Original article

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ToF-SIMS imaging reveals that *p*-hydroxybenzoate groups specifically decorate the lignin of fibres in the xylem of poplar and willow

<https://doi.org/10.1515/hf-2020-0130>

Received May 29, 2020; accepted August 11, 2020; published online September 18, 2020

Abstract: Esterified phenolics occur as γ -linked lignin acylations in many plant taxa but little is known about the distribution or function of such groups. In the family Salicaceae, *p*-hydroxybenzoate groups are present in the lignin of poplars (*Populus* spp.) and willows (*Salix* spp.). In this study, the distribution of cell wall-bound *p*-hydroxybenzoate was examined in different tissue and cell types of poplar and willow trees. This analysis showed that *p*-hydroxybenzoate groups were most prevalent in juvenile stems, and were elevated in tension wood and following nitrogen fertilisation. Closer examination of stem cross-sections using time-of-flight secondary-ion mass spectrometry (ToF-SIMS) directly showed that *p*-hydroxybenzoate groups occur predominantly in the cell walls of fibres and are largely absent from vessel elements. These results point to a role for ester-linked groups in modifying the syringyl-rich lignin of fibres, perhaps to strengthen the cell walls of these structural elements or to increase lignification rates.

Keywords: cell wall-bound phenolics; ester-linked pendant groups; lignin acylations; mass spectrometry imaging; *p*-hydroxybenzoic acid.

1 Introduction

Lignin is a primary component of the secondary cell walls of vascular plants. It contributes greatly to the mechanical strength of stems and branches, and it plays important roles in water conduction and plant defence (Novo-Uzal et al. 2012). Although lignin is indispensable for plant vitality, it poses a major challenge for the industrial processing of plant biomass. As such, lignin chemistry and biosynthesis have received considerable research attention, and great strides have been made in modifying lignin structures through plant biotechnology (Mottiar et al. 2016).

Lignin is a complex phenolic polymer that is largely resistant to chemical degradation (Figure 1a). While the recalcitrance of lignin presents a challenge for efficient biomass deconstruction, it also provides opportunities for the development of biochemicals and biomaterials (Glasser 2019). Traditionally, lignin was seen as a low-value byproduct of industrial processing and was typically burned for energy. Today, new processes are being developed to preserve phenolic structures during lignocellulose deconstruction in a strategy termed ‘lignin-first bio-refining’ (Schutyser et al. 2018). Various biomaterials are envisioned from recovered polymers and oligomers, and lignin-derived phenolics could be fed to microbes for fermentation into diverse secondary products just as cellulose and hemicellulose can be converted into biofuels (Beckham et al. 2016; Kai et al. 2016).

Lignin is assembled primarily from three cinnamic acid derivatives known as monolignols: *p*-coumaryl, coniferyl and sinapyl alcohol (Boerjan et al. 2003). Once incorporated into a lignin polymer, these precursors give

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Figure 1: (a) Model of lignin showing ester-linked *p*-hydroxybenzoate groups ($-pHB$) in orange. (b) Acylated monolignol conjugates of coniferyl-*p*-hydroxybenzoate and sinapyl-*p*-hydroxybenzoate. (c) Fragmentation ions of *p*-hydroxybenzoyl ($-pHB$) and *p*-hydroxyphenyl (H unit) moieties in lignin (following Saito et al. 2006).

rise to *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin units. The monolignols are produced in the cytosol and then exported from the cell to the apoplast where peroxidase and laccase enzymes generate phenoxyl radicals that undergo coupling and cross-coupling reactions (Ralph et al. 2004). As the resulting polymers arise through combinatorial assembly, perturbations in the supply of monomers lead to differences in lignin composition and structure.

The plasticity of lignification extends beyond the three primary monolignols, and various non-canonical lignin monomers are found in diverse plant taxa. For example, hydroxycinnamaldehydes and hydroxybenzaldehydes hyper-accumulate in the lignin of pine and tobacco plants deficient in the biosynthetic enzyme cinnamyl alcohol dehydrogenase (Kim et al. 2003). A linear lignin polymer built entirely from caffeyl alcohol has been identified in cactus seed coats and vanilla bean pods (Chen et al. 2012). And, lignin monomers can even be recruited from other biosynthetic pathways, such as the stilbenoids resveratrol and piceatannol, which have been found in the lignin of palm fruits (Del Río et al. 2017).

Some non-canonical lignins naturally arise from the incorporation of acylated (ester-linked) monomers. In commelinid grasses, for example, *p*-coumarate groups are linked to the lignin backbone via the γ position (Karlen et al. 2018; Ralph et al. 1994; Shimada et al. 1971). It has been shown that members of the BAHD family of acyltransferases are responsible for this biochemistry by producing ester-linked conjugates of coniferyl-*p*-coumarate and sinapyl-*p*-coumarate which can, via the monolignol moieties, participate in the radical-coupling reactions that assemble lignin polymers (Hatfield et al. 2009; Withers et al. 2012).

Analogously to the better-studied *p*-coumarate, ester-linked *p*-hydroxybenzoate groups (henceforth denoted as $-pHB$ to emphasise the pendant nature) occur in poplars, willows, palms, and at particularly high levels in the seagrass *Posidonia oceanica* (Lu et al. 2015; Pearl et al. 1957; Rencoret et al. 2020; Smith 1955). These $-pHB$ groups are also linked via the γ position and arise through the incorporation of acylated monolignol conjugates (Figure 1b; Lu et al. 2004; Lu et al. 2015; Morreel et al. 2004; Nakamura and Higuchi 1978).

The *p*-hydroxybenzoyl moieties of these conjugates prefer radical transfer over radical coupling, and therefore remain as pendant groups and are not integrated into lignin polymer backbones (Ralph 2006). As they are ester-linked, mild alkaline hydrolysis (saponification) releases the phenolic pendant groups as a ‘clip-off’ which can be separated for use in fermentation or directly as a platform chemical (Rinaldi et al. 2016). Furthermore, the presence of ester-linked pendant phenolics may improve deconstruction and saccharification rates (Grabber et al. 2004; Sibout et al. 2016). Accordingly, cell wall-bound phenolics have become an important target in lignin engineering although little is known about their biological function or distribution.

Previous work with UV microscopy indicated that $-pHB$ groups may occur primarily in the xylem fibres of poplar wood (Musha and Goring 1975). Analysis of plant tissues by time-of-flight secondary-ion mass spectrometry (ToF-SIMS) offers many benefits over classical imaging techniques including high surface sensitivity (top 1–2 nm), low detection limits (ppb–ppm), high spatial resolution (~ 50 – 300 nm), and rich spectral data with high mass resolution (Goacher et al. 2014). No tagging or labelling is required for ToF-SIMS imaging, and microtomed plant sections can be analysed directly without applied matrices. Moreover, characteristic ToF-SIMS secondary ions unique to lignin and polysaccharides have been reported previously (Goacher et al. 2011; Goacher et al. 2012; Goacher et al. 2013).

