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THE STRUCTURAL CHANGES OF LIGNIN AT DIFFERENT STAGES OF GROWTH OF NAPIER GRASS

The aim of this study was to investigate the lignin composition and structure during Napier grass growth. Napier grass showed an increase in lignin, xylan, glucan content and a decrease in acetone extractives and ash content with its growth, while the content of cellulose and holocellulose reached the highest value after four months growth. In addition, milled-wood lignins (MWL) isolated from Napier grass were characterized with 2D-NMR and Py-GC-MS. The results obtained showed that GSH-type substructures were included in Napier grass lignin, whereas the S-G ratio decreased and the abundance of H-substructures increased during lignification. H-units were deposited at the earlier stages, whereas Napier grass was enriched in G-lignin during the late lignification, and S-units were reduced at the final stage. The differences in deposition of lignin units influenced the distribution of the different linkages of lignin units during Napier grass growth. All lignin samples showed the most abundant β -O-4', β - β' resinol linkages, whereas the data obtained by 2D-NMR indicated the similar development in GSH-type substructures with by Py-GC-MS during all stages of lignification.

Keywords: Napier grass, composition, lignin, structure, lignification

Introduction

Lignin is the main component in secondary cell walls. It exists in wood tissue and hardens the cell wall through the formation of a network. Lignin is mainly located between cellulose fibres, playing a role in protecting plants against pressure. Woody plants contain 25% of lignin, which is the second most abundant organic matter in the world. The content and composition of lignin in plants are different varying with plant species, cell type, cell layer, and growth conditions [Buxton and Russell 1988; Manoj et al. 2016].

Lignin is a complex polymer synthesized mainly by the coupling of aromatic alcohols (p-coumaryl, coniferyl alcohol and sinapyl alcohol). These monolignols

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give rise to three types of lignin units by polymerization called p-hydroxyphenyl [H], guaiacyl (G), and syringyl (S) units, respectively. Gymnosperms contain almost exclusively G type lignin and dicotyledonous plants, mainly contain G-S type lignin while monocotyledon plants contain G-S-H type lignin [Martínez et al. 2008; Guerriero et al. 2016]. The lignin monomers are dehydrogenated under the action of peroxidase/H₂O₂ or laccase/O₂ formed in the cell wall, forming phenolic free radicals and their resonance forms. These radicals are combined with each other, or added by an H₂O or a lignin monomer to condense a dimer. These dimers are then condensed again and again with H₂O or lignin monomer by free radical coupling mechanisms generating a series of linkages to form a complex polymer. Lignin was hence deposited at different stages of xylem cell differentiation through polymerization of monolignols. Lignin composition and structure will be changing in timing of deposition during lignification [Gang et al. 1999; Ralph et al. 2004; Barsberg et al. 2006].

Napier grass (*Pennisetum purpureum*) is an important perennial herbaceous plant in (sub)tropical geographies of the world and it has gained an increasing interest in several applications including papermaking, bioenergy, and ecology. Lignin content in Napier grass was slightly lower than that of reed (21%), rice straw (22%), wheat straw (23%), but higher than sorghum stalk (13%) [Gençer and Şahin 2015]. Due to the high calorific value, biomass productivity, a number of studies on Napier grass have already been carried out in many fields [Ashori 2006; Nadji et al. 2009; Basso et al. 2014; Devin and Khanal 2015; Jiao et al. 2016]. It has been found lignin composition and structure greatly affects its reactions [Grabber 2005; Holopainen-Mantila et al. 2013]. Therefore, it is important to elucidate the lignin composition and structure of the Napier grass by a combination of 2D-NMR and Py-GC-MS over its natural course of growth for pulp, chemical, or biofuel production in this study.

Materials and methods

Samples

Napier grass samples were collected at different stages of growth corresponding to two, four, and eight months, from an experimental field in Kunming, Yunnan Province. The stalks were cut into small piece and then dried in an oven at 60°C for 15 h, and ground to pass through a 1 mm sieve. The powders were then Soxhlet-extracted with acetone to remove the extracts. The sample was dried in an oven at 60°C for 15 h and then stored in a vacuum dryer.

Chemical composition

The chemical composition of Napier grass was determined: ash (TAPPI T211 om-02), extractives (TAPPI T204 cm-07); lignin (klason lignin and acid-soluble lignin) (TAPPI T222 om-11, UM 205 om-83). The carbohydrate analysis was

performed on a Dionex ICS-3000 High Pressure Ion Chromatography using the hydrolysate from the lignin analysis [Blumenkrantz and Asboe-Hansen 1973].

