc) containing wound reaction compounds with relatively low absorbance (★).

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Cellular UV-microspectrophotometry was used to determine the UV-absorbance intensities and spectral characteristics of lignified cell walls and deposits of aromatic compounds within the boundary layer. UV-image profiles obtained with a constant wavelength of 278 nm showed that the envelopes of the balloon-like vessel deposits had absorbance values between 0.1 and 0.3 (Figure 3A). Inner balloon-structures, if present, often displayed significantly higher absorbance values of 0.8 or even more, whereas absorbance values were distinctly lower along their outer margins (Figure 3B). Some vessel deposits did not show UV-absorbances in the inner portions (Figures 3A & 3B). Similar results were obtained for the topochemical distribution and characterisation of wound reaction compounds in axial and ray parenchyma cells. Some of these compounds were characterised by high absorbance values in inner portions and low absorbance values along the outer margin (Figure 3C). However, other axial or ray parenchyma cells, either additionally or exclusively, contained dispersed material with rather low absorbance values (Figures. 3C & 3D). Walls of vessels with wound-associated deposits in the lumina displayed typical absorbance characteristics with values of around 0.3 in cell corners, around 0.2 in the middle lamella, and between 0.1 and 0.2 in the secondary wall (Figure 3C), which is a normal value regularly recorded for control tissue of several hardwood species (e.g. Koch & Kleist, 2001; Frankenstein et al., 2006). Aggregated wound-reaction compounds attached to a vessel wall, however, show higher absorbance values of up to 0.7 (Figure 3C). More details on the UV-absorbance behaviour of individual cell wall layers and deposits were obtained by recording spectra using the point measurement mode with a wavelength varying between 240 nm and 700 nm. These spectra were used to characterise different aromatic compounds, including various types of condensed phenolics and also lignin. Condensed phenolics all have a maximum absorbance beyond 300 nm (Koch & Grünwald, 2004; Koch et al., 2006). Lignin occurs only in cell walls and, in hardwoods, shows a single maximum at 278 nm (Fergus & Goring, 1970).

Spectra taken from the strongly absorbing deposits from cell lumens showed a distinct absorbance maximum at about 370 nm (Figure 4). According to Koch (2004), this particular absorbance corresponds to highly condensed aromatic compounds with a flavonoid skeletal structure. The spectra of deposits in both axial and ray parenchyma cells also showed such a distinct absorbance maximum at about 370 nm (Figure 4). In addition, these deposits showed a second absorbance maximum at 278 nm (Figure 4). This cannot be due to lignin since lignin does not occur in lumen deposits. For substances in cell lumens with both maxima, the maximum at 278 nm indicates the occurrence of precursor compounds of highly condensed flavonoids, which are regularly synthesised in the parenchyma cells and later on deposited in vessels and fibres

