STRUCTURAL CHARACTERIZATION OF THE LIGNIN IN THE CORTEX AND PITH OF ELEPHANT GRASS (PENNISETUM PURPUREUM) STEMS

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ABSTRACT

The structure of the lignin in the cortex and pith of elephant grass (*Pennisetum purpureum*) stems were studied both in situ and in isolated milled 'wood' lignins by several analytical methods. The presence of *p*-coumarate and ferulate in the cortex and pith, as well as in their isolated lignins, was revealed by pyrolysis in the presence of TMAH, and by 2D-NMR, and indicated that ferulate acylates the carbohydrates while *p*-coumarate acylates the lignin polymer. 2D-NMR showed a predominance of β–O–4 alkyl aryl ether linkages (82% of total inter-unit linkages), with low amounts of 'condensed' substructures, such as resinols, phenylcoumarans, and spirodienones. Moreover, the NMR also indicated that these lignins are extensively acylated at the γ-carbon of the side-chain. DFRC analyses confirmed that *p*-coumarate groups acylate the γ-OHs of these lignins, and predominantly on syringyl units.

I. INTRODUCTION

Elephant grass (*Pennisetum purpureum*) is a s pecies from the Poaceae native to the tropical grasslands of Africa and now introduced into most tropical and subtropical countries. The species is a robust grass with perennial stems, reaching over 3-m high, and is widely recognized as having the highest biomass productivity among herbaceous plants, attaining up to 45 Mg ha-1 yr^{-1}, and therefore has been considered an excellent alternative feedstock to provide abundant and sustainable resources of lignocellulosic biomass for the production of biofuels (Sommerville et al., 2010). However, there is a lack of studies regarding the chemistry of the lignin of elephant grass. Previous papers have only reported the lignin contents of different cultivars of elephant grass and their variation with growth development (Xie et al., 2011), but did not provide any information about its composition and structure.

In this paper, we therefore report the detailed chemical composition and structural characteristics of the lignin in elephant grass. For this purpose, the cortex and the pith of elephant grass stems were separated manually and analyzed independently by an array of analytical techniques. Among them, we used pyrolysis-gas chromatography-mass spectrometry (Py-GC/MS). However, the presence of *p*-hydroxycinnamates (*p*-coumarate and ferulate), which are abundant in grasses, constitutes a complication for lignin analysis by analytical pyrolysis since they yield products similar to those of corresponding lignin units due to decarboxylation reactions. This problem can however be partially solved by using pyrolysis in the presence of tetramethylammonium hydroxide (TMAH), that avoids decarboxylation and releases intact methylated *p*-hydroxycinnamates (del Río et al., 1996, 2007). Additional information regarding the different units and interunit linkages present in the lignin polymer was provided by 2D-NMR spectroscopy, which provides information of the structure of the whole macromolecule and is a powerful tool for lignin structural characterization (Ralph and Landucci, 2010; del Río et al., 2008), and DFRC (derivatization followed by reductive cleavage), which gives information on the nature and extent of γ-acylation of the lignin side-chain (Lu and Ralph, 1997; del Río et al., 2008). The knowledge of the composition and structure of the lignin of elephant grass will help to maximize the exploitation of this interesting crop for biomass and biofuel production.

II. EXPERIMENTAL

Samples. Elephant grass was collected at an age of 150 days-old. The stems were air-dried and then separated into the cortex and pith fractions, which were analyzed separately. Klason lignin content was estimated according to Tappi test method T222 om-88 (Tappi, 2004). The milled "wood" lignins (MWLs) were obtained according to the classical procedure (Björkman, 1956).

Py-GC/MS. Pyrolysis were performed with a 2020 micro-furnace pyrolyzer (Frontier Labs) 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 μm film thickness) and an Agilent 5973 mass selective detector (EI at 70 eV). The pyrolysis was performed at 500 ºC. The GC oven temperature was programmed from 50 ºC (1 min) to 100 ºC at 30 ºC/min and then to 290 ºC (10 min) at 6 ºC/min. Helium was the carrier gas. For Py/TMAH, 100 μg of sample were mixed with approximately 0.5 μL TMAH (25%, w/w, in methanol) and the pyrolysis was carried out as described above.

NMR spectroscopy. For the NMR of the whole cell walls, around 100 mg of finely divided (ball-milled) extractives-free samples were swollen in 0.75 mL DMSO-d_6. In the case of the isolated MWLs, around 40 mg were dissolved in 0.75 mL of DMSO-d_6. NMR spectra were recorded at 25 ºC on a Bruker AVANCE 600 MHz instrument equipped with a cryogenically-cooled z-gradient triple-resonance probe.

DFRC (derivatization followed by reductive cleavage). The DFRC degradation was performed according to the developed protocol, as previously described (Lu and Ralph, 1997; del Río et al., 2008). To assess the presence of naturally acetylated lignin units, the described modification of the standard DFRC method using propionylating instead of acetylating reagents (DFRC´) was used in the present study (Ralph and Lu, 1998).