In this study, the occurrence of $-p\text{HB}$ groups was surveyed in various tissues of poplar and willow by chemical analysis, and ToF-SIMS was then used to examine the distribution of $-p\text{HB}$ in wood. To our knowledge, this report represents the first use of ToF-SIMS to examine the distribution of non-canonical lignin monomers.

2 Materials and methods

2.1 Plant samples

Samples for chemical analysis were harvested from trees grown in a glasshouse. Dormant cuttings of *Populus nigra* cv. 'Italica' (Lombardy poplar), *Populus trichocarpa* cv. 'Nisqually-1' (black cottonwood) and *Salix eriocephala* DUK-E5 (heart-leaf willow) were harvested from mature trees, dipped in rooting powder (0.8% indole-3-butyric acid), planted in two-gallon pots with peat-based potting soil and irrigated as needed with water containing Hoagland's solution (Hoagland and Arnon 1950). After six months of growth, the main stems were divided into 50 cm sections (i.e., young, juvenile, and mature) while the roots were separated into young and mature roots. Young and mature leaves were also collected, as were branches and bark which was peeled from the main stems. Stem samples were also harvested from glasshouse-grown *Populus tremula* $\times$ *alba* INRA 717-IB4 (hybrid poplar) fed with low nitrogen (0.1 mM ammonium nitrate) and high nitrogen (10 mM ammonium nitrate) fertilisers. Samples of tension wood, opposite wood, and normal wood from the same genotype were described previously (Gerttula et al. 2015). All samples were comminuted using a Wiley mill to pass a 40-mesh screen and then Soxhlet-extracted for 24 h with hot acetone prior to analysis. Three biological replicates were analysed for each genotype.

Wood samples for ToF-SIMS were harvested from the same Lombardy poplar and heart-leaf willow trees described above. Transversal xylem cross-sections were cut from the main stems to a thickness of roughly 100 μm using a sliding-block microtome and then allowed to air dry. Prior to ToF-SIMS analysis, several cross-sections were solvent-extracted to remove small-molecule extractives (Goacher et al. 2013). Sections were placed in cellulose thimbles and Soxhlet-extracted for 8 h with 95% ethanol, 8 h with 70% ethanol/30% toluene, and 4 h with distilled water, following ASTM standard D1105–96 (2007) but with a doubled ethanol extraction time. Subsequently, some of the untreated and extracted microtomed sections underwent mild alkaline hydrolysis to cleave ester-linked $-p\text{HB}$ by soaking in 2 M sodium hydroxide for 24 h at 30 °C with shaking at 60 rpm (Goldstein 1984). After extraction and/or alkaline hydrolysis, the cross-sections were rinsed with 20 mL aliquots: once with distilled water, four times with 1 M acetic acid, and finally 10 times with distilled water (Braham and Goacher 2015). Samples of wood powder from *Betula alleghaniensis* (yellow birch), an angiosperm genus which is devoid of $-p\text{HB}$ groups (Bardet et al. 1985a), were used as a negative control for peak identification purposes.

2.2 Chemical analysis of $-p\text{HB}$ groups and lignin

The amount of ester-linked cell wall-bound $-p\text{HB}$ groups was determined by mild alkaline hydrolysis of extractive-free powdered

samples. Duplicate 20 mg samples of oven-dried powder were weighed into 2 mL screw-cap vials and 1 mL of 2 M sodium hydroxide was added along with 100 μL of 1 mg mL^{-1} *o*-anisic acid as an internal standard. Tubes were incubated at 30 °C for 24 h. The saponification reactions were terminated by the addition of 100 μL of 72% sulphuric acid. After incubation on ice for 5 min, the supernatants were collected by centrifugation and filtered through 0.45 μm nylon syringe filters prior to analysis using reversed phase HPLC.

Using a Dionex Summit HPLC apparatus equipped with a diode array detector (Thermo Scientific, Waltham, Massachusetts, USA), 20 μL samples were injected onto a Symmetry C-18 column (4.6 $\times$ 250 mm, 5 μm particle size, Waters Inc., Milford, Massachusetts, USA) maintained at 35 °C. Good peak resolution was achieved using non-isocratic separation with a gradient over 30 min from 5 to 45% of eluent A (0.1% trifluoroacetic acid in 70:30 acetonitrile:methanol) in eluent B (0.1% trifluoroacetic acid in water) at a flow rate of 0.7 mL min^{-1} . Integration of *p*-hydroxybenzoic acid and *o*-anisic acid peaks was performed at the UV maxima of 255 and 296 nm respectively, and a seven-point calibration curve from 1 to 100 ng mL^{-1} prepared with analytical-grade standards (at least 99% pure, Sigma-Aldrich Corp., St. Louis, Missouri, USA) was used for quantification.

The amount and composition of lignin was determined using the Klason lignin method and thioacidolysis procedure followed by derivatisation and gas chromatography as described previously (Mottiar et al. 2020). Heat maps of $-p\text{HB}$ content (normalised per g lignin) were generated using Adobe Illustrator (Adobe Systems Inc., Mountain View, California, USA).

2.3 ToF-SIMS

ToF-SIMS data was acquired using two ToF-SIMS 5 instruments equipped with reflectron-type mass analysers (ION-TOF GmbH, Münster, Germany). Analysis was performed using Bi_3^{++} primary ions at a 45° incident angle with 20 eV electron flooding for charge compensation. The untreated, extracted, and alkaline-treated sections were analysed using high-current conditions for fast spectral acquisition (50 keV Bi_3^{++} , 0.7 pA pulsed current, 65 μs cycle time, 256 $\times$ 256 pixels rastered randomly over either 100 $\times$ 100 or 500 $\times$ 500 μm^2 areas). High resolution images were obtained of the extracted sections using burst alignment settings with 0.11 pA current, 50 keV Bi_3^{++} at a 65 μs cycle time, and 256 $\times$ 256 pixels rastered randomly over a 200 $\times$ 200 μm^2 area; or with 0.06 pA current, 60 keV Bi_3^{++} at a 100 μs cycle time, and 512 $\times$ 512 pixels rastered randomly over a 200 $\times$ 200 μm^2 area. The pressure during analysis was less than 3×10^{-7} mbar and delayed extraction was used to minimise the effects of surface topography.

