ABSTRACT

Wood cell walls are naturally fluorescent due to the presence of lignin. Auto-fluorescence offers a more specific method for localising lignin than staining and can potentially be used to assess cell wall modification resulting from a range of biological, chemical and physical treatments. In order to optimise conditions for imaging lignin by auto-fluorescence and to evaluate possible differences in fluorescence between softwood and hardwood lignin, wood sections of radiata pine and poplar were examined by confocal laser scanning microscopy to measure fluorescence spectra in a range of mounting media. Glycerol/buffer mixtures at three different pH values were compared with immersion oil and thiodiethanol using both UV and visible excitation. Glycerol/buffer at pH 9 produced the strongest lignin fluorescence at visible excitation indicating that this is the optimal mounting medium for imaging and spectroscopy. For UV excitation, thiodiethanol gave increased brightness relative to glycerol. Poplar lignin was four times brighter than pine lignin with excitation at 488 nm at pH 9, and showed characteristic differences in spectral emission under these conditions. This characteristic fluorescence was localised to the inner secondary wall of fibres, expressed as a gradient from the outer S₂ region increasing towards the lumen, as visualised by spectral unmixing. Comparison with sapwood from other hardwood species suggests that this fluorescence emission is characteristic of poplar and willow (Salicaceae). Members of the Salicaceae family are known to contain characteristic syringyl p-hydroxybenzoate lignin and it is likely that this special lignin type is responsible for the characteristic pH dependent fluorescence of poplar fibre walls.

Keywords: Fluorescence, lignin, cell wall, tension wood, syringyl p-hydroxybenzoate, spectral unmixing.

INTRODUCTION

Lignin can be localised microscopically using a wide range of techniques (Donaldson 2001) including UV absorption microscopy (Fergus et al. 1969; Fergus & Goring 1970; Koch & Kleist 2001), fluorescence microscopy (Kutscha & McOrmond 1972; Donaldson et al. 1999, 2010; Donaldson & Bond 2005; Radotić et al. 2006; Djikanović et al. 2007; Donaldson & Knox 2012), transmission electron microscopy combined with
potassium permanganate staining (Kutscha 1968; Bland et al. 1971; Donaldson 1994) or with immunocytochemistry (Kim & Koh 1997; Joseleau et al. 2004; Kukkola et al. 2004; De Micco et al. 2012), elemental tagging combined with energy dispersive X-ray spectroscopy (EDS) (Saka & Thomas 1982; Eriksson et al. 1988), Raman microscopy (Agarwal 2006; Gierlinger & Schwanninger 2006), and time-of-flight secondary-ion mass spectrometry (TOF-SIMS) microscopy (Tokareva et al. 2007). There are also several histochemical tests to indicate lignification or lignin type including phloroglucinol and maule reagent (Lin & Dence 1992). Because of the importance of lignin to both biological and industrial studies involving wood, microscopy has been used extensively to visualise and measure lignin content and composition on microscopic sections (Donaldson 2001).

Wood cell walls exhibit natural fluorescence due to the presence of lignin (Kutscha & McOrmond 1972; Castellan & Davidson 1994; Olmstead & Gray 1997; Albinsson et al. 1999). Lignin has a broad range of fluorescence emission and can be excited with both UV and visible light, emitting blue, green and red fluorescence (Donaldson et al. 1999, 2010; Donaldson & Bond 2005; Radotić et al. 2006; Djikanović et al. 2007; Donaldson & Knox 2012). Autofluorescence has been used to localise lignin in wood and pulp in order to investigate the effects of industrial processing (Davidson et al. 1991; Beyer et al. 1993; Billa et al. 1999, 2000), to study the photodegradation of wood surfaces (Pandey 2005), or to study lignin distribution in various wood types (Donaldson et al. 1999; Donaldson et al. 2010; De Micco & Aronne 2012; De Micco et al. 2012) or during wood formation (Kutscha & McOrmond 1972; Mast et al. 2009). Lignin fluorescence can provide specific chemical information and has been used, for example, to detect compression wood and to measure its severity (Donaldson et al. 1999; Donaldson et al. 2004; Donaldson et al. 2010).

