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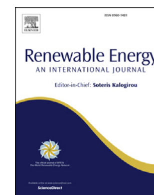

Lignin plays a key role in determining biomass recalcitrance in forage grasses

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A B S T R A C T

Lignocellulosic biomass is an abundant renewable feedstock, rich in polysaccharides that are covalently linked with lignin. In this study, biomass composition of nine forage grasses revealed the role of lignin in biomass recalcitrance. We determined the profiles of cell wall-bound phenolics, lignin, monosaccharides, enzymatic saccharification, and the chemical fingerprints using Fourier transform infrared (FTIR) and Raman spectroscopies. Coastcross and Tifton 85, both bermuda grass cultivars, showed lower lignin content and higher saccharification at 2 h and 72 h of enzymatic hydrolysis, supporting their use as valuable sources of carbohydrates for ethanol production. Principal component analysis (PCA) of thirteen different cell wall traits revealed that lignin was a hierarchical factor in reduced saccharification of forage grasses. As such, lignin content could be used as a marker for the selection of grass cultivars for genetic engineering programs for improved sustainable biofuel production.

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1. Introduction

Lignocellulosic biomass from forest residues, agro-waste and energy grasses is extensively exploited for bioenergy production [1,2]. In terms of potential, forage grasses have been considered as renewable sources for energy applications due to their high annual biomass yields, reduced levels of sulfur, disease resistance, relative economic advantages, low fertilizer requirement, and ability to grow in a wide range of soil and environmental conditions [3–5]. The high productivity of these grasses is mainly to their C4 photosynthetic metabolism, which also provides for nitrogen use efficiency [6]. The use of forage grasses for renewable energy is emerging as an alternative to reduce CO_2 emissions as society attempts to transition from fossil fuels to sustainable energy sources [7,8].

Miscanthus spp. and switchgrass have been widely used in Europe and the United States as dedicated bioenergy crops due to their high biomass yields [4]. Bermuda grass (*Cynodon dactylon*), found in the southern United States, where it is a cheap feedstock used for nutrient management in animal farms and it can be considered a promising biomass feedstock for bioethanol production [9]. In Brazil, India and China, sugarcane (*Saccharum* spp.) has been efficiently used for bioethanol production, and some forage grasses have already been characterized and evaluated as potential biomass sources [10–12]. Brazil has a large cultivated pasture area, of about 174 million hectares [13], where *Urochloa brizantha* (previously *Brachiaria brizantha*) occupies around 50% of the these total area, followed by *U. decumbens* (35%), *Panicum maximum* (10%) and *Pennisetum purpureum*, which have been extensively used for animal feeding [14].

Grass cell walls are mainly composed mainly of cellulose,

Abbreviations: AIR, alcohol insoluble residue; CW, cell wall; DM, dry matter; FA, ferulic acid; FTIR, Fourier transform infrared spectroscopy; *p*CA, *p*-coumaric acid; PCA, Principal component analysis; *p*HBald, *p*-hydroxybenzaldehyde; VAN, vanillin.

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hemicelluloses, lignin, phenolic compounds, and low amounts of pectin. The hemicellulose of grasses is composed primarily of xylan and β-glucan. The xylan backbone consists of a linear chain of β-(1,4)-D-xylosyl residues (Xyl*p*) and makes up between 20% and 35% of the total cell wall [15]. Arabinofuranose residues (Ara*f*) may be α-(1,2)- or α-(1,3)-linked to the xylan backbone forming arabinoxylan (AX), which may be further substituted with ferulic (FA) or *p*-coumaric acid residues (*p*CA) [16]. Both FA and *p*CA have a carboxylic group at the end of their propenyl group, providing the ability to esterify hemicelluloses [17]. FA ester-linked to AX can polymerize cross-linking vicinal FA-AX residues or lignin, connecting cell wall polymers. As a result, FA performs the key roles in cell metabolism, cessation of cell growth, anchoring lignin to cell wall polysaccharides, restricting the access of plant pathogens and lignocellulose degradation [18–20].