SurfaceLab 6.3 and 6.6 (IONTOF GmbH, Münster, Germany) software versions were used to calibrate the ToF-SIMS mass spectra to C_3H_5^+ (m/z 41.03), C_4H_7^+ (m/z 55.05), C_7H_7^+ (m/z 91.05) and $\text{C}_5\text{H}_5\text{O}_2^+$ (m/z 97.03), and to generate images of characteristic ions. Mass interval lists were prepared with high mass resolution to export data to Matlab R2016b (MathWorks, Natick, MA, USA) for multivariate statistical analysis using the PLS Toolbox 7.0 and MIA Toolbox 3.0 (Eigenvector Research Inc., Manson, WA, USA). Pre-processing of spectra for principal component analysis (PCA) included Poisson (square root mean) scaling, normalisation, and mean centring. Curve-smoothing was performed for the spectra presented in the m/z 121 peak

envelope using Microsoft Excel (Microsoft Corp., Redmond, Washington, USA) with a damping factor of 0.9. Images were pre-processed using Poisson scaling and mean centring for PCA, while only Poisson scaling was applied for image analysis via multivariate curve resolution (MCR). Figures were assembled and scale bars were added using Adobe Photoshop and Adobe Illustrator (Adobe Systems Inc.).

3 Results and discussion

3.1 Occurrence of *-p*HB groups in poplar and willow

To begin, the distribution of *-p*HB was surveyed throughout the vegetative tissues of poplar and willow. As *-p*HB moieties are ester-linked to the lignin backbone, mild alkaline hydrolysis was used to liberate these groups prior to analysis by HPLC. This survey revealed substantial variability between tissues, but also among the different taxa more broadly (Figure 2a–c). In agreement with previous reports, Lombardy poplar had the highest *-p*HB levels, while heart-leaf willow had the lowest (Pearl et al. 1957; Venverloo 1971).

In all three genotypes examined, the stems contained the greatest amounts of *-p*HB with levels ranging from 5 to 29 mg *-p*HB/g lignin, or in other words, 0.5–2.9% of the lignin (Figure 2a–c). By examining a developmental gradient, it became clear that the juvenile stems had the most *-p*HB (i.e. not the youngest stems, nor the oldest). This may help explain the discord in the literature where some reported higher levels in younger stems (Chen et al. 2017), while others found more in mature stems (Ververloo 1969; Wang et al. 2020) and at least one group reported no differences whatsoever (Nakano et al. 1961). In the present study, more *-p*HB was observed in mature roots compared to young roots, particularly in Lombardy poplar where *-p*HB represented 2.2% of the total lignin in the mature roots. Bark contained much less *-p*HB (at most 0.2% of the lignin), with the leaves having only trace levels. As *-p*HB was most prevalent in the stems, the effect of perturbations in stem development were considered next.

Nitrogen fertilisation of trees has been shown to lead to changes in development and resource allocation, generally resulting in fast-growing stems with wider vessels and reduced total lignin content (Li et al. 2012). In the hybrid poplar samples analysed in the present study, *-p*HB groups were more abundant in the xylem of trees fertilised with more nitrogen, in agreement with previous observations (Figure 2d; Pitre et al. 2007). As nitrogen fertilisation is known to reduce lignin content in glasshouse-grown

poplar (Novaes et al. 2009), it should be emphasised that this represents a decrease in *-p*HB per g of lignin, not merely per g of biomass.

Reaction wood, formed as a gravitropic response in leaning stems, is another example of wood with “natural” perturbations in xylem morphology and cell wall composition. In poplar, tension wood has fibres with a thick G-layer comprising more cellulose and less lignin, as well as vessels that are narrower and fewer in number (Jourez et al. 2001). In the hybrid poplar samples analysed in the present study, tension wood had 2.3 times more *-p*HB than opposite wood, and 3.5 times more than normal wood (Figure 2d). This observation corroborates previous NMR studies on the lignin of reaction wood (Foston et al. 2011; Hedenstrom et al. 2009).

As lignin levels are lower following nitrogen fertilisation and in tension wood, it is conceivable that the metabolic shift away from lignin leads to greater production of *p*-hydroxybenzoate, the key precursor of *-p*HB groups. Although the biosynthesis of *p*-hydroxybenzoate has not yet been fully elucidated in plants, radiotracer studies have shown a link to lignin biosynthesis (Terashima et al. 1975).

Figure 2: Levels of cell wall-bound *p*-hydroxybenzoate groups (mg *-p*HB/g lignin) in vegetative tissues of (a) Lombardy poplar, (b) black cottonwood, and (c) heart-leaf willow, as well as in (d) stems of hybrid poplar grown with low and high levels of nitrogen fertilisation, and in tension wood, opposite wood and normal wood. Tissue types depicted in a–c include stems (in 50 cm increments), branches, roots (young and mature), leaves (young and mature), and bark. Gradient scales show the lignin-normalised amount of *-p*HB with yellow representing more *-p*HB and red representing less. All measurements were performed in duplicate with extractive-free whole cell wall material using three biological replicates.

Alternatively, variations in $-pHB$ could be due to the presence/absence of $-pHB$ in different cell types, as both reaction wood and nitrogen fertilisation result in altered proportions of normal fibre and vessel cell walls in wood.

3.2 ToF-SIMS detection of $-pHB$ groups

In order to better understand the occurrence of $-pHB$ in wood, ToF-SIMS imaging was employed to examine the distribution of these groups in the juvenile stems of poplar and willow. Principal component analysis (PCA) was initially used to identify characteristic secondary ions (Supplementary Material; Supplementary Figure S1). This work uncovered a putative signature for $-pHB$ at m/z 121.03. It was also observed that solvent extraction was important in the sample preparation, as has been reported previously (Goacher et al. 2011; Goacher et al. 2013).

Examination of high mass resolution scans showed several peaks in the vicinity of m/z 121, namely m/z 121.03, 121.07 and 121.10. As a quality control measure to test whether these were spurious peaks caused by sample roughness or topography, the ToF-SIMS extractor bias was altered from 0 to +50 V to reject any stray ions originating from cell sidewalls. None of the peaks at m/z 121 were influenced by the extractor voltage and were therefore all deemed representative of real chemistry and not instrumental artefacts.

Several sample treatments were devised to examine the nature of these three ions. First, solvent extraction resulted in decreased intensity of m/z 121.10 in Lombardy poplar (Figure 3a), consistent with the PCA analysis. The reduction of m/z 121.10 was also observed with solvent extraction of heart-leaf willow samples (data not shown). Therefore, the peak at m/z 121.10 likely corresponds to a small-molecule aliphatic extractive, and its mass is consistent with a peak assignment of $C_9H_{13}^+$.