Lignin isolation

Lignin was isolated from Napier grass according to the Björkman procedure [Björkman 1956]. Powders (10 g) were ball-milled in a ceramic ball mill at room temperature (70 r/min) for 3 days. The ball-milled powders were treated with aqueous *dioxane* containing 0.05mol/l HCl (*dioxane*/water, 80/20, v/v) under a nitrogen environment at 85°C for 4 h. The mixture was filtered to obtain the filtrate and the precipitate. The filtrate was neutralized with 1 mol/L NaOH to pH 5.5, vacuum-distilled, precipitated by adding 95% ethanol. The filtrate was *filtered* from the *solution*, vacuum-distilled, then neutralized with 6 mol/LHCl to pH 2.0 to obtain the precipitate. The precipitate was washed with acidic water (pH 2.0) to obtain the lignin sample. The lignin sample was freeze-dried overnight, and then stored at 5°C.

Results and discussion

Chemical composition at different stages of growth

The contents of the main components (i.e. acetone extractives, cellulose, holocellulose, lignin, ash, xylan, glucan) in Napier grass at different stages of growth are listed in table 1. The lignin content was found to increase in the growing seasons and reached its maximal value after 8 months, whereas Napier grass showed a decrease in the content of acetone extractives and ash with its growth. The amount of cellulose, holocellulose has the highest content (36.34%, 59.13%) after 4 months, and showed a decrease 8 months later, while that of xylan, glucan showed a progressive increase in the growth period.

Table 1. Chemical composition of Napier grass at different stages of growth

Constituent	Content [% o.d. mass]	
	2 months	4 months
Acetone extractives	15.93	13.51
Cellulose	32.06	36.34
Holocellulose	37.63	59.13
Klason lignin	12.03	14.47
Acid-soluble lignin	2.03	2.81
Total lignin	14.06	17.28
Ash	14.03	9.27
Xylan	16.46	18.69
Glucan	34.65	37.86

Py-GC/MS of lignin at different stages of growth

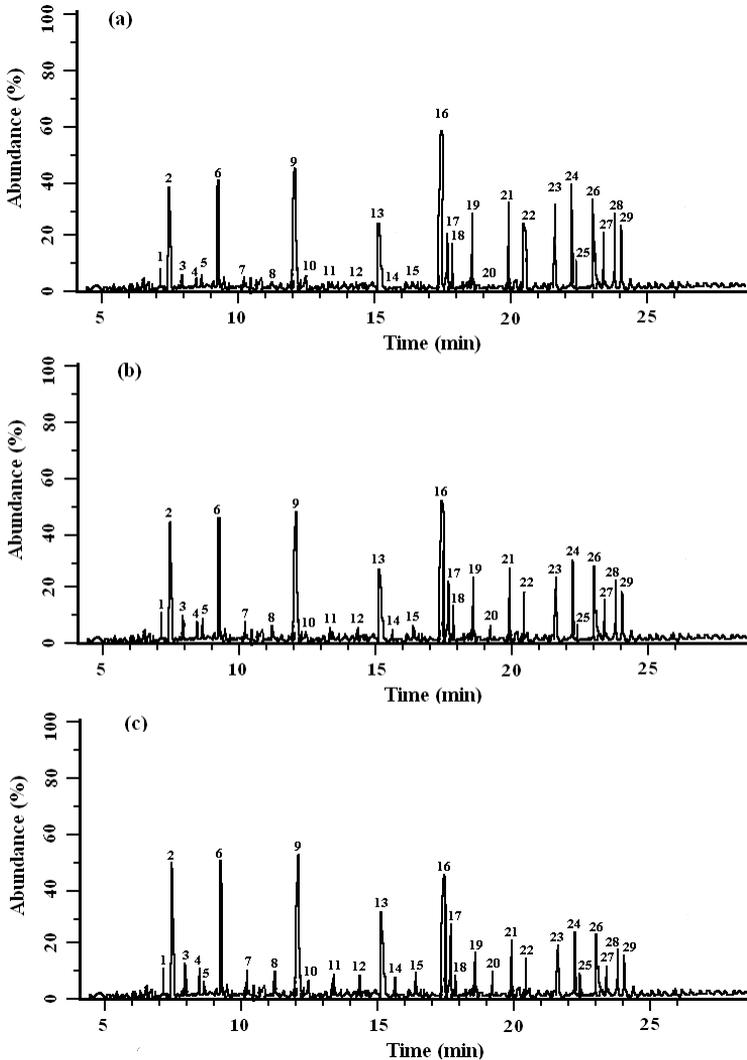


Fig. 1. Py-GC-MS chromatogram of Napier grass lignin at stage of (a) 2 months, (b) 4 months, and (c) 8 months