Lignin is a heterogeneous and complex polymer synthesized by the oxidative radical coupling of lignin monomers, mainly the three canonical monolignols: *p*-coumaryl, coniferyl and sinapyl alcohol, which differ in their degree of methoxylation [21]. Lignin polymer reinforces and waterproofs plant cell walls, occluding the cellulose microfibrils and protecting it physically from enzymatic degradation [22]. Apart from its role for plant development, lignin is also a barrier to efficient biomass saccharification, receiving significant attention in the biofuels field with regard for improving the efficient conversion of biomass. Due to the complex structure of lignocellulosic substrates, its hydrolysis is considered the rate-limiting step for the production of liquid biofuels [23]. However, pretreatments of lignocellulosic biomass can be used to remove of most lignin fraction leading the hydrolysis of polysaccharide fractions much more efficient [24]. Unravelling the influence of lignin on digestibility of grasses contributes to emerging a possible model to explain how it is associated with biomass digestibility. Genetic manipulation of lignin biosynthesis in plants with naturally lower lignin content is a potential approach to engineering crops that match the industrial requirements for cellulosic ethanol and biorefineries [17,22,25].

Differently from sugarcane, maize and *Miscanthus*, few comparative studies were conducted to evaluate the characteristics of forage grasses and their utilization as biomaterials and biofuels [11,26–29]. In this context, the primary focus of this study was to gain a better understanding of the influence of lignin on the biomass recalcitrance of different forage grasses. Characterization of biomass before and after alkaline pretreatment was performed to determine the profile of cell wall polymers and enzymatic saccharification, increasing the range of potential feedstocks for Brazilian and tropical bioethanol production. In addition, the chemical fingerprints of the biomasses were characterized using Fourier transform infrared spectroscopy (FTIR) and Raman spectroscopies. Lignin played a key role in the biomass recalcitrance of forage grasses, and its content was a marker for the selection of potential grasses for cellulosic ethanol production.

2. Material and methods

2.1. Plant material

Nine forage grasses were evaluated in this study, consisting of two cultivars of bermuda grass (*C. dactylon* cvs. Tifton 85 and Coastcross), two cultivars of guinea grass (*P. maximum* cvs. Mombaza and Tanzania), two cultivars of elephant grass (*P. purpureum* cvs. Napier and Pioneiro), one cultivar of *Urochloa decumbens* (previously *Brachiaria decumbens* cv. Basilisk) and two cultivars of *U. brizantha* (cvs. Marandu and Piata). Leaves with stalks were harvested at the vegetative stage with 4-month-old plants, from the experimental station of the State University of Maringá, Brazil (23° 25′ S, 51° 57′ W, 550 m above sea level). The samples were dried (60 °C for 48 h), ball-milled to a fine powder and stored in plastic boxes at 4 °C.

2.2. Cell wall preparation and determination of extractives

Dry matter (500 mg) of each sample was subjected to three consecutive extractions with 20 mL of 80% (*v/v*) ethanol at 55 °C for 4 h in a shaker at 200 rpm. Each extraction was followed by centrifugation (6750×*g*, 4 °C, 10 min) [30], with the supernatant being collected for the quantification of soluble extractives. The final alcohol insoluble residue (AIR) was washed with 5 mL acetone and dried at 60 °C for 24 h. The difference between the initial and final weight of dry matter was used to quantitate the soluble extractives fraction. The absorbance of aromatic compounds in soluble extract was measured at 280 nm, and the concentration was calculated as previously described [25]. Protein was determined by absorbance at 595 nm [31], using bovine serum albumin as a standard.

2.3. Profile of cell wall-bound phenolics

AIR samples (50 mg) were suspended in 2.5 mL of 0.5 M NaOH and incubated at 96 °C for 2 h. After centrifugation (2180×*g* at 4 °C for 15 min) the supernatant was acidified to pH 2.0 with 6 M HCl, partitioned twice with anhydrous ethyl ether and dried at 40 °C. The residue after evaporation was dissolved in methanol/4% acetic acid (30/70, *v/v*) and filtered through a 0.45-μm filter. Quantification of cell wall-bound phenolics was carried out on HPLC system (Shimadzu® Liquid Chromatograph, Tokyo, Japan), equipped with LC-10AD pump, CBM-101 Communications Bus Module, Rheodyne® injector, and SPD-10A UV-VIS detector. The compounds were separated at 40 °C on C18 column (250 mm × 4.6 mm, 5 μm; Supelco Discovery®) with equivalent pre-column (10 × 4.6 mm). The mobile phase was methanol/4% acetic acid (30/70, *v/v*), with a flow rate of 0.8 mL/min in isocratic mode. Absorption of FA, *p*CA, *p*-hydroxybenzaldehyde (*p*HBald) and vanillin (VAN) were detected at 322, 309, 280 and 280 nm, respectively, and quantified according to standard values. The results were expressed as mg/g AIR.