The aromatic ring structure of $-pHB$ is identical to H-lignin units (Figure 1c). As such, it was important to also consider peaks arising from H-lignin in this study. Previously reported ToF-SIMS analysis of a synthetic 8-O-4' H-type polymer resulted in the assignment of three characteristic ions for H-lignin: m/z 107.05 for $C_7H_7O^+$, m/z 121.03 for $C_7H_5O_2^+$, and m/z 121.07 for $C_8H_9O^+$ (Saito et al. 2006). Of these, $-pHB$ would most likely fragment as m/z 121.03 due to cleavage of the C–O bond (Figure 1c). The ions at m/z 107.05 and 121.07 are not expected for $-pHB$ as they would require a rearrangement of $-pHB$ and its associated lignin monomer unit to form.

As $-pHB$ groups are ester-linked (but H-lignin units are not), mild alkaline hydrolysis should lead to a reduction in

Figure 3: ToF-SIMS positive-ion mass spectra of the m/z 121 peak envelope for (a) various treatments of Lombardy poplar wood, and for (b) solvent-extracted samples of three different species where all spectra were normalised to total ion intensity.

any $-pHB$ -related peaks. Indeed, treatment with sodium hydroxide was observed to reduce the intensity of the peak at m/z 121.03 for samples of Lombardy poplar (Figure 3a), confirming this ion as characteristic of $-pHB$. The residual signal observed at m/z 121.03 after treatment with sodium hydroxide could be due to remaining, uncleaved $-pHB$ groups or could arise from H-lignin. Notably, the intensity of the peak at 121.07 was not altered after solvent extraction or sodium hydroxide treatment. Such insensitivity to solvents and alkaline hydrolysis would be expected for peaks corresponding to H-lignin units.

Chemical analysis showed that juvenile stems of Lombardy poplar had seven times as much $-pHB$ as heart-leaf willow but a similar amount of H-lignin (Supplementary Table S1), thus a comparison of the peak envelopes for these samples could also be informative. The intensity of the peak at m/z 121.03 was only about four times greater in Lombardy poplar than in heart-leaf willow (Figure 3b). And, while m/z 121.03 had

greater relative intensity than m/z 121.07 in both poplar and willow, the opposite was true for yellow birch, which lacks $-p\text{HB}$ but does have H-lignin units. Moreover, the contribution of H-lignin to m/z 121.03 can be estimated since yellow birch lacks $-p\text{HB}$ but does have H-lignin units. Following this approach, the $-p\text{HB}$ component of the m/z 121.03 peak was estimated to be 7.5 times greater in poplar than willow, closer in line with the chemical analysis. These observations provide further support for the assignment of m/z 121.03 as originating from $-p\text{HB}$ (with some contribution from H-lignin), m/z 121.07 as originating entirely from H-lignin units, and m/z 121.10 as corresponding to an aliphatic extractive compound.

3.3 Distribution of $-p\text{HB}$ groups in wood

Chemical images of the m/z 121.03 peak originating from $-p\text{HB}$ were prepared using false-colour overlays where the $-p\text{HB}$ signal intensity is plotted in magenta and the intensity of two aromatic ions at m/z 77.04 (phenyl cation) and m/z 91.05 (tropylium cation) representing the overall signal from lignin are summed and plotted in green (Figure 4). The scales represent relative, not absolute, concentrations. The ToF-SIMS chemical images for Lombardy poplar indicate that $-p\text{HB}$ is enriched in the cell walls of fibres and is largely absent in areas surrounding vessel elements, as well as in some regions adjacent to ray parenchyma (Figure 4a). Although this contrast is more evident in poplar where there is a higher concentration of $-p\text{HB}$, the same pattern can also be observed in willow (Figure 4b). Additional false-colour ion images are provided (Supplementary Figure S2) to support the interpretation that $-p\text{HB}$ is largely absent from vessels. However, it should be emphasised that some portion of the m/z 121.03 intensity in these ion images may originate from H-lignin depending on its relative abundance.

Accordingly, the ToF-SIMS images were further examined using MCR analysis. This chemometric technique deconstructs complex multivariate datasets into model components to help elucidate the contributions of minor chemical constituents (Graham and Castner 2012). MCR analysis for the ion images of Lombardy poplar (Figure 5a) and heart-leaf willow (Figure 5b) uncovered two model components of interest. The first of these, plotted in blue in these false-colour images, corresponds to several lignin-related peaks including G-lignin peaks at m/z 137.05 and 151.03 (Figure 5c, d). Not surprisingly, this model

Figure 4: ToF-SIMS false-colour ion images with $-p\text{HB}$ shown in magenta (m/z 121.03) and lignin shown in green (m/z 77.04 and 91.05) for extractive-free transversal xylem cross-sections of juvenile (a) Lombardy poplar (b) and heart-leaf willow stems. Gradient scales show the ion intensities for relative quantification. Scale bars represent 20 μm .

component was evenly dispersed throughout the cell walls of both fibres and vessels in the xylem of poplar and willow.

The second component of interest in the MCR modelling, plotted in yellow, was comprised primarily of the peak at m/z 121.03 related to $-p\text{HB}$ as well as m/z 167.03 and 181.05 peaks which originate from S-lignin (Figure 5e, f). This component was found exclusively in the cell walls of fibres. The deficiency of S-lignin signatures in vessels is well known (Gorzás et al. 2011; Musha and Goring 1975; Zhou et al. 2011), and this observation helps validate the methodology. While the distribution of m/z 121.03 in the ion images could not be attributed entirely to $-p\text{HB}$ as discussed earlier, the absence here of other peaks related to H-lignin (i.e., m/z 107.05 and 121.07) indicates that H-lignin is not a contributor to this model component. In other words, the response at m/z 121.03 in this model component likely originates entirely from $-p\text{HB}$. Thus, MCR analysis of the ToF-SIMS spectral images provides confirmation that –

Figure 5: Multivariate curve resolution modelling for extractive-free transversal xylem cross-sections of juvenile (a, c, e) Lombardy poplar and (b, d, f) heart-leaf willow stems identified model components (c, d) comprising peaks related mostly to lignin (shown in blue) dispersed throughout the xylem, whereas separate components (e, f) comprising peaks related to $-pHB$ and S -lignin (shown in yellow) were confined to the cell walls of fibres. Gradient scales show the scores intensity of each model component for relative quantification. Scale bars represent 20 μm.

pHB occurs primarily, if not exclusively, in the cell walls of fibres in poplar and willow.

Musha and Goring (1975) used UV microscopy to examine the distribution of $-pHB$ groups in poplar wood. Since lignin monomers have UV maxima around 280 nm while p -hydroxybenzoate absorbs strongly near 255 nm, they interpreted differences in the UV spectra of vessels and fibres as evidence that $-pHB$ was present only in fibres. Interestingly, another study employed the same methodology to describe a decrease in the amount of $-pHB$ in poplar xylem following wounding (Frankenstein et al. 2006). Using a spectral unmixing approach, Donaldson (2013) identified a signature in the autofluorescence of poplar and willow fibres consistent with $-pHB$. Building on these studies, the results presented herein provide the first direct evidence using mass spectrometry that $-pHB$ groups occur predominantly in the cell walls of fibres.