Py-GC-MS has been widely used to explore the composition of lignin in plants [Rodrigues et al. 1999; Rencoret et al. 2007; Chen et al. 2015]. Py-GC-MS chromatograms of lignin samples at different growth stages are shown in figure 1, and the compounds and their relative molar abundances are listed in table 2. Among the compounds as identified by Py-GC-MS, S- and G-lignin units were included, such as 4-vinylsyringol (no. 16), propylsyringol (no. 18), allylsyringol (no. 19), sinapaldehyde (no. 21), acetosyringone (no. 24), syringylacetone (no. 26), sinapyl alcohol (no. 28), syringaldehyde (no. 29),

guaiacol (no. 2), 4-methylguaiacol (no. 6), 4-vinylguaiacol (no. 9), vanillin (no. 13), vanillic acid (no. 15). Moreover, some compounds were also available in *considerable amounts, which were* derived from H lignin units, such as phenol (no. 1), 2-methylphenol (no. 3), 4-methylphenol (no. 4), 4-ethylphenol (no. 5), 2,4-dimethylphenol (no. 7) [Ralph and Hatfield 1991; Abdulkhani et al. 2010; Laskar et al. 2012].

Table 2 Relative molar abundance of the lignin-derived units of Napier grass lignin at the different growth stages

No.	Compounds	Relative molar abundance [%]	
		2 months	4 months
1	Phenol	4.84	6.27
2	Guaiacol	6.57	7.72
3	2-Methylphenol	1.22	2.29
4	4-Methylphenol	1.32	2.46
5	4-Ethylphenol	1.54	2.58
6	4-Methylguaiacol	6.28	6.82
7	2,4-Dimethylphenol	1.76	3.07
8	4-Ethylguaiacol	1.47	1.83
9	4-Vinylguaiacol	7.70	8.47
10	Eugenol	0.38	0.33
11	Propylguaiacol	0.36	0.47
12	Isoeugenol	0.64	1.24
13	Vanillin	3.55	3.62
14	Homovanillin	0.36	0.61
15	Vanillic acid	0.53	0.83
16	4-Vinylsyringol	12.47	9.33
17	Guaiacylacetone	1.46	1.82
18	Propylsyringol	3.68	2.17
19	Allylsyringol	4.72	3.18
20	Dihydroconiferyl alcohol	0.77	1.34
21	Sinapaldehyde	6.98	6.36
22	Homosyringaldehyde	2.47	1.77
23	Syringic acid	3.74	2.65
24	Acetosyringone	5.38	4.67
25	Coniferaldehyde	1.66	1.75
26	Syringylacetone	5.37	4.84
27	Dihydrosinapyl alcohol	3.15	2.87
28	Sinapyl alcohol	5.06	4.72
29	Syringaldehyde	4.57	3.92
Total G		31.73	36.85
Total S		57.59	46.48
Total H		10.68	16.67

GSH type structures were found in all lignins at the different growth stages, according to the relative molar abundance of the lignin-derived units, as shown in table 2. At all growth stages, the abundance of S-type units decreased and that of G-type units increased with the growing seasons, with an S-G ratio ranging from 1.81 in the 2 months' lignin to 0.84 in the 8 months' lignin. Contrarily, an increase in lignin S-G ratio was reported in lignin as kenaf growing [Mazumder et al. 2005]. This difference could be due to the variation in monolignol deposition in the timing of growth [Lee et al. 2013; Yuree et al. 2013]. Besides, the S-G ratio of 8 months' lignin was similar to what happened with cardoon lignin [0.79] [Lourenço et al. 2015].

The abundance of H-type compounds increased from 10.68% to 17.08% during growth. H-units were deposited, followed by G-units, and S-units were then reduced at the final stage, which was opposite to Rencoret's findings obtained in young versus adult eucalyptus lignin [Rencoret et al. 2011].

HSQC-NMR of lignin at different stages of growth

The lignin samples were also characterized using 2D-NMR (fig. 2). The relative abundance of the substructures found in the different lignin samples are listed in table 3. All lignin samples showed distinct β -O-4' structures (A), as shown in figure 2, which was an important linkage in lignin structure. Moreover, other substructures such as resinol (B), phenylcoumaran (C) and spirodienone (D) were also found in all the spectra.