2.4. Alkaline pretreatment

AIR samples (200 mg) were pretreated in screw-capped glass tubes with 8 mL of 0.25 M NaOH at 130 °C for 40 min as previous described [32] with minor modifications. After cooling on ice, the samples were transferred to 15 mL centrifuge tubes and centrifuged at 4000×*g* for 10 min. The supernatant was discarded and the solid fraction was washed three times with 80% (*v/v*) ethanol following the centrifugation (4000×*g* for 10 min), until the pH of the mixtures was 6.0–7.0. Next, the alkaline insoluble biomass was dried at 60 °C for 24 h for the biochemical experiments.

2.5. Acetyl bromide soluble lignin

AIR samples (150 mg) were washed by successive stirring and centrifugation with 1% Triton X-100 (*v/v*) in 0.05 M potassium phosphate buffer pH 7.0 (four times), 1 M NaCl in buffer pH 7.0 (three times), distilled water (three times) and acetone (twice) [33] with minor modifications. The final pellets were dried at 60 °C for 24 h and then cooled in a vacuum desiccator. Total lignin content was determined using the acetyl bromide method [34]. Twenty mg of protein-free cell wall was placed in a screwcap centrifuge tube containing 0.5 mL freshly prepared acetyl bromide solution (25% acetyl bromide/glacial acetic acid, *v/v*) and incubated at 70 °C for 30 min. After complete digestion, the sample was ice-cooled and

then mixed with 0.9 mL of 2 M NaOH, 0.1 mL of 5 M hydroxylamine-HCl and 6 mL glacial acetic acid for complete solubilization of the lignin fraction. After centrifugation (1400×g, 5 min), the absorbance of the supernatant was measured at 280 nm. A standard curve was generated with alkali lignin (Sigma-Aldrich, St. Louis, MO, USA), and the results were expressed as mg lignin/g cell wall.

2.6. Monosaccharide profile

Non-cellulosic monosaccharide analysis was performed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Five mg of AIR was hydrolyzed with 1 mL of 2 M trifluoroacetic acid (TFA) for 1 h at 100 °C. The acid was evaporated under vacuum and the monosaccharides were resuspended in 1 mL ultra-purified water. Monosaccharide profiles were analyzed by HPAEC-PAD on CarboPac SA10 column (DX-500 system, Dionex®) using 99.2% water/0.8% 150 mM NaOH (*v/v*) as eluent at 1 mL/min. Monosaccharides were detected with a post-column addition of 500 mM NaOH (1 mL/min). Monosaccharide standards included fucose, rhamnose, arabinose, mannose, galactose, glucose, and xylose. In order to verify the response factors, a standard calibration was performed before analysis of each batch of samples.

2.7. FTIR and Raman spectroscopy

AIR samples (2 mg) were mixed with 200 mg potassium bromide, compressed into the pellets at a pressure of ~10 ton. The spectra were obtained with a Bruker Vertex 70 FTIR spectrometer equipped with an attenuated total reflectance accessory. The scanning ranged from 4000 to 400 cm^{-1}, with the resolution of 2 cm^{-1} and 128 scans per sample. Peak heights and areas of the FTIR spectra were determined using Opus software version 6.5 normalized by maximum and minimum peaks. Raman experiments were carried out with a MultiRAM FT-Raman Spectrometer (Bruker, Billerica, MA, USA). The resolution was set to 2 cm^{-1}, 256 scans were recorded for each analysis, the scanning ranged from 400 cm^{-1}–4000 cm^{-1}, and the laser power at the sample was 150 mW. A Nd:YAG laser was used for excitation at 1064 nm.

2.8. Production of fungal enzymes and activities

Aspergillus fumigatus var. *niveus* (previously *Aspergillus niveus*) was cultured in Petri dishes containing commercial potato dextrose agar (PDA, Sigma-Aldrich, USA) for five days at 37 °C. Spore suspensions from sporulated cultures were obtained by adding 5 mL of distilled water. Enzyme production was carried out in 125 mL Erlenmeyer flasks containing 25 mL of modified Czapek growth medium, pH 6.0, consisting of: 3 g/L $NaNO_3$, 1 g/L KH_2PO_4, 1 g/L $MgSO_4$.7 H_2O, 0.5 g/L KCl, 0.01 g/L $FeSO_4$.7 H_2O and 10 g/L sugarcane bagasse, as a carbon source. This medium was inoculated with the spore suspension (10^7 spores), and the flasks were incubated for five days at 37 °C, without agitation. Following incubation, the medium was vacuum-filtered using Whatman No. 1 filter paper with the crude filtrate being lyophilized and then suspended in 50 mM sodium acetate buffer pH 5.0 for enzymatic analysis.