This observation may help explain the abundance of $-pHB$ in different plant tissues. Compared to mature wood, juvenile stems have more fibres and wider vessels (Zobel and Sprague 1998). On the other hand, very young stems have more vessels as well as fibres with thinner cell walls. In this way, juvenile stems have the highest overall proportion of fibre cell walls per gram, thus accounting for the higher concentrations of $-pHB$. While the elevated levels in tension wood and following nitrogen fertilisation may also relate to changes in the amount of normal fibre cell walls, reduced lignin levels offer another explanation. It could be that more p -hydroxybenzoate precursors become available, or perhaps there is greater expression of the BAHD acyltransferase responsible for generating acylated monolignol conjugates. One clue is provided by radiotracer experiments which showed that exogenous p -hydroxybenzoate incorporates into lignin

more readily in earlier stages of cell wall development (Terashima et al. 1979). Alternatively, perhaps *p*-hydroxybenzoate is deployed to the wall to use up any remaining phenolics prior to programmed cell death or as a way of promoting plant defence. In any case, it remains unclear whether acylations are used by plants to intentionally alter lignin structure, or merely as a consequence of differences in phenylpropanoid metabolism between cell types.

3.4 The importance of *-pHB* as lignin decorations

Although lignin acylations have been the focus of considerable efforts in plant biotechnology (Petrik et al. 2014; Sibout et al. 2016; Smith et al. 2015), little is known about any potential biological role for these pendant phenolics. As *p*-hydroxybenzoyl moieties readily transfer radicals to monolignols rather than undergoing radical coupling reactions directly, these conjugates could serve to increase polymerisation rates (Ralph 2006). For instance, sinapyl alcohol is not efficiently oxidised by many plant peroxidases (Marjamaa et al. 2009), so conjugation with *p*-hydroxybenzoate could be a particularly useful strategy to enhance lignification for S-rich lignins such as those in the fibres of poplar and willow. To this point, there is evidence that *-pHB* occurs almost exclusively on S-lignin units in poplar (Regner et al. 2018; Stewart et al. 2009), so perhaps the BAHG acyltransferase responsible for conjugating *p*-hydroxybenzoate prefers sinapyl alcohol over coniferyl alcohol. However, one recent study reported a negative correlation between S-lignin and *-pHB* in a collection of natural poplar variants (Yoo et al. 2018). The co-localisation of *-pHB* with S-lignin in fibres is supported by the MCR analysis herein (Figure 5), although these results cannot distinguish conjugation of *-pHB* to S-lignin units specifically.

As *-pHB* groups are linked via the γ position, these acylations lead to structural changes in lignin. For example, during β - β coupling of monolignols, the γ hydroxyl is normally used to trap the quinone methide intermediate leading to the formation of resinol structures in lignin (Ralph et al. 2004). Since such structures are not possible when *-pHB* is present, alternative linkages such as β -O-4 prevail in highly γ -acylated S-rich lignin polymers (Martinez et al. 2008). Greater abundance of pendant groups might also lead to reductions in polymer length. For example, lignification with *-pHB* and *p*-hydroxybenzaldehyde in transgenic *Arabidopsis* led to lignin polymers with lower average molecular weight

(Eudes et al. 2012). Similarly, the degree of polymerisation was reduced in synthetic dehydrogenative lignin polymers due to the incorporation of *p*-coumarate (Djikanović et al. 2012). However, the impacts of acylated monolignols on lignin molecular weight have not yet been conclusively demonstrated.

Although what benefits these lignin alternations may offer to fibres remains an open question, the industrial significance of *-pHB* is much clearer. Lignin polymers with abundant β -O-4 ether bonds are more amenable to deconstruction (Mottiar et al. 2016; Stewart et al. 2006). Interestingly, it has also been suggested that liberation of *-pHB* promotes acid-catalysed reactions and can act as a blocking agent to prevent condensation reactions that counteract lignin depolymerisation (Bardet et al. 1985b; Chua and Wayman 1979). However, the most attractive aspect is that these ester-linked phenolics can be readily 'clipped-off' by mild alkaline hydrolysis.

As a platform chemical, *p*-hydroxybenzoate can be used to make diverse valuable commodity chemicals that would otherwise be derived from fossil fuels (Wang et al. 2018). Perhaps most notably, parabens (a class of preservatives widely used in cosmetics and pharmaceuticals) are produced via *p*-hydroxybenzoate by esterification with alcohols such as methanol, butanol or benzyl alcohol (Yang et al. 2018). Terephthalate, the key precursor for PET plastics, is traditionally synthesised from petroleum-derived *p*-xylene but could also be produced via carboxylation of *p*-hydroxybenzoate (Bai et al. 2016). Novel processes are also being devised to synthesise high-value compounds from *p*-hydroxybenzoate such as paracetamol (acetaminophen) (Ralph et al. 2019). Furthermore, engineered microorganisms have been developed that can metabolically upgrade *p*-hydroxybenzoate into sundry high-value products (Becker and Whittmann 2019).

The potential of 'clip-off' phenolics will undoubtedly encourage future plant engineering efforts to increase the levels of *-pHB* groups in industrially useful biomass feedstock species, and the ToF-SIMS methodology developed here will be useful in evaluating the effectiveness of this work.

4 Conclusions

While the biological role of cell wall-bound phenolics such as *-pHB* remains obscured, there is considerable industrial potential for such 'clip-offs' in lignin valorisation. Accordingly, ToF-SIMS imaging was used to examine the distribution of *-pHB* groups in the xylem of

poplar and willow stems. The results presented here corroborate historical UV-microscopy studies and show that *-p*HB is found predominantly, if not exclusively, in the cell walls of fibres. This observation helps explain the occurrence of *-p*HB in different plant tissues and perhaps also in reaction wood and following nitrogen fertilisation. An improved understanding of *-p*HB could help elucidate the biological role of lignin decorations and may inform future efforts in plant biotechnology and lignin engineering.

Abbreviations

G lignin	guaiacyl lignin unit
H lignin	<i>p</i> -hydroxyphenyl lignin unit
MCR	multivariate curve resolution
PCA	principal component analysis
<i>-p</i> HB	ester-linked <i>p</i> -hydroxybenzoate group
S lignin	syringyl lignin unit
ToF-SIMS	time-of-flight secondary-ion mass spectrometry

Acknowledgements: Thanks to L. Tran (University of Victoria) and A. Groover (University of California Davis) for providing the nitrogen-fertilised and reaction wood hybrid poplar samples.

Author contribution: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: We gratefully acknowledge funding from the Natural Sciences and Engineering Research Council of Canada to Y.M. and S.D.M., from the Great Lakes Bioenergy Research Center, U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research under Award Numbers DE-SC0018409 and DE-FC02-07ER64494, to S.D.M., and from the Niagara University Academic Center for Integrated Sciences to R.E.G.

