

# CHANGES IN THE COMPOSITION AND STRUCTURE OF LIGNIN DURING THE GROWTH OF A *EUCALYPTUS GLOBULUS* CLONE

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## ABSTRACT

The content, composition and structure of lignin in a *Eucalyptus globulus* clone were studied at different growth stages (1 month, 18 months, and 9 years). The lignin content in the eucalypt woods increase during growth and the changes in its composition and structure were studied “in situ” by Py-GC/MS and 2D-NMR of the whole wood. In addition, milled-wood lignins were isolated and analyzed by Py-GC/MS, 2D-NMR and thioacidolysis followed by Raney Nickel desulfurization. The data obtained indicated that H and G lignin units are deposited first at the earlier stages, whereas the woods are enriched in S lignin during late lignification. The main linkages present were  $\beta$ -O-4' alkyl-aryl ether and resinol  $\beta$ - $\beta'$ , whereas other substructures (such as  $\beta$ -5' phenylcoumaran and  $\beta$ -1' spirodienones) were present in lower abundances during all stages of lignification. The differences in monomer deposition affect the distribution of the different linkages during lignification.

## I. INTRODUCTION

Lignin is a complex polymer synthesized by radical coupling of mainly three hydroxycinnamyl alcohol monomers, differing in their degree of methoxylation: *p*-coumaryl, coniferyl and sinapyl alcohols (Ralph et al., 2004). Each of these monolignols gives rise to a different type of lignin unit called H (*p*-hydroxyphenyl), G (guaiacyl) and S (syringyl) units, respectively, when incorporated into the lignin polymer. The amount and composition of lignins vary among taxa, cell types, individual cell-wall layers and environmental conditions. Softwood contains exclusively G-type lignin, whereas hardwood lignin consists mainly of S units and lower amounts of G units. After their synthesis, the lignin monomers are transported to the cell wall where they are polymerized in a combinatorial fashion by free radical coupling mechanisms generating a variety of linkages (Ralph et al., 2004). Lignin deposition is one of the final stages of xylem cell differentiation and mainly takes place during secondary thickening of the cell wall. Previous studies have suggested that the three monolignols are incorporated at different stages of cell-wall formation (Terashima et al., 1993). The difference in timing of monolignol deposition would imply that the lignin composition and structure will be changing during lignification. The aim of this paper is, therefore, to study the changes produced in the structure of the lignin during the lignification process in eucalypt. For this purpose, samples of wood from the same *Eucalyptus globulus* clone were collected at different stages of growth, and the structure investigated by Py-GC/MS, 2D-NMR and thioacidolysis followed by Raney Nickel desulfurization.

## II. EXPERIMENTAL

### E. globulus wood samples and isolation of their milled wood lignins

Samples from a eucalypt (*E. globulus*) clone (334-1-AR) at different stages of growth (1 month, 18 months and 9 years) were provided by ENCE (Pontevedra, Spain). Whole stems of 1-month old eucalypt plants and, previously debarked 18-months and 9-years old eucalypt wood chips, were air-dried and subsequently milled. The content of water solubles, and acetone extractives, Klason and acid-soluble lignin, holocellulose and ash were estimated according to Tappi rules (Tappi, 2004). Milled-wood lignins (MWL) were obtained according to the classical procedures from grounded wood.

### Py-GC/MS

Pyrolysis of the woods and their MWL (approximately 100  $\mu$ g) were performed at 500°C in a 2020 micro-furnace pyrolyzer (Frontier Laboratories Ltd.) connected to an Agilent 6890 GC/MS system equipped with a DB-1701 fused-silica capillary column (30 m x 0.25 mm i.d., 0.25  $\mu$ m film thickness). The oven temperature was programmed from 40°C (1 min) to 300°C at 6°C min<sup>-1</sup> (10 min).

### NMR spectroscopy

For the analysis of the whole cell walls, around 100 mg of finely divided and extractives-free wood samples were swollen in dimethylsulfoxide (DMSO)-*d*<sub>6</sub>/pyridine-*d*<sub>5</sub> (4:1, v:v) solvent system, according to the method developed by Kim and Ralph (2010). In the case of the MWL, around 40 mg of MWL were dissolved in 0.75 mL of (DMSO)-*d*<sub>6</sub>. 2D-NMR spectra were recorded in HSQC mode at 25 °C in a Bruker AVANCE 500 MHz

spectrometer fitted with a cryogenically cooled 5 mm gradient probe with inverse geometry. The  $^1J_{CH}$  used was 140 Hz and the  $J$ -coupling evolution delay was set to 500 ms. HSQC cross-signals were assigned by comparing with the literature (Rencoret et al., 2008; Kim and Ralph, 2010). A semiquantitative analysis of the intensities of the HSQC cross-signal intensities was performed separately in the different regions of the spectrum.