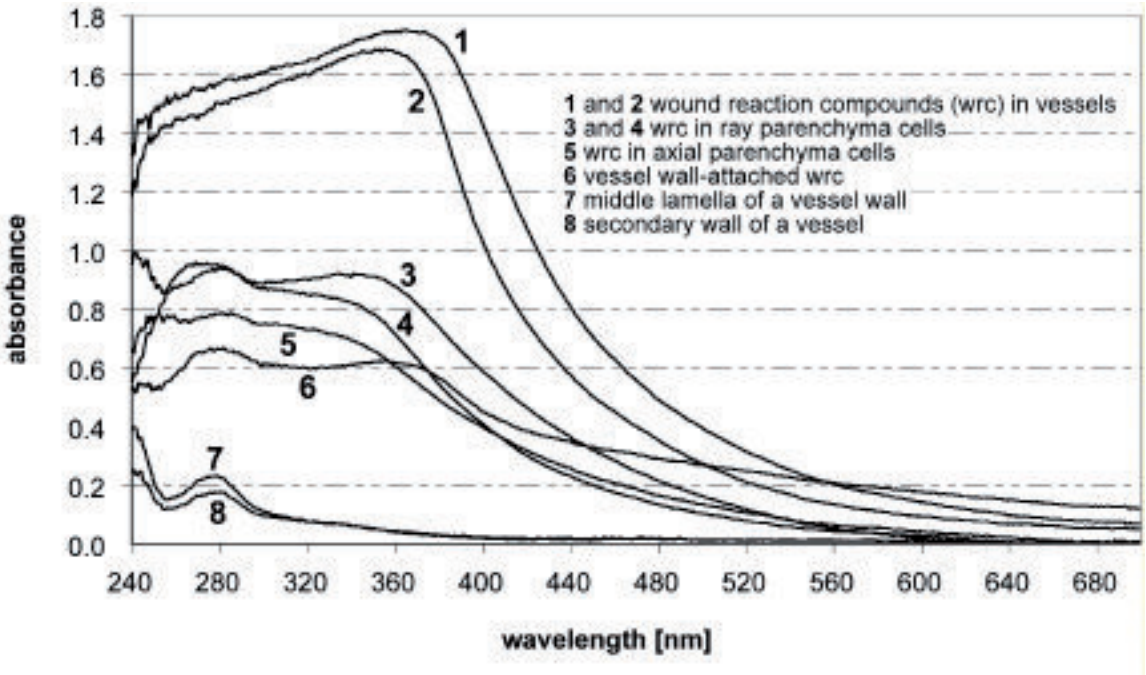

FIGURE 4: UV-absorbance spectra representative of vessel walls and wound reaction compounds obtained by the point measurement mode with varying wavelength. Wound reaction compounds in vessel lumina show a characteristic maximum at about 370 nm (spectra 1 and 2). In ray parenchyma cells wound reactions compounds display an additional maximum at 270-280 nm pointing to the occurrence of precursor compounds of highly condensed flavonoids (spectra 3-6). Lignin in woody cell walls shows distinct lower absorbances at wavelengths of around 278 nm and no additional maximum beyond 300 nm (spectra 7 and 8).

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with subsequent poly-condensation. Flavonoids are normally synthesised via the phenyl-propanoid metabolism. Melcher et al. (2003) who analysed *T. americana* wound tissue by thermal desorption-GC/MS revealed an increasing synthesis of aromatic compounds similar to the derivatives of coniferyl aldehyde with increasing duration of wound response. The absorbance maximum at wavelengths above 300 nm can be explained by the formation of compounds with chromophoric groups such as conjugated double bonds attributed to highly condensed phenolics as described in the literature for discoloured wood (Goldschmid, 1971; Koch et al., 2003; Koch, 2004). When comparing axial with ray parenchyma cells, it became evident that wound reaction substances in ray cells had a higher degree of condensation than those in axial parenchyma cells as semiquantitatively inferred from the increased UV-absorbances (Figure 4). Absorbance maxima at 278 nm and 370 nm were also recorded for deposits along the vessel walls. However, the intensities were much lower than in the lumen deposits (Figure 4). Vessel walls with these attached deposits did not show any difference in their UV-absorbance to vessel walls of the control tissue. Middle lamella regions and secondary walls had absorbance values (slightly above 0.2 and slightly below 0.2, respectively) of the same magnitude as in the controls (Figure 4). These findings show that the wound reaction compounds are not impregnating the vessel wall. Koch et al. (2003) made similar observations for phenolic extractives in vessels of discoloured beechwood. During regular heartwood formation, however, vessel walls become impregnated with those extractives as demonstrated by Koch and Kleist (2001) for sapeli (*Entandrophragma cylindricum*) and by Koch et al. (2006) for afzelia (*Afzelia* sp.) and merbau (*Intsia* sp.). On the other hand, Melcher et al. (2003) found that low molecular weight phenolic extractives produced in *T. americana* during xylem wound reactions penetrated into inner fibre wall regions.

In conclusion, the combination of structural and topochemical investigations clarifies the close relationships between the synthesis of wound reaction compounds in parenchyma cells and their deposition in adjacent vessels and fibres. Parenchyma cells contain precursors of flavonoid compounds deposited as highly condensed compounds in lumina of fibres and vessels during wound tissue formation. In the early stages, the phenolic extractives in vessels mostly form balloon-like structures and wall-attachments, but do not impregnate the vessel wall.

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