Conflict of interest statement: The authors declare no conflicts of interest regarding this article.

References

- ASTM standard D1105–96. (2007). *Standard test method for preparation of extractive-free wood*. ASTM International, West Conshohocken, PA, USA.
- Bai, Z., Phuan, W.C., Ding, J., Heng, T.H., Luo, J., and Zhu, Y. (2016). Production of terephthalic acid from lignin-based phenolic acids by a cascade fixed-bed process. *ACS Catal.* 6: 6141–6145.
- Bardet, M., Foray, M.-F., and Robert, D. (1985a). Use of the DEPT pulse sequence to facilitate the ^{13}C NMR structural analysis of lignins. *Makromol. Chem.* 186: 1495–1504.
- Bardet, M., Robert, D.R., and Lundquist, K. (1985b). On the reactions and degradation of the lignin during steam hydrolysis of aspen wood. *Svensk Papperstidning* 6: R61–R67.
- Becker, J. and Wittmann, C. (2019). A field of dreams: lignin valorization into chemicals, materials, fuels, and health-care products. *Biotechnol. Adv.* 37: 107360.
- Beckham, G.T., Johnson, C.W., Karp, E.M., Salvachúa, D., and Vardon, D.R. (2016). Opportunities and challenges in biological lignin valorization. *Curr. Opin. Biotechnol.* 42: 40–53.
- Boerjan, W., Ralph, J., and Baucher, M. (2003). Lignin biosynthesis. *Annu. Rev. Plant Biol.* 54: 519–546.
- Braham, E.J. and Goacher, R.E. (2015). Identifying and minimizing buffer interferences in ToF-SIMS analyses of lignocellulose. *Surf. Interface Anal.* 47: 120–126.
- Chen, F., Tobimatsu, Y., Havkin-Frenkel, D., Dixon, R.A., and Ralph, J. (2012). A polymer of caffeoyl alcohol in plant seeds. *Proc. Natl. Acad. Sci. U.S.A.* 109: 1772–1777.
- Chen, T.-Y., Wang, B., Wu, Y.-Y., Wen, J.-L., Liu, C.-F., Yuan, T.-Q., and Sun, R.C. (2017). Structural variations of lignin macromolecule from different growth years of triploid of *Populus tomentosa* Carr. *Int. J. Biol. Macromol.* 101: 747–757.
- Chua, M.G.S. and Wayman, M. (1979). Characterization of autohydrolysis aspen (*P. tremuloides*) lignins. Part 1. Composition and molecular weight distribution of extracted autohydrolysis lignin. *Can. J. Chem.* 57: 1141–1149.
- Del Río, J.C., Rencoret, J., Gutiérrez, A., Kim, H., and Ralph, J. (2017). Hydroxystilbenes are monomers in palm fruit endocarp lignins. *Plant Physiol.* 174: 2072–2082.
- Djikanović, D., Simonović, J., Savić, A., Ristić, I., Bajuk-Bogdanović, D., Kalauzi, A., Cakić, S., Budinski-Simendić, J., Jeremić, M., and Radotić, K. (2012). Structural differences between lignin model polymers synthesized from various monomers. *J. Polym. Environ.* 20: 607–617.
- Donaldson, L. (2013). Softwood and hardwood lignin fluorescence spectra of wood cell walls in different mounting media. *IAWA J.* 34: 3–19.
- Eudes, A., George, A., Mukerjee, P., Kim, J.S., Pollet, B., Benke, P.I., Yang, F., Mitra, P., Sun, L., Cetinkol, O.P., et al. (2012). Biosynthesis and incorporation of side-chain-truncated lignin monomers to reduce lignin polymerization and enhance saccharification. *Plant Biotechnol. J.* 10: 609–620.
- Foston, M., Hubbell, C.A., Samuel, R., Jung, S., Fan, H., Ding, S.-Y., Zeng, Y., Jawdy, S., Davis, M., Sykes, R., et al. (2011). Chemical, ultrastructural and supramolecular analysis of tension wood in *Populus tremula* x *alba* as a model substrate for reduced recalcitrance. *Energy Environ. Sci.* 4: 4962–4971.
- Frankenstein, C., Schmitt, U., and Koch, G. (2006). Topochemical studies on modified lignin distribution in the xylem of poplar (*Populus* spp.) after wounding. *Ann. Bot.* 97: 195–204.
- Gerttula, S., Zinkgraf, M., Muday, G.K., Lewis, D.R., Ibatullin, F.M., Brumer, H., Hart, F., Mansfield, S.D., Filkov, V., and Groover, A. (2015). Transcriptional and hormonal regulation of gravitropism of woody stems in *Populus*. *Plant Cell* 27: 2800–2813.
- Glasser, W.G. (2019). About making lignin great again some lessons from the past. *Front. Chem.* 7: 565.
- Goacher, R.E., Jeremic, D., and Master, E.M. (2011). Expanding the library of secondary ions that distinguish lignin and polysaccharides in time-of-flight secondary ion mass spectrometry analysis of wood. *Anal. Chem.* 83: 804–812.