III. RESULTS AND DISCUSSION

In this work, we separated the cortex (84%) and pith (16%) fractions of the stem and studied them separately. The lignin content was 18.5% in the cortex and 15.5% in the pith. The compositions of the lignin in both parts of elephant grass were analyzed in situ by Py-GC/MS and 2D-NMR. Additionally, for a m ore detailed structural characterization, the MWLs were isolated and subsequently analyzed by Py-GC/MS, 2D-NMR and DFRC.

Py-GC/MS released phenolic compounds derived from lignin moieties as well as from *p*-hydroxycinnamates. The most prominent compound in the pyrograms of the whole cell wals and the MWLs was 4-vinylphenol, derived largely from the *p*-coumarate esters acylating lignin side chains. The estimation of the S/G ratios was 1.5 and 1.4 in the MWL of the cortex and pith. The presence of *p*-hydroxycinnamates in the isolated lignins were addressed by pyrolysis in the presence of tetramethylammonium hydroxide (TMAH). Py/TMAH of the cortex and pith released high amounts (over 45% of the total peak area) of the methylated derivative of *p*-coumaric acid. The relative abundances of *p*-hydroxycinnamates present in the cortex and pith and in their MWLs, were estimated by Py/TMAH, and revealed additional features. Both, *p*-coumarate and ferulate were found in the whole cell-walls of the cortex and pith, while only *p*-coumarate was found in the isolated MWLs. This indicates that ferulate is mostly attached to the carbohydrates while *p*-coumarate is primarily attached to the lignin polymer.

The whole cell walls of the elephant grass cortex and pith fractions were analyzed in situ by gel-state 2D-NMR, and the spectra were compared with those from the MWLs isolated from the same samples (**Figure 1**). The spectra showed prominent signals corresponding to β–O–4 alkyl-aryl ether linkages **A**, although other substructures were also observed in lower amounts, including phenylcoumarans (**B**), resinols (**C**) and spirodienones (**D**). Other small signals in the side-chain region of the HSQC spectra corresponded to c innamyl alcohol end-groups (**I**) and γ-acylated cinnamyl alcohol end-groups (**I'**), and α-keto-β–O–4-substructures (**E**). In addition, the spectra clearly showed the presence of intense signals corresponding to γ-acylated units (including structure **A'**), together with the presence of signals from normally hydroxylated γ-carbons in β–O–4-units **A**. The percentage of γ-acylation of the lignin side-chain ranged from 39% in the cortex to 55% in the pith lignin. In the aromatic region, signals from *p*-hydroxycinnamyl (**H**), guaiacyl (**G**) and syringyl (**S**) units were observed. In addition, prominent signals corresponding to *p*-coumarate (**PCA**) and ferulates (**FA**) were observed in the spectra.