For brightfield microscopy, a range of staining techniques is available to differentiate lignified and un lignified tissues in plants. The most common stain for botanical tissue is safranine (safranine O) (Ma et al. 1993; Vazquez-Cooz & Meyer 2002; Angeles et al. 2004; Bond et al. 2008). Safranine is typically used in conjunction with a counterstain such as fast green (Berlyn & Miksche 1976; Ma et al. 1993) or astra blue (Srebotnik & Messner 1994; Vazquez-Coos & Meyer 2002), so that lignified tissue is stained red while un lignified tissues are stained green or blue. Such stains can also be used for fluorescence microscopy (Haseloff 2003). Safranine is not specific for lignin but will also stain a range of other materials found in plant tissues such as nuclei, cutin, suberin, cellulose, and gums and resins (Kasten 1989; Ruzin 1999).

Lignin can also be localised with fluorescent stains such as acriflavin (Donaldson 2002), basic fuchsin (Donaldson & Bond 2005), safranine (De Micco & Aronne 2007; Bond et al. 2008) and berberine sulphate (Brundrett et al. 1988; Serrato-Valenti & Riveros 1995). Such stains may not be entirely specific but are bright compared to lignin autofluorescence and hence make it easier to image samples with great sensitivity. However, the lack of specificity is a disadvantage compared to lignin autofluorescence.

In order to optimise lignin autofluorescence for microscopy applications and to investigate potential differences between softwood and hardwood lignin for histochemi-
cal purposes, sections of radiata pine and poplar were mounted in a range of media including glycerol/buffer at high, low and neutral pH, as well as two organic media. A comparison of UV and visible excitation, and measurement of subsequent emission spectra was used to optimise the brightness of lignin autofluorescence for imaging, and to investigate the potential to differentiate lignin subunits. This study provides a benchmark for determining the response of lignin fluorescence to a range of biological, chemical and physical treatments as a way of measuring the effects of such treatments.

MATERIALS AND METHODS

A summary of samples, treatments and conditions for fluorescence spectroscopy is shown in Table 1. Samples of Pinus radiata D.Don and Populus deltoides Marshall wood were stored in formalin aceto-alcohol (FAA) fixative prior to sectioning in the transverse plane at a thickness of 60 µm. After washing in distilled water, sections were infiltrated in 50% glycerol in 0.01 M phosphate buffer at pH 5, pH 7, or pH 9 for 15 mins prior to mounting in the same medium on a microscope slide. Wet sections were briefly blotted dry and infiltrated in 99% 2,2’-thiodiethanol (TDE) for 15 mins followed by mounting in the same medium on a microscope slide (Staudt et al. 2007). Other sections were washed in ethanol and air dried under vacuum overnight while clamped between two microscope slides to prevent curling. These sections were infiltrated in immersion oil (IO) under vacuum for 15 mins before mounting in the same medium.

Sections were examined using a Leica TCS SP5 II spectral confocal microscope using UV (355 nm), blue (488 nm) or green (561 nm) excitation. Emission spectra were acquired from 400–700 nm at intervals of 5 nm and using a bandwidth of 10 nm for UV excitation, and from 500–700 nm and 575–700 nm respectively for blue and green excitation. Average spectra were calculated from three replicates acquired from adjacent regions of the sample. The relative brightness of each preparation was measured at a standardised gain by measuring the average brightness of comparable images on

Table 1. Sample preparation and fluorescence spectroscopy methods. For each combination of mounting medium and species, fluorescence spectra were acquired using UV (355 nm), blue (488 nm) and green (561 nm) excitation.