- Goacher, R.E., Edwards, E.A., Yakunin, A.F., Mims, C.A., and Master, E.R. (2012). Application of time-of-flight-secondary ion mass spectrometry for the detection of enzyme activity on solid wood substrates. *Anal. Chem.* 84: 4443–4451.
- Goacher, R.E., Tsai, A.Y.-L., and Master, E.R. (2013). Towards practical time-of-flight secondary ion mass spectrometry lignocellulolytic enzyme assays. *Biotechnol. Biofuels* 6: 132.
- Goacher, R.E., Selig, M.J., and Master, E.R. (2014). Advancing lignocellulose bioconversion through direct assessment of enzyme action on insoluble substrates. *Curr. Opin. Biotechnol.* 27: 123–133.
- Goldstein, I.S. (1984). Degradation of wood by chemicals. In: Rowell, R. (Ed.). *The chemistry of solid wood*. American Chemical Society, Washington, DC, USA, pp. 575–586.
- Gorzsás, A., Stenlund, H., Persson, P., Trygg, J., and Sundberg, B. (2011). Cell-specific chemotyping and multivariate imaging by combined FT-IR microspectroscopy and orthogonal projections to latent structures (OPLS) analysis reveals the chemical landscape of secondary xylem. *Plant J.* 66: 903–914.
- Grabber, J.H., Ralph, J., Lapierre, C., and Barrière, Y. (2004). Genetic and molecular basis of grass cell-wall degradability. I. Lignin-cell wall matrix interactions. *C. R. Biol.* 327: 455–465.
- Graham, D.J. and Castner, D.G. (2012). Multivariate analysis of ToF-SIMS data from multicomponent systems: the why, when, and how. *Biointerphases* 7: 49.
- Hatfield, R.D., Marita, J.M., Frost, K., Grabber, J., Ralph, J., Lu, F., and Kim, H. (2009). Grass lignin acylation: *p*-coumaroyl transferase activity and cell wall characteristics of C₃ and C₄ grasses. *Planta* 229: 1253–1267.
- Hedenström, M., Wiklund-Lindström, S., Oman, T., Lu, F., Gerber, L., Schatz, P., Sundberg, B., and Ralph, J. (2009). Identification of lignin and polysaccharide modifications in *Populus* wood by chemometric analysis of 2D NMR spectra from dissolved cell walls. *Mol. Plant* 2: 933–942.
- Hoagland, D.R. and Arnon, D.I. (1950). The water-culture method for growing plants without soil. *Calif. Agric. Exp. Stn. Circ.* 347: 1–32.
- Jourez, B., Riboux, A., and Leclercq, A. (2001). Anatomical characteristics of tension wood and opposite wood in young inclined stems of poplar (*Populus euramericana* cv 'Ghoy'). *IAWA J.* 22: 133–157.
- Kai, D., Tan, M.J., Chee, P.L., Chua, Y.K., Yap, Y.L., and Loh, X.J. (2016). Towards lignin-based functional materials in a sustainable world. *Green Chem.* 18: 1175–1200.
- Karlen, S.D., Free, H.C.A., Padmakshan, D., Smith, B.G., Ralph, J., and Harris, P.J. (2018). Commelinid monocotyledon lignins are acylated by *p*-coumarate. *Plant Physiol.* 177: 513–521.
- Kim, H., Ralph, J., Lu, F., Ralph, S.A., Boudet, A.-M., MacKay, J.J., Sederoff, R.R., Ito, T., Kawai, S., Ohashi, H., et al. (2003). NMR analysis of lignins in CAD-deficient plants. Part 1. Incorporation of hydroxycinnamaldehydes and hydroxybenzaldehydes into lignins. *Org. Biomol. Chem.* 1: 268–281.
- Li, H., Li, M., Luo, J., Cao, X., Qu, L., Gai, Y., Jiang, X., Liu, T., Bai, H., Janz, D., et al. (2012). N-fertilization has different effects on the growth, carbon and nitrogen physiology, and wood properties of slow-and fast-growing *Populus* species. *J. Exp. Bot.* 63: 6173–6185.
- Lu, F., Ralph, J., Morreel, K., Messens, E., and Boerjan, W. (2004). Preparation and relevance of a cross-coupling product between sinapyl alcohol and sinapyl *p*-hydroxybenzoate. *Org. Biomol. Chem.* 2: 2888–2890.
- Lu, F., Karlen, S.D., Regner, M., Kim, H., Ralph, S.A., Sun, R.-C., Kuroda, K.-I., Augustin, M.A., Mawson, R., Sabarez, H., et al. (2015). Naturally *p*-hydroxybenzoylated lignins in palms. *Bioenergy Res.* 8: 934–952.
- Marjamaa, K., Kukkola, E.M., and Fagerstedt, K.V. (2009). The role of xylem class III peroxidases in lignification. *J. Exp. Bot.* 60: 367–376.
- Martínez, Á.T., Rencoret, J., Marques, G., Gutiérrez, A., Ibarra, D., Jiménez-Barbero, J., and del Río, J.C. (2008). Monolignol acylation and lignin structure in some nonwoody plants: a 2D NMR study. *Phytochemistry* 69: 2831–2843.
- Morreel, K., Ralph, J., Kim, H., Lu, F., Goeminne, G., Ralph, S., Messens, E., and Boerjan, W. (2004). Profiling of oligolignols reveals monolignol coupling conditions in lignifying poplar xylem. *Plant Physiol.* 136: 3537–3549.
- Mottiar, Y., Vanholme, R., Boerjan, W., Ralph, J., and Mansfield, S.D. (2016). Designer lignins: harnessing the plasticity of lignification. *Curr. Opin. Biotechnol.* 37: 190–200.
- Mottiar, Y., Gierlinger, N., Jeremic, D., Master, E.R., and Mansfield, S.D. (2020). Atypical lignification in eastern leatherwood (*Dirca palustris*). *New Phytol.* 226: 704–713.
- Musha, Y. and Goring, D.A.I. (1975). Distribution of syringyl and guaiacyl moieties in hardwoods as indicated by ultraviolet microscopy. *Wood Sci. Technol.* 9: 45–58.
- Nakamura, Y. and Higuchi, T. (1978). Ester linkage of *p*-coumaric acid in bamboo lignin. III. Dehydrogenative polymerization of coniferyl *p*-hydroxybenzoate and coniferyl *p*-coumarate. *Cellul. Chem. Technol.* 12: 209–221.
- Nakano, J., Ishizu, A., and Migita, N. (1961). Studies on lignin XXXII. Ester groups of lignin. *TAPPI* 44: 30–32.
- Novo-Uzal, E., Pomar, F., Gómez Ros, L.V., Espiñeira, J.M., and Ros Barceló, A. (2012). Evolutionary history of lignins. *Adv. Bot. Res.* 61: 311–350.
- Novaes, E., Osorio, L., Drost, D.R., Miles, B.L., Boaventura-Novaes, C.R., Benedict, C., Dervinis, C., Yu, Q., Sykes, R., Davis, M., et al. (2009). Quantitative genetic analysis of biomass and wood chemistry of *Populus* under different nitrogen levels. *New Phytol.* 182: 878–890.
- Pearl, I.A., Beyer, D.L., Johnson, B., and Wilkinson, S. (1957). Alkaline hydrolysis of representative hardwoods. *TAPPI* 40: 374–378.
- Petrik, D.L., Karlen, S.D., Cass, C.L., Padmakshan, D., Lu, F., Liu, S., Le Bris, P., Antelme, S., Santoro, N., Wilkerson, C.G., et al. (2014). *p*-Coumaroyl-CoA:monolignol transferase (PMT) acts specifically in the lignin biosynthetic pathway in *Brachypodium distachyon*. *Plant J.* 77: 713–726.
- Pitre, F.E., Pollet, B., Lafarguette, F., Cooke, J.E.K., Mackay, J.J., and Lapierre, C. (2007). Effects of increased nitrogen supply on the lignification of poplar wood. *J. Agric. Food Chem.* 55: 10306–10314.
- Ralph, J. (2006). What makes a good monolignol substitute? In: Hayashi, T. (Ed.). *The science and lore of the plant cell wall biosynthesis, structure and function*. Brown Walker Press, Boca Raton, FL, USA, pp. 285–293.
- Ralph, J., Hatfield, R.D., Quideau, S., Helm, R.F., Grabber, J.H., and Jung, H.-J.G. (1994). Pathway of *p*-coumaric acid incorporation into maize lignin as revealed by NMR. *J. Am. Chem. Soc.* 116: 9448–9456.
- Ralph, J., Lundquist, K., Brunow, G., Lu, F., Kim, H., Schatz, P.F., Marita, J.M., Hatfield, R.D., Ralph, S.A., Christensen, J.H., et al. (2004). Lignins: natural polymers from oxidative coupling of 4-hydroxyphenylpropanoids. *Phytochem. Rev.* 3: 29–60.