Cellulase, xylanase, pectinase, arabinanase and mannanase activities were measured by determining levels of reducing sugars by colorimetric assay using carboxymethyl cellulose, xylan from beechwood, polygalacturonic acid sodium salt, linear arabinan and locust bean gum as substrates, respectively [35,36]. The reaction mixture consisted of 50 μL of enzyme solution and 50 μL of 1% substrate (*w/v*) in 50 mM sodium acetate buffer pH 5.0. The reaction was incubated at 50 °C for 30 min, and stopped by adding 100 μL of 3,5-dinitrosalicylic acid reagent (DNS) followed the immediate boiling for 5 min [37]. After cooling, the reducing sugars released by enzyme activity were estimated by measurement of absorbance at 540 nm. Sugar concentrations were interpolated from standard curves of glucose, xylose, galacturonic acid, arabinose and mannose for cellulase, xylanase, pectinase, arabinanase and mannanase activities, respectively. One unit of enzymatic activity (U) was defined as the amount of enzyme required of releasing 1 μmol of reducing sugars per minute, under the experimental conditions used.

Two mM solutions of synthetic substrates (*p*-nitrophenyl-α-L-arabinofuranoside, *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-xylopyranoside) were also used in the same assay conditions as the natural substrates [38]. The assays were stopped by adding 100 μL of 0.2 M sodium carbonate. Spectrophotometric readings were performed at 410 nm, using *p*-nitrophenol for a standard curve (0−0.6 μmol/mL). One unit of enzymatic activity (U) was defined as the amount of enzyme required of releasing 1 μmol of *p*-nitrophenol per minute, under the experimental conditions used.

2.9. Enzymatic hydrolysis

Reaction mixtures consisted of 15 mg of AIR, 20 U/mL of xylanase and 0.50 U/mL of cellulase from *A. fumigatus* var. *niveus* extract (see Table 1 for full details of units used in the enzymatic hydrolysis), 0.02% (*w/v*) sodium azide to inhibit microbial contamination, and 50 mM sodium acetate pH 5.0 in a final volume of 1 mL [30]. Mixtures were incubated at 50 °C and were sampled for analysis at 2 h and 72 h of hydrolysis. The supernatant from samples was collected by centrifugation (12,000×*g*, 5 min) and quantitation of the reducing sugars released was determined by the DNS method [37].

2.10. Data analysis

Data were expressed as the mean of five replicates ± standard error of the means (SEM). Analysis of variance (ANOVA) was performed to test the significance of the observed differences using the Sisvar software package (version 5.4, Universidade Federal de Lavras, MG, Brazil). Differences between parameters were evaluated by the Scott-Knott test, and P values ≤ 0.05 were considered statistically significant.

Principal component analysis (PCA) was performed to determine the distribution of forage grass cultivars in relation to biomass pretreatment and biomass composition. The variables measured were: ferulic acid (FA), *p*-coumaric acid (*p*CA), *p*-hydroxybenzaldehyde (*p*HBald), vanillin (VAN), lignin, saccharification at 72 h of hydrolysis, and cell wall monosaccharide composition (glucose, fucose, galactose, arabinose, xylose, rhamnose, and mannose levels). The synthetic variables were tested by the general linear model (GLM) to verify significant differences in relation to pretreatment, forage grass cultivar and interactions between both of these components ($P \leq 0.05$). These analyses were performed using Minitab-14.1 software.

3. Results and discussion

3.1. Ethanol-soluble extractives

The levels of compounds in ethanol-soluble extractives, aromatic compounds and soluble proteins differed markedly among cultivars (Table 2). We applied the Scott-Knott test for statistical analysis because it was able to analyze and organize well-defined groups without ambiguity, detecting small differences between the means. Yields of ethanol-soluble extractives of forage grasses of

Table 1
Ethanol-soluble extractives, aromatics and protein contents of forage grasses ranked by their lignin content. Mean values ± SEM ($n = 4$) marked with different letters are significantly different ($P \leq 0.05$, Scott-Knott test).