<table>
<thead>
<tr>
<th>Mounting media</th>
<th>Excitation/Emission</th>
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<tbody>
<tr>
<td>Pine</td>
<td></td>
</tr>
<tr>
<td>50% Glycerol pH 9</td>
<td>355 nm Ex/400 – 700 Em</td>
</tr>
<tr>
<td>50% Glycerol pH 7</td>
<td>488 nm Ex/500 – 700 Em</td>
</tr>
<tr>
<td>50% Glycerol pH 5</td>
<td>561 nm Ex/575 – 700 Em</td>
</tr>
<tr>
<td>Immersion oil (IO)</td>
<td></td>
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<tr>
<td>Thiodiethanol (TDE)</td>
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<tr>
<td>Poplar</td>
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<tr>
<td>50% Glycerol pH 9</td>
<td>355 nm Ex/400 – 700 Em</td>
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<td>50% Glycerol pH 7</td>
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<td>Immersion oil (IO)</td>
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<td>Thiodiethanol (TDE)</td>
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five replicate fields of view per sample (Moëll & Donaldson 2001), and this was used to compare fluorescence spectra on an intensity basis. Spectra were plotted as either normalised or relative intensity using measurements in glycerol-buffer at pH 9 as the reference sample.

Spectral unmixing is a technique for decomposing an image containing multiple but overlapping fluorophores (Zimmermann 2005). This procedure can be based on known reference spectra or can be calculated by blind procedures such as non-negative matrix factorisation (Neher et al. 2009). In the resulting unmixed image, the location of each fluorophore is shown in a different colour.

Spectral unmixing was performed on a spectral image sequence from 500–700 nm with 488 nm excitation using the blind unmixing plugin Poisson NMF for ImageJ software (Neher et al. 2009). We compared the results of blind unmixing with linear unmixing using reference spectra from pine secondary wall (guaiacyl rich lignin) and poplar fibre wall (syringyl-rich lignin) using Leica LCS software. In order to confirm the histochemical localisation of guaiacyl and syringyl rich lignins, sections were treated with Maule reagent (Lin & Dence 1992). The differential localisation of lignin subunits was also examined using spectral unmixing in several additional hardwood species including sapwood of Populus heterophylla L.; Salix nigra Marshall, Salix chilensis Molina (Salicaceae), Eucalyptus nitens H. Deane & Maiden and Acacia melanoxylon R. Br. (Myrtaceae).

RESULTS

Optimisation of lignin fluorescence

With UV excitation, pine and poplar wood both have a similar fluorescence emission profile, with a $\lambda_{\text{max}}$ of 455 nm (Fig. 1 & 2), but with blue excitation, pine emits at slightly longer wavelengths ($\lambda_{\text{max}}$ 545 nm vs 530 nm) and is more obviously multimodal (Fig. 2) compared to poplar. With green excitation both pine and poplar have more or less identical emission profiles with a $\lambda_{\text{max}}$ of 580 nm (Fig. 1 & 2).

Both wood types show significant variation in fluorescence intensity with mounting medium (Fig. 3). With UV excitation, pine shows greater emission intensity when mounted in immersion oil or thiodiethanol compared to glycerol, with a small pH effect resulting in slightly greater emission at pH 9 compared to lower pH. Poplar shows much less variation among media with UV excitation.

With blue excitation there is a marked increase in emission with glycerol at pH 9 in both species compared to other media, with approximately double the intensity at pH 9 compared to lower pH (Fig. 3 & 4). A similar but smaller difference is seen with green excitation in both species. Poplar lignin fluorescence is about four times brighter than pine with 488 nm excitation in pH 9 medium (Fig. 5a).

Differentiation of lignin subunits

Figure 2 clearly shows that 488 nm excitation is the optimum wavelength for detecting differential fluorescence between pine and poplar. Comparing fluorescence emission in different morphological regions at 488 nm excitation shows that poplar fibre secondary wall has a lower $\lambda_{\text{max}}$ (530 nm) and hence a shorter wavelength emis-
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Figure 1. Fluorescence emission spectra for pine and poplar wood at 355, 488, and 561 nm excitation for 5 different mounting media showing intensity relative to glycerol/buffer at pH 9, which represents the preferred mounting medium.