- Ralph, J., Karlen, S., and Mobley, J. (2019). *Synthesis of paracetamol (acetaminophen) from biomass-derived p-hydroxybenzamide*. United States Patent US10286504B2.
- Regner, M., Bartuce, A., Padmakshan, D., Ralph, J., and Karlen, S.D. (2018). Reductive cleavage method for quantitation of monolignols and low-abundance monolignol conjugates. *ChemSusChem* 11: 1600–1605.
- Rencoret, J., Marques, G., Serrano, O., Kaal, J., Martínez, A. T., del Río, J.C., and Gutiérrez, A. (2020). Deciphering the unique structure and acylation pattern of *Posidonia oceanica* lignin. *ACS Sustain. Chem. Eng.* 8: 12521–12533.
- Rinaldi, R., Jastrzebski, R., Clough, M.T., Ralph, J., Kennema, M., Buijtinckx, P.C., and Weckhuysen, B.M. (2016). Paving the way for lignin valorisation: recent advances in bioengineering, biorefining and catalysis. *Angew. Chem. Int. Ed.* 55: 8164–8215.
- Saito, K., Kato, T., Takamori, H., Kishimoto, T., Yamamoto, A., and Fukushima, K. (2006). A new analysis of the depolymerized fragments of lignin polymer in the plant cell walls using ToF-SIMS. *Appl. Surf. Sci.* 252: 6734–6737.
- Schutyser, W., Renders, T., Van den Bosch, S., Koelewijn, S.F., Beckham, G.T., and Sels, B.F. (2018). Chemicals from lignin: an interplay of lignocellulose fractionation, depolymerisation, and upgrading. *Chem. Soc. Rev.* 47: 852–908.
- Shimada, M., Fukuzuka, T., and Higuchi, T. (1971). Ester linkages of *p*-coumaric acid in bamboo and grass lignins. *TAPPI* 54: 72–78.
- Sibout, R., Le Bris, P., Legée, F., Cézard, L., Renault, H., and Lapierre, C. (2016). Structural redesigning *Arabidopsis* lignins into alkali-soluble lignins through the expression of *p*-coumaroyl-CoA: monolignol transferase PMT. *Plant Physiol.* 170: 1358–1366.
- Smith, D.C.C. (1955). *p*-hydroxybenzoate groups in the lignin of aspen (*Populus tremula*). *J. Chem. Soc.* 3: 2347–2351.
- Smith, R.A., Gonzales-Vigil, E., Karlen, S.D., Park, J.-Y., Lu, F., Wilkerson, C.G., Samuels, L., Ralph, J., and Mansfield, S.D. (2015). Engineering monolignol *p*-coumarate conjugates into poplar and *Arabidopsis* lignins. *Plant Physiol.* 169: 2992–3001.
- Stewart, J.J., Kadla, J.F., and Mansfield, S.D. (2006). The influence of lignin chemistry and ultrastructure on the pulping efficiency of clonal aspen (*Populus tremuloides* Michx.). *Holzforschung* 60: 111–122.
- Stewart, J.J., Akiyama, T., Chapple, C., Ralph, J., and Mansfield, S.D. (2009). The effects on lignin structure of overexpression of ferulate 5-hydroxylase in hybrid poplar. *Plant Physiol.* 150: 621–635.
- Terashima, N., Mori, I., and Kanda, T. (1975). Biosynthesis of *p*-hydroxybenzoic acid in poplar lignin. *Phytochemistry* 14: 1991–1992.
- Terashima, N., Okada, M., and Tomimura, Y. (1979). Heterogeneity in formation of lignin. I. Heterogeneous incorporation of *p*-hydroxybenzoic acid into poplar lignins. *Mokuzai Gakkaishi* 25: 422–426.
- Venverloo, C.J. (1969). The lignin of *Populus nigra* L. cv. 'Italica': a comparative study of the lignified structure in tissue cultures and the tissues of the tree. *Acta Bot. Neerl.* 18: 241–314.
- Venverloo, C.J. (1971). The lignin of *Populus nigra* L. cv. 'Italica' and some other *Salicaceae*. *Holzforschung* 25: 18–24.
- Wang, H.-M., Ma, C.-Y., Li, H.-Y., Chen, T.-Y., Wen, J.-L., Cao, X.-F., Wang, X.-L., Yuan, T.-Q., and Sun, R.-C. (2020). Structural variations of lignin macromolecules from early growth stages of poplar cell walls. *ACS Sustain. Chem. Eng.* 8: 1813–1822.
- Wang, S., Bilal, M., Hu, H., Wang, W., and Zhang, X. (2018). 4-Hydroxybenzoic acid a versatile platform intermediate for value-added compounds. *Appl. Microbiol. Biotechnol.* 102: 3561–3571.
- Withers, S., Lu, F., Kim, H., Zhu, Y., Ralph, J., and Wilkerson, C.G. (2012). Identification of grass-specific enzyme that acylates monolignols with *p*-coumarate. *J. Biol. Chem.* 287: 8347–8355.
- Yang, H., Zhang, F., and Wu, H. (2018). Review on life cycle of parabens: synthesis, degradation, characterization and safety analysis. *Curr. Org. Chem.* 22: 769–779.
- Yoo, C.G., Dumitrache, A., Muchero, W., Natzke, J., Akinosho, H., Li, M., Sykes, R.W., Brown, S.D., Davison, B., Tuskan, G.A., et al. (2018). Significance of lignin S/G ratio in biomass recalcitrance of *Populus trichocarpa* variants for bioethanol production. *ACS Sustain. Chem. Eng.* 6: 2162–2168.
- Zobel, B.J. and Sprague, J.R. (1998). *Juvenile wood in forest trees*. Springer-Verlag, Berlin Heidelberg.
- Zhou, C., Li, Q., Chiang, V.L., Lucia, L.A., and Griffis, D.P. (2011). Chemical and spatial differentiation of syringyl and guaiacyl lignins in poplar wood via time-of-flight secondary ion mass spectrometry. *Anal. Chem.* 83: 7020–7026.

Supplementary material: Supplementary material to this article can be found online at (<https://doi.org/10.1515/hf-2020-0130>).