Sample	Total extractives (mg/g dry matter)	Compounds (mg/g extractive)	
		Aromatics	Soluble proteins
Tifton 85	147.79 ± 7.43^{a}	157.69 ± 12.55^{c}	28.13 ± 1.61^{b}
Coastcross	116.34 ± 6.81^{b}	213.70 ± 4.82^{b}	36.78 ± 3.72^{b}
Decumbens	153.01 ± 4.45^{a}	201.86 ± 12.90^{b}	32.79 ± 1.64^{b}
Marandu	150.06 ± 3.54^{a}	220.87 ± 8.04^{b}	36.92 ± 1.67^{b}
Piata	127.51 ± 6.72^{b}	231.70 ± 9.25^{b}	38.51 ± 3.05^{b}
Napier	142.50 ± 7.18^{a}	249.75 ± 16.03^{b}	47.26 ± 2.05^{a}
Pioneiro	110.75 ± 6.02^{b}	315.77 ± 24.64^{a}	50.20 ± 1.83^{a}
Mombaza	122.49 ± 4.55^{b}	213.55 ± 4.80^{b}	35.74 ± 3.61^{b}
Tanzania	125.77 ± 4.02^{b}	225.31 ± 13.14^{b}	31.17 ± 2.25^{b}

Table 2
Enzymatic activities from *Aspergillus fumigatus* var. *niveus* extract.

Enzymatic activity	Substrate	U/mL	U/mg protein*	U/mL applied in the saccharification
Xylanase	Xylan from beechwood	221.7	88.69	20.0
β-glucosidase	*p*-Nitrophenyl glucopyranoside	26.8	10.71	2.41
Arabinanase	Linear arabinan	12.1	4.85	1.10
Cellulase	Carboxymethyl cellulose	5.6	2.24	0.50
Mannanase	Locust bean gum	3.4	1.36	0.31
Arabinofuranosidase	*p*-Nitrophenyl arabinopiranoside	1.6	0.64	0.15
β -xylosidase	*p*-Nitrophenyl xylopyranoside	0.8	0.33	0.07
Laccase	ABTS	ND	ND	ND
Pectinase	Polygalacturonic acid	ND	ND	ND

ND, not detected. * Protein dosage: 2.5 mg protein/mL.

110.75 mg/g dry matter (DM) – 153.01 mg/g DM were similar to those obtained from wheat straw (129.5 mg/g DM) and switchgrass (138.0–169.9 mg/g DM) [1], and were higher than those for sugarcane bagasse (16–75 mg/g DM) [25].

To differentiate the classes of compounds present in the ethanol-soluble extractives, we evaluated the content of aromatic compounds and proteins. Proteins comprised <5% of the total extractives, whereas the aromatic fraction represented by 15–31% (Table 2). Cultivar Pioneiro showed the highest content of aromatics (315.77 mg/g extractive) and soluble proteins (50.20 mg/g extractive), whereas Tifton 85 presented the lowest content of aromatics (157.69 mg/g extractive) and proteins (28.13 mg/g extractive). In addition, there was no clear correlation between the pattern of compounds in the extractives, aromatics and proteins. The high amount of extracted compounds in biomasses of forage grasses suggests the potential use of proteins, carbohydrates, organic acids, and other organic compounds as agro-industrial by-products [39].

3.2. Profile of cell wall-bound phenolics

Hydroxycinnamic acids accounted for a significant fraction of phenolic compounds cross-linking cell wall polysaccharides in grasses (Fig. 1). We observed that FA varied between 1.77 and 3.36 mg/g AIR, with three distinct quantitative groups (Fig. 1A): the highest FA content, Coastcross < Decumbens < Tifton 85 < Piata; the intermediate FA content, Marandu < Napier < Pioneiro; and the lowest FA content, Mombaza < Tanzania. The *p*-coumaric acid (*p*CA) contents (3.29–4.21 mg/g AIR) were not significantly different between cultivars, but higher than the FA content (Fig. 1A). In grasses, FA is mostly attached to hemicelluloses, acylating the C5–OH of arabinosyl moieties in arabinoxylans (AXs), although small quantities of *p*CA can also acetylate AXs [16,40]. *p*CA predominates in *P. purpureum* stems [41] and sugarcane bagasse [25], whereas FA is the main hydroxycinnamic acid in *Phalaris aquatic, Lolium perenne* [42], and *Hordeum vulgare* [43].