The fluorescence emission profile of pine secondary wall, with a maximum at 545 nm (5a), is distinct from that of poplar secondary wall (Fig. 5b). Pine secondary wall and poplar middle lamella have similar fluorescence spectra while the poplar vessel wall has a slightly wider emission profile (Fig. 5b). Thus poplar fibre secondary wall has a different emission profile from other wall regions, corresponding to the known enrichment with syringyl lignin compared to guaiacyl lignin in this region.

In order to visualise this difference, spectral unmixing was applied to spectral image sequences of both pine and poplar. This technique is used to decompose the individual fluorophores present in the spectral image sequence and thus identify their location. We compared spectral unmixing using reference spectra from pine secondary wall (guaiacyl rich) and poplar fibre secondary wall (syringyl rich) on images of both species, with
Figure 2. Comparison of fluorescence emission spectra for pine and poplar wood mounted in glycerol/buffer at pH 9 for excitation at A: 355, B: 488, and C: 561 nm. Spectra are similar for the two species at 355 and 561 nm excitation but poplar shows a more distinctly green fluorescence than pine at 488 nm excitation.

Figure 3. Comparison of fluorescence intensity among mounting media and species with excitation wavelength relative to glycerol/buffer at pH 9. For UV excitation, thiodiethanol (TDE) and immersion oil (IO) treatments are somewhat brighter than glycerol/buffer and there is a reduced effect of pH. For visible excitation the glycerol/buffer at pH 9 treatment is generally much brighter than for all other media, especially in poplar. Error bars show the 95% confidence interval.
Figure 4. Comparison of lignin fluorescence intensity in pine and poplar with pH of mounting medium at 488 nm excitation at fixed gain within species. Brightness declines with reducing pH to a greater extent in poplar than in pine. Images within species are acquired at the same gain to show the effect of pH but poplar is significantly brighter than pine (Fig. 5A) and is hence shown at reduced gain to make the brightness comparable between species. — Scale bars = 60 µm.
blind unmixing as described by Neher et al. (2009), producing identical results. Since blind unmixing allows the application of this technique to other species without having to re-measure calibration spectra we only present these results in Figure 6.

In pine all of the cell wall shows the same emission profile and hence appears the same colour (magenta) indicative of guaiacyl-rich lignin (Fig. 6). In poplar, both middle lamella and vessel secondary wall regions have similar emission profiles to pine secondary wall and hence appear magenta reflecting the similarity in guaiacyl content to pine lignin (Fig. 6). The fibre wall has a different emission profile showing a gradient from grey in the outer wall, suggesting a mixture of guaiacyl and syringyl enriched lignins, with increasing green colouration towards the cell lumen, suggesting syringyl-rich lignin. These colours are entirely arbitrary but have been chosen to reflect the shorter wavelength emission of fibre secondary walls compared to other cell wall regions. The green/magenta combination also facilitates interpretation by readers.

Figure 5. – A: Comparison of fluorescence intensity for pine and poplar with 488 nm excitation at pH 9 showing the 4× greater brightness of poplar lignin on a relative intensity scale. – B: Comparison of pine secondary wall (S2), poplar vessel wall, poplar middle lamella (ML), and poplar fibre wall at normalised intensity. Poplar fibre wall has a significantly shorter wavelength emission.
with both normal and modified colour perception. Areas with both green and magenta will appear grey. Spectral unmixing appears to suggest a histochemical separation of guaiacyl and syringyl rich regions in poplar based on lignin autofluorescence.

The characteristic emission profile in poplar fibres has a strong dependence on pH. In radiata pine, images taken at different pH but with optimised gain to remove differences in brightness, have similar contrast, but in poplar, the image at pH 9 appears strikingly different from those at other pH values (Fig. 7).