Table 3. Relative abundance of the lignin side-chains involved in different linkages from Napier grass lignin at the different growth stages

Linkages	Relative abundance [%]	
	2 months	4 months
β -O-4' aryl ether (A)	67.70	69.27
Resinol (B)	21.26	20.05
Phenylcoumaran (C)	2.49	3.26
Spirodienones (D)	1.27	1.68
β -1' structures (E)	1.76	1.25
β -O-4' oxidized at C _{α} (F)	1.26	2.13
Cinnamyl alcohol end-groups (I)	4.26	2.36
G	30.28	37.01
S	60.26	47.16
H	9.46	15.83

The data in table 3 indicated the same trend in change with the data found by Py-GC/MS with respect to the H-, G-, S-substructures. All lignins contained the most abundance of β -O-4', β - β' resinol linkage in the substructures. The lower

amounts of units such as phenylcoumaran (C) (2.49-4.47%), spirodienones (D) (1.27-2.34%), β -1' structures (E) (1.06-1.76%), cinnamyl alcohol end-groups (I) (1.73-4.26%) were observed in the lignins in a range from 1-5% during growth.

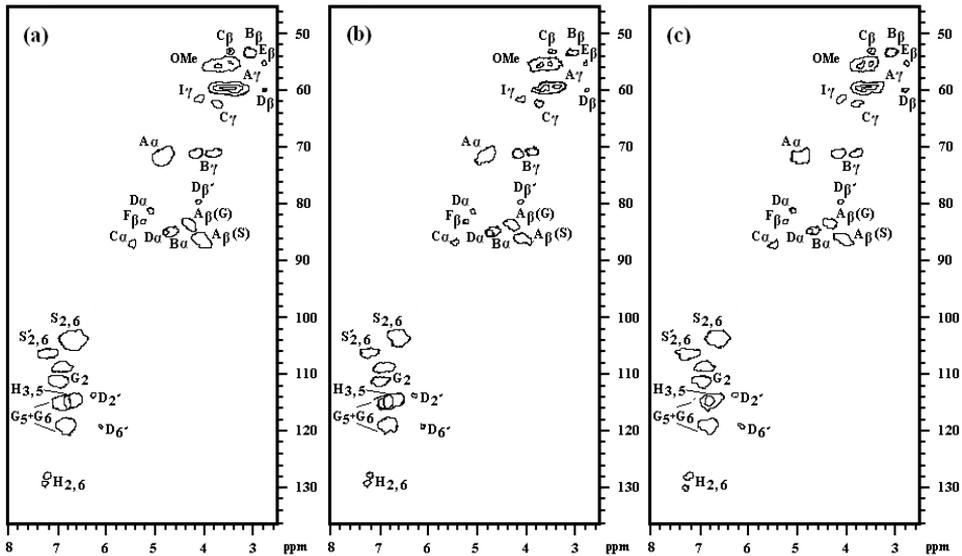


Fig. 2. HSQC NMR spectra of of Napier grass lignin at stage of (a) 2 months, (b) 4 months, and (c) 8 months. (A) β -O-4'; (B) resinol (β - β'); (C) phenylcoumaran (β -5'); (D) spirodienone (β -1'/ α -O- α'); (E) β -1'; (F) C α -oxidized β -O-4'; (G) guaiacyl units; (H) *p*-hydroxyphenyl units; (I) *p*-hydroxycinnamyl alcohol end-groups; (S) syringyl units; and (S') oxidized syringyl units containing a carbonyl group at C α

In addition, the content of phenylcoumaran units increased, which indicated an increase of G-units with growth. With respect to the change in G-, S-types, the abundance of β -1' structures decreased while the abundance of spirodienones increased with lignification. A small number of substructures were found to be oxidized during growth. β -O-4' linkages oxidized at C α (F) and occurred during lignification of Napier grass at the different growth stages.

Conclusions

The change in compositional content and lignin structure in Napier grass at different stages of growth were studied. The compositional content changed during lignification of Napier grass. The H-monolignols were first deposited, then the G-units, and finally the S-units with growth. The inter-unit linkages in the lignin structure were hence distributed following the deposition order of monolignols. Phenylcoumaran structures increased, which resulted in the increase of the G-units during lignification. Simultaneously, the decrease of β - β'

resinol units derived from S-structures gave rise to the decrease of S-structures with lignification.

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