#### **Thioacidolysis**

Thioacidolysis of 5 mg of MWL were performed as described by Rolando *et al.* (1992) using 0.2 M  $BF_3$  etherate in dioxane/ethanethiol 8.75:1. The reactions products were extracted with  $CH_2Cl_2$ , dried and concentrated. The thioacidolysis products was subsequently desulfurized as described by Lapierre *et al.* (1991).

### **III. RESULTS AND DISCUSSION**

The content of the main constituents of the wood from the *E. globulus* clone at different stages of growth are summarized in **Table 1**. The lignin content in the wood increased during growth (from 15.7% in the 1-month sample to 24.5 % in the 9-years old wood).

**Table 1.** Abundance of the main constituents (%) of the woods from the same *E. globulus* clone at different growth stages

	1 month	18 months	9 years
Acetone extractives	8.6	0.5	0.6
Water soluble extracts	6.6	1.4	2.2
Klason lignin	13.0	17.5	19.8
Acid-soluble lignin	2.7	5.2	4.7
Holocellulose	64.5	74.7	72.3
Ashes	4.6	0.7	0.4

#### **Py-GC/MS of wood and MWL during eucalypt growth**

The pyrolysis of the different eucalypt woods released carbohydrate and lignin-derived compounds. Among the lignin-derived compounds, G- and S-type phenols were released. In addition, important amounts of compounds derived from H lignin-units could be detected in the youngest eucalypt samples. The molar H:G:S composition of the lignin in the different woods, together with the lignin S/G ratios obtained from the molar areas of all the lignin-derived compounds, are shown in **Table 2**.

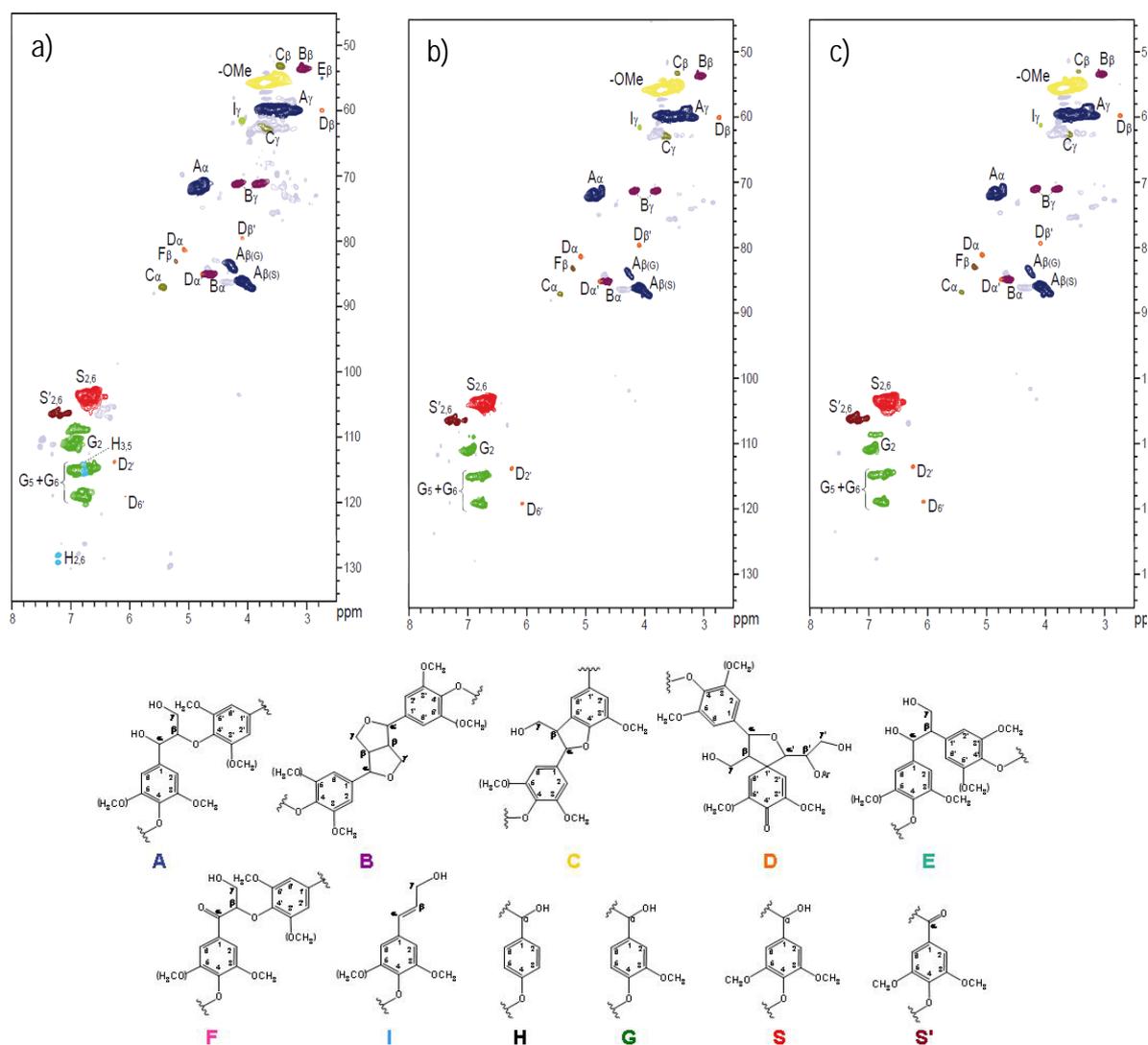
**Table 2.** Relative molar abundance (%) of the lignin-units obtained upon Py-GC/MS of the *E. globulus* wood clone at the different growth stages