In order to confirm this apparent differentiation of lignin type we examined several samples of other poplar and willow species, as well as two other unrelated hardwoods (eucalypt and acacia) known to have a similar guaiacyl/syringyl ratio to poplar. Spectral unmixing reveals a similar contrast between fibre secondary walls and other cell wall regions in *Salix* to that shown by poplar (Fig. 6). However, *Acacia* and *Eucalyptus* show similar fluorophores in all cell wall regions (Fig. 6). All of the poplar and willow samples examined show the same pH dependent fluorescence pattern in fibre walls shown in Figure 7. However, for the acacia and eucalypt wood samples no such pH dependent contrast change between cell wall regions was observed (Fig. 7), confirming that the characteristic fluorescence of poplar fibres is not due to a simple guaiacyl/syringyl difference.

**DISCUSSION**

**Optimisation of lignin fluorescence**

Lignin has a characteristic uni- or bimodal fluorescence with UV excitation but is multimodal with visible excitation, the secondary peaks occurring with increasingly longer emission steps reflecting variations in bond length and conjugation within the lignin molecule (Radotić *et al.* 2006; Donaldson *et al.* 2010). Pine and poplar have similar emission profiles with UV excitation but are somewhat different with blue excitation where poplar has a shorter $\lambda_{\text{max}}$ and less prominent secondary peaks at longer wavelengths (Fig. 2). The fluorescence emission in poplar is thus more distinctly green than the yellow emission of pine. Poplar fluorescence at blue excitation is also significantly brighter than that for pine. However, this difference is of limited use in histochemical differentiation because it can only be detected by careful measurement.

Lignin autofluorescence is relatively weak in terms of quantum yield which can make it difficult to acquire images depending on the sensitivity of the microscope hardware available. It is therefore of interest to find some way of increasing the fluorescence intensity of lignin, both for imaging and spectroscopy, in order to improve signal to noise ratio and to reduce the time required for data acquisition. Like many fluorophores, lignin fluorescence is sensitive to pH (Burlakov *et al.* 1979) with a significant increase in brightness at pH 9 compared to lower pH values (Fig. 2). While emission resulting from visible excitation is optimised by high pH, emission resulting from UV excitation appears to be much less sensitive to pH. Mounting media such as immersion oil and thiodiethanol have a higher refractive index than glycerol/buffer mixtures and hence can act as clearing agents allowing deeper imaging with confocal microscopy. Thiodiethanol is water miscible and can easily infiltrate wood sections whereas immersion oil is not water miscible and is more difficult to infiltrate, with air bubbles especially...
Figure 6. Fluorescence images at 488 nm excitation, 500–700 nm emission and pH 9. – A & B: Monochrome fluorescence images of pine and poplar, respectively. – C & D: Corresponding images generated from a spectral image sequence after blind linear unmixing using the Poisson NMF algorithm. These images show regions estimated to have different fluorescence emission spectra in different colours. Magenta regions may represent guaiacyl-rich lignin while green may represent syringyl p-hydroxybenzoate-rich lignins. Poplar fibre walls thus show evidence of a different fluorescence spectrum from vessel secondary walls and middle lamella, while
within ray cells, causing problems. The need to dry the sections is also problematic as drying may cause cell walls in sections to delaminate. Thiodiethanol produces brighter emission with UV excitation, especially in pine but its use is somewhat limited with green excitation because of a weak fluorescence background produced by this mounting medium (Fig. 1). None of the other mounting media show any evidence of fluorescence at any excitation wavelength.

Under some conditions the use of glycerol/buffer based mounting media must be avoided. For example when imaging embedded wood tissue the cell walls may undergo severe distortion due to glycerol induced swelling that is resisted by the embedding plastic. In this situation immersion oil must be used as the mounting medium accepting the associated reduction in intensity for visible excitation (Donaldson & Knox 2012).