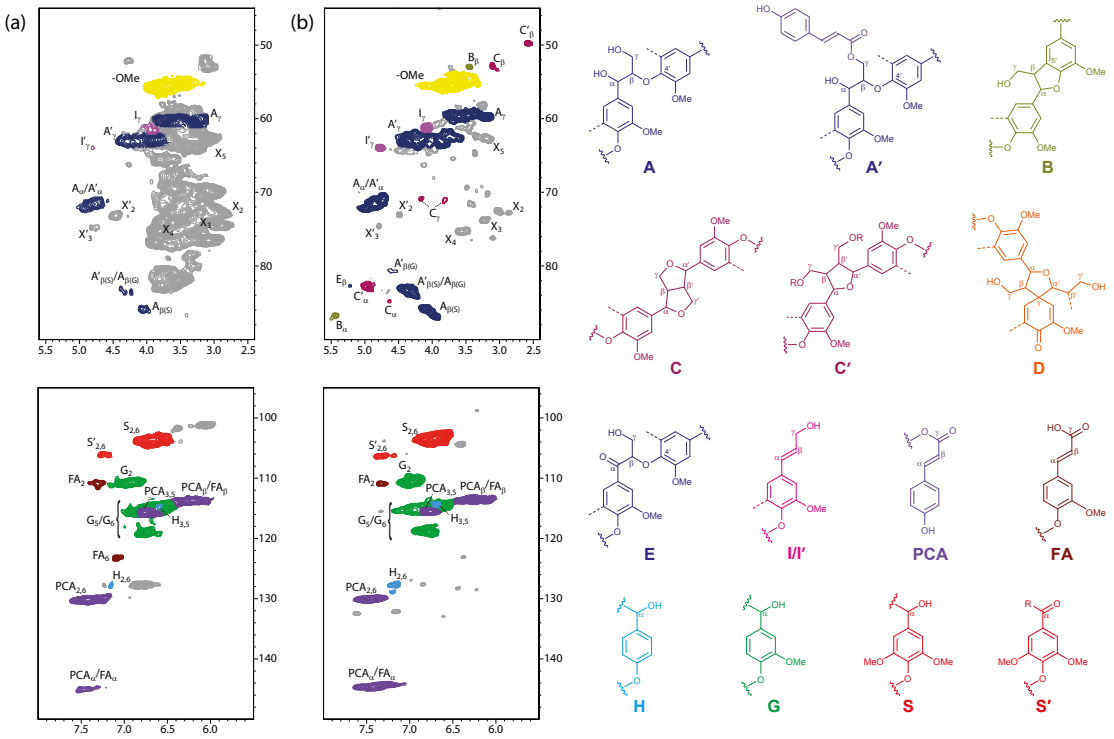

Figure 1. Side-chain (δ_C/δ_H 45-90/2.40-5.60) and aromatic (δ_C/δ_H 95-150/5.50-8.00) regions in the 2D HSQC NMR spectra of elephant grass (*P. purpureum*) cortex (a) and its isolated MWL (b).

The molar composition of different lignin units (H, G, S) and *p*-hydroxycinnamates (*p*-coumarate, ferulate) in the cortex and pith, as well as in their isolated MWLs, is reflected in **Table 1**. The data indicated a similar lignin composition in the cortex and pith, with similar S/G ratio around 1.3. On the other hand, *p*-coumarate is highly abundant in the whole cell-walls of the cortex and pith, as well as in their isolated MWLs, while the abundance of ferulate is much lower in the isolated MWL. This fact indicates that ferulate is mostly or entirely attached to the carbohydrates, while *p*-coumarate is predominantly attached to the lignin moiety, in agreement with Py-GC/MS results.

Table 1. Structural characteristics (lignin inter-unit linkages, end-groups, percentage of γ-acylation, relative molar composition of the lignin aromatic units, S/G ratio and *p*-coumarate/ferulate ratio) from the HSQC spectra of whole cell-walls of cortex and pith of elephant grass (*P. purpureum*) and their isolated MWLs.

	Cortex	MWL cortex	Pith	MWL pith
Lignin inter-unit linkages (%)				
β–O–4′ substructures (**A/A'**)	-	82	-	82
β–5′ phenylcoumaran substructures (**B**)	-	8	-	7
β–β′ resinol substructures (**C**)	-	2	-	1
β–β′ tetrahydrofuran substructures (**C′**)	-	3	-	5
β–1′ spirodienone substructures		2	-	2
α-oxidized β–O–4′ substructures(**E**)	-	2	-	3
Lignin end-groups				
cinnamyl alcohol end-groups (**I**)	-	6	-	7
γ-acylated cinnamyl alcohol end-groups (**I'**)	-	3	-	4
Lignin side-chain γ-acylation (%)	-	39	-	55
Lignin aromatic units				
H (%)	0	3	3	3
G (%)	44	40	42	39
S (%)	56	57	55	58
S/G ratio	1.3	1.4	1.3	1.5
p-Hydroxycinnamates				
p-coumarates (%)	26	29	39	40
ferulates (%)	11	3	16	4
p-coumarates/ferulates ratio	2.4	9.7	2.4	10.0

In both MWLs, the main lignin substructure present is the β–O–4 aryl ether, that accounts for up to 82% of all inter-unit linkages in the cortex and in the pith, while condensed linkages (β–β resinols, β–5 phenylcoumarans, β–1 spirodienones) are present in minor amounts. In particular, there is a strikingly low proportion of resinol structures, which account for only 2% of inter-unit linkages in the cortex and only 1% in the pith lignin. Interestingly, tetrahydrofuran structures C' formed by β–β-homo-coupling of two γ-acylated monolignols are also present in these lignins, being especially abundant in the pith (5% of all inter-unit linkages). The presence of these tetrahydrofuran substructures in the lignin is indicative of the occurrence of *p*-coumaroylated monolignol conjugates that participate in coupling and cross-coupling reactions during lignification. Further analysis by DFRC confirmed that *p*-coumarate groups are attached to the γ-carbon of these lignins, and predominantly on syringyl units.

IV. CONCLUSIONS

The analyses of the lignins from the cortex and pith of elephant grass indicate that they have a typical G:S lignin, with low amounts (~3%) of H-units, and a S/G ratio of 1.3-1.5. The analyses also indicate the presence of high amounts of *p*-coumarate groups which acylate the γ-OH of the lignin side-chains, and preferentially on syringyl units. The main inter-unit linkage present is the β–O–4 alkyl aryl ether (82% of all inter-unit linkages), with lower amounts of condensed linkages: resinols and tetrahydrofurans (β–β), phenylcoumarans (β-5), and spirodienones (β-1). The presence of a tetrahydrofuran structure formed from the β–β-homo-coupling of two γ-acylated monolignols, presumably two γ-p-coumaroylated sinapyl alcohols, was observed in significant amounts, being especially abundant in the pith (5% of all inter-unit linkages).

V. ACKNOWLEDGEMENT

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