	1 month		18 months		9 years	
	Wood	MWL	Wood	MWL	Wood	MWL
Total H	9.4	2.6	1.9	0.7	1.9	0.8
Total G	38.5	42.0	21.2	27.5	20.4	24.3
Total S	52.1	55.4	76.9	71.8	77.6	74.9
S/G ratio	1.4	1.3	3.6	2.6	3.8	3.1
H/G ratio	0.24	0.06	0.09	0.03	0.09	0.03

In all samples, the S-type phenols were predominant, with a S/G ratio ranging from 1.4 in the youngest wood sample (1 month) to 3.8 in the oldest sample (9 years). The high content of H-lignin units in the youngest wood sample decreases during lignification (from 9.4% to 1.9% in the woods from 18-months and 9-years, respectively). In addition, the abundances of the S-lignin increase whereas the G-lignin decreases with lignification. These data indicate that H-units are deposited first, followed by G- and then by S-units, and are in agreement with previous studies using microautoradiography and UV-microspectrometry (Terashima et al., 1986). The pyrolysis of the MWL released a similar distribution of lignin-derived compounds, although the content of H-lignin units was lower (**Table 2**), this being especially evident in the MWL from the 1-month wood. In any case, the same trend observed in the pyrolysis of woods, that indicate an increase of S-lignin and a decrease of H- and G-lignin with lignification, were also observed in the pyrolysis of MWL, in agreement with a monolignol deposition order of H, G and then S during lignification.

### HSQC-NMR of wood and MWL during eucalypt growth

The woods and their respective MWL were also analyzed by 2D-NMR (**Figure 3**). All the spectra showed prominent signals corresponding to  $\beta$ -O-4' ether linkages (**A**). In addition, signals for resinol (**B**), phenylcoumaran (**C**) and spirodienone (**D**) substructures could also be observed.



**Figure 1.** HSQC NMR spectra of the MWL isolated from the different *E. globulus* wood samples at different growth stages: **a)** 1 month; **b)** 18 months; and **c)** 9 years. The main lignin structures identified are **A)**  $\beta$ -O-4'; **B)** resinol ( $\beta$ - $\beta'$ ); **C)** phenylcoumaran ( $\beta$ -5'); **D)** spirodienone ( $\beta$ -1'/ $\alpha$ -O- $\alpha'$ ); **E)**  $\beta$ -1'; **F)** C $\alpha$ -oxidized  $\beta$ -O-4'; **G)** guaiacyl units; **H)** p-hydroxyphenyl units; **I)** p-hydroxycinnamyl alcohol end-groups; **S)** syringyl units; and **S')** oxidized syringyl units bearing a carbonyl group at C $\alpha$ .