Fluorescence microscopy as a method for visualising lignin distribution has a number of advantages over some other methods. Fluorescence microscopy requires almost no sample preparation apart from sectioning, compared to UV absorbance microscopy which requires time consuming embedding and sectioning with a diamond knife (Koch & Kleist 2001). This allows fluorescence to be used, for example, to rapidly screen samples for compression wood severity (Donaldson et al. 2004). However, if quantitative measurements are required then UV microscopy has the advantage – fluorescence intensity is not directly proportional to the amount of lignin but is also affected by molecular environment and chemical structure.

**Differentiation of lignin subunits**

A comparison of the spectral emission profiles of pine and poplar reveals a significant difference in fluorescence emission for the fibre secondary wall at 488 nm excitation and this can be highlighted visually using spectral unmixing. This characteristic fluorescence emission of poplar fibres seems to correspond to the known location of syringyl-rich lignin in the fibre secondary wall (Musha & Goring 1975). However, when other unrelated hardwood species are examined no such difference in emission is observed making it unlikely that the characteristic fluorescence of the fibre secondary wall in poplar is directly related to the presence of syringyl subunits.

Members of the Salicaceae family have been reported to contain an unusual lignin type based on syringyl \(\gamma\)-p-hydroxybenzoate (Lu et al. 2004; Morreel et al. 2004). Comparison of fluorescence from other poplar and willow species confirms that fibre walls in all these samples from the same family have the same or similar characteristic fluorescence, which is absent from acacia and eucalypt wood. This suggests that the pine shows uniform spectral emission among wall layers. Gelatinous fibres (GF) in poplar show weak fluorescence of mixed green/magenta colour (grey) in the \(S_2\) layer and green at the inner margin of the g-layer. – E: Spectral image of *Salix chilensis* showing similar differentiation of vessels and middle lamella from fibre walls to that found in poplar. – F: Spectral image of *Acacia melanoxylon* showing a uniform spectral distribution among cell walls. – G: Spectral image of *Eucalyptus nitens* showing a uniform spectral distribution among cell walls. — Scale bars = 60 \(\mu\)m.
Figure 7. Comparison of fluorescence among species over a range of pH at optimised gain to remove the effect of pH on brightness. Excitation is 488 nm and emission is 500–550 nm. Pine shows uniform contrast at different pH while poplar shows a very different contrast at pH 9 with bright fibre walls, and dark middle lamellae and vessel walls, compared to lower pH, where the contrast is reversed. *Salix* shows a similar change in contrast with pH to poplar, while *Acacia* and *Eucalyptus* show no change in contrast with pH. — Scale bars = 60 µm.
observed characteristic emission in the fibre secondary wall of poplar may be due to
the presence of syringyl  \( \gamma \)-p-hydroxybenzoates. This characteristic fluorescence is
very dependent on pH. Figure 7 reveals that the bright fibre walls at 488 nm excitation
are only observed at pH 9 and this contrast is entirely absent at pH 7 and pH 5.
\( \alpha \)-Hydroxybenzoic acid is reported to be non-fluorescent while the \( \beta \)- and m-isomers
show differential fluorescence dependant on pH (Thommes & Leininger 1958). Presumably \( \alpha \)-hydroxybenzoic acid becomes fluorescent when incorporated in syringyl lignin or it may alter the fluorescence of syringyl lignin itself.

Musha and Goring (1975) examined a range of softwood and hardwood species by
UV absorbance microscopy including eastern cottonwood (\textit{P. deltoides}). By studying
the UV absorbance of \( \alpha \)-hydroxy model compounds compared to pure guaiacyl lignin
and pure syringyl lignin they were able to differentiate \( \alpha \)-hydroxybenzoic acid from
syringyl and guaiacyl lignin and to determine that such groups are associated with syrin-
gyl lignin in the fibre wall rather than with guaiacyl lignin in the vessel wall. This
approach is quite similar to the spectral unmixing approach used in the present study.

It seems to be a general conclusion that \( \alpha \)-hydroxy components of lignin show
differences in both absorbance and fluorescence. Donaldson \textit{et al.} (2010) found char-
acteristic differences in UV fluorescence of compression wood lignin which is rich in
\( \alpha \)-hydroxyphenyl groups. In the case of compression wood, \( \alpha \)-hydroxyphenyl groups
are associated with guaiacyl lignin and show an increase in longer wavelength fluo-
rescence, while in poplar, \( \alpha \)-hydroxybenzoate is associated with syringyl lignin and
shows increased shorter wavelength fluorescence.

In grasses, phenolic acids bound to cell walls may show pH dependant fluorescence
(Harris & Hartley 1976, 1980). However, such compounds have not been demonstrated
microscopically in either pine or poplar wood. Extractives may also infiltrate cell
walls in heartwood and are often highly fluorescent at longer yellow or red emission
wavelengths compared to lignin. Extractives are therefore unlikely to account for the
shorter wavelength fluorescence of poplar fibres, especially in sapwood.

Other studies have also demonstrated the distribution of specific lignin groups in
hardwoods. Studies using histochemistry and UV absorbance microscopy have shown
that vessel cell walls and middle lamellae are rich in guaiacyl lignins while fibres and
parenchyma cell walls contain lignin enriched in syringyl units (Fergus & Goring 1970;
composition and topochemistry of hardwood lignin varies significantly among species
(Fujii \textit{et al.} 1987). In a survey of 25 hardwood species from China, Wu \textit{et al.} (1992)
found that lignin composition is related to cell type, location within a growth ring, vessel
arrangement and tree habitat. This study found three topochemical patterns related to
wood type. In vessel-less hardwoods and some tropical diffuse-porous woods, all cell
walls contained predominantly guaiacyl lignin, while in most diffuse porous woods,
vessel cell walls were enriched in guaiacyl lignin, and fibres were enriched in syringyl
lignin. In ring-porous and radial-porous woods the distribution of guaiacyl and syringyl
lignins was variable. Vessel secondary walls are typically more lignified than fibre or
parenchyma cell walls allowing them to resist the large compressive forces associated
Using a polyclonal antibody to syringyl units, Joseleau et al. (2004) showed that syringyl units increase towards the inner $S_2$ region but are greatly reduced in the $S_2$ region of gelatinous fibres in poplar. This observation of gradients in syringyl units is similar to that observed by lignin fluorescence in the current study as shown in Figure 6. This gradient can only be seen at pH 9 whereas images apparently showing lignin concentration without this gradient can be seen at lower pH. This suggests that the gradient at high pH reflects chemical composition such as the number of syringyl $p$-hydroxybenzoate units, while at lower pH only the total amount of lignin is shown. Therefore it is possible to produce images of lignin composition or lignin concentration using fluorescence by raising or lowering the pH of the mounting medium, at least in poplar.

CONCLUSIONS

Softwood and hardwood lignins show similar fluorescence behaviour in a range of mounting media with quantum yield optimised in glycerol/buffer at pH 9 for visible excitation suggesting that this medium is the best general purpose mountant for wood fluorescence. Fluorescence resulting from UV excitation is less sensitive to pH but may be increased in organic mounting media, especially for softwood lignin. Fluorescence spectroscopy and spectral unmixing demonstrate a characteristic difference in fluorescence resulting from visible excitation in poplar fibre secondary cell walls compared to other wall regions and to pine, which is strongly pH dependant. It seems likely that this novel fluorescence is due to the presence of $p$-hydroxybenzoate groups attached to syringyl lignin characteristic of poplar and willow, but absent in other hardwoods. The ability of lignin fluorescence to change in response to its molecular environment suggests applications in assessment of biological, chemical and physical modification of cell walls.

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