The abundances of the main inter-unit linkages in the different eucalypt woods and their MWL, as well as the H:G:S composition and the S/G ratio, are shown in **Table 3**. The H:G:S composition and S/G ratio are in agreement with the Py-GC/MS data and indicated a decrease of H- and G-units and an increase of S-units during lignification. With respect to the linkage types present, all the lignins showed a predominance of  $\beta$ -O-4' ether linkages (70-72%), followed by  $\beta$ - $\beta'$  resinol-type linkages (16-19%) and lower amounts of  $\beta$ -5' phenylcoumaran-type (2-4%) and  $\beta$ -1' spirodienone-type linkages (1-4%). The conventional open  $\beta$ -1' structures (**E**) are observed only in the MWL and ranged from 1-3%. The changes in monolignol composition during growth affects the abundances of the different linkages. Hence, the abundance of phenylcoumaran structures decreases with lignification, which is related to the decrease of the G-lignin units during growth. In addition, the content of open  $\beta$ -1' structures decreases with lignification while the content of spirodienones increases. This trend can be better reflected by the ratio spirodienones/ $\beta$ -1' that ranges from 0.8 at the earlier stages to 3.2 in the oldest wood samples. Finally, a small but continuous oxidation of the C $\alpha$  of the lignin side chain occurs during lignification.

**Table 3.** Percentage of lignin side-chains forming different inter-unit linkages **A-F** and cinnamyl alcohol end-groups **I**, referred to as the total side-chains; H, G and S contents, and S/G ratio from the *E. globulus* wood at the different growth stages, and their MWL

	1 month		18 months		9 years	
	Wood	MWL	Wood	MWL	Wood	MWL
<u>Linkage relative abundance (% side-chains involved):</u>						
$\beta$ -O-4' aryl ether ( <b>A</b> )	72	67	72	70	70	71
Resinol ( <b>B</b> )	17	20	19	20	17	18
Phenylcoumaran ( <b>C</b> )	4	5	1	2	2	2
Spirodienones ( <b>D</b> )	1	2	2	3	4	4
$\beta$ -1' structures ( <b>E</b> )	-	1	-	1	-	1
$\beta$ -O-4' oxidized at C $\alpha$ ( <b>F</b> )	1	1	2	2	4	3
Cinnamyl alcohol end-groups ( <b>I</b> )	5	4	4	2	3	1
Spirodienones/ $\beta$ -1' ratio	-	1.6	-	3.2	-	3.2
<u>Percentage of lignin units (%):</u>						
H	5	2	0	0	0	0
G	41	42	26	27	23	24
S	53	56	74	73	77	76
S/G ratio	1.3	1.3	2.9	2.7	3.3	3.2

### Thioacidolysis

The thioacidolysis monomer composition showed a predominance of S over G units in the etherified lignin, and the absence of H units. The monomer yield increases with lignification (from 1400  $\mu\text{mol g}^{-1}$  lignin to nearly 3000  $\mu\text{mol g}^{-1}$  lignin at the oldest stage) revealing a progressive enrichment of etherified structures during growth. The dimers obtained were of 5-5', 4-O-5',  $\beta$ -1',  $\beta$ -5',  $\beta$ - $\beta'$  and phenylisochroman types, the  $\beta$ - $\beta'$  structures being the most prominent. The abundances of the  $\beta$ - $\beta'$  structures increase with lignification, a trend that was not clearly observed in the HSQC spectra. Interestingly, most of the  $\beta$ - $\beta'$  dimers released were of syringaresinol type, pinoresinol being completely absent, and the G-S resinol structure appearing only in trace amounts.

### IV. CONCLUSIONS

The content, composition and structure of the lignin in *E. globulus* wood at different growth stages were studied. The lignin content increases with growth and its composition changes with lignification, the order of monolignol deposition being H, G, and then S at a late stage. This deposition order affects the abundances of the inter-unit linkages in the lignin structure. Thus, an increase of the  $\beta$ -O-4' structures were observed during lignification due to the enrichment of S-units. Similarly,  $\beta$ - $\beta'$  resinol structures, which are formed almost exclusively of syringaresinol, also increases with lignification. On the contrary,  $\beta$ -5' and 5-5' biphenyl structures decrease during lignification due to the parallel decrease of G-units.

### V. ACKNOWLEDGEMENTS

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