It is important to note that FA ester-linked to the arabinosyl residue of AX can dimerize with another FA-AX, connecting adjacent AX chains. Inter-molecular crosslinking of AXs with lignin contribute to the recalcitrance of grass biomass leading the reduced enzymatic saccharification [44]. Studies have demonstrated that FA released from AIR by mild-alkali hydrolysis is mainly from the arabinosyl residue of AX. In fact, as monolignol ferulates are firmly established monomers in the lignification of monocots, such compounds could in principle also result from the lignin itself; however, as ferulates are incorporated into lignins by radical coupling reactions, form carbon-carbon or ether linkages, the amounts of FA released from lignin are extremely low and can be ignored here [45].

Forage grasses presented low contents (<0.28 mg/g AIR) of alkali-extractable *p*-hydroxybenzaldehyde (*p*HBald) and vanillin (VAN), although VAN content was higher than *p*HBald in all plants (Fig. 1B). These phenolic aldehydes have also been found in cell walls of wheat, rice, rye, barley straw [46] and wild rice (*Zizania aquatica*) [47]. Therefore, it is possible that these phenolic aldehydes are natural constituents of grass cell walls, and are linked through an alkali-labile bond to nitrogen bases of structural proteins, or esterified by their hydroxyl groups to uronic acids; however, their exact roles in the cell wall remain unclear [47].

3.3. Lignin profile

Several studies have demonstrated that the efficiency of enzymatic hydrolysis of untreated biomass is typically below that 35%, whereas chemical alterations in the biomass composition that has undergone pretreatments significantly improve the enzymatic hydrolysis [24,25,29]. To evaluate the relationship between lignin and saccharification, we measured lignin content using acetyl bromide method due to its simplicity, good reproducibility, and high recovery of lignin [34]. A previous study demonstrated the consistent negative correlation between lignin and saccharification in grasses [48].

Lignin in forage grasses varied from 223.48 mg/g cell wall (CW)

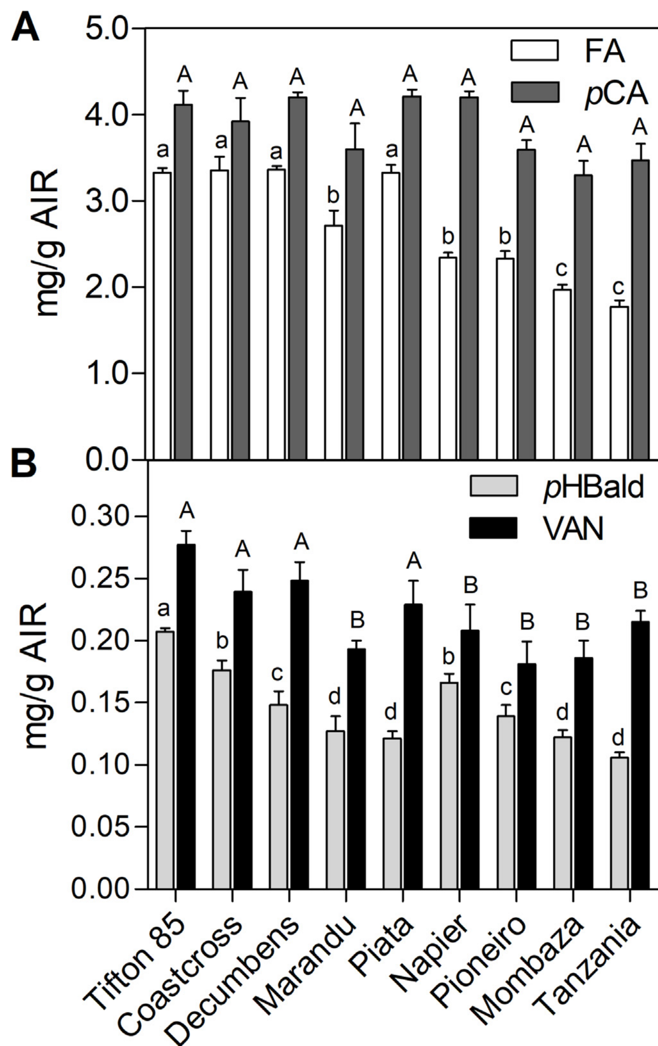

Fig. 1. Profiles of cell wall-bound phenolics of forage grasses. A) Ferulic acid (FA) and *p*-coumaric acid (*p*CA), B) *p*-hydroxybenzaldehyde (*p*HBald) and vanillin (VAN). Mean values ± SEM ($n = 5$) marked with different letters are significantly different ($P \leq 0.05$, Scott-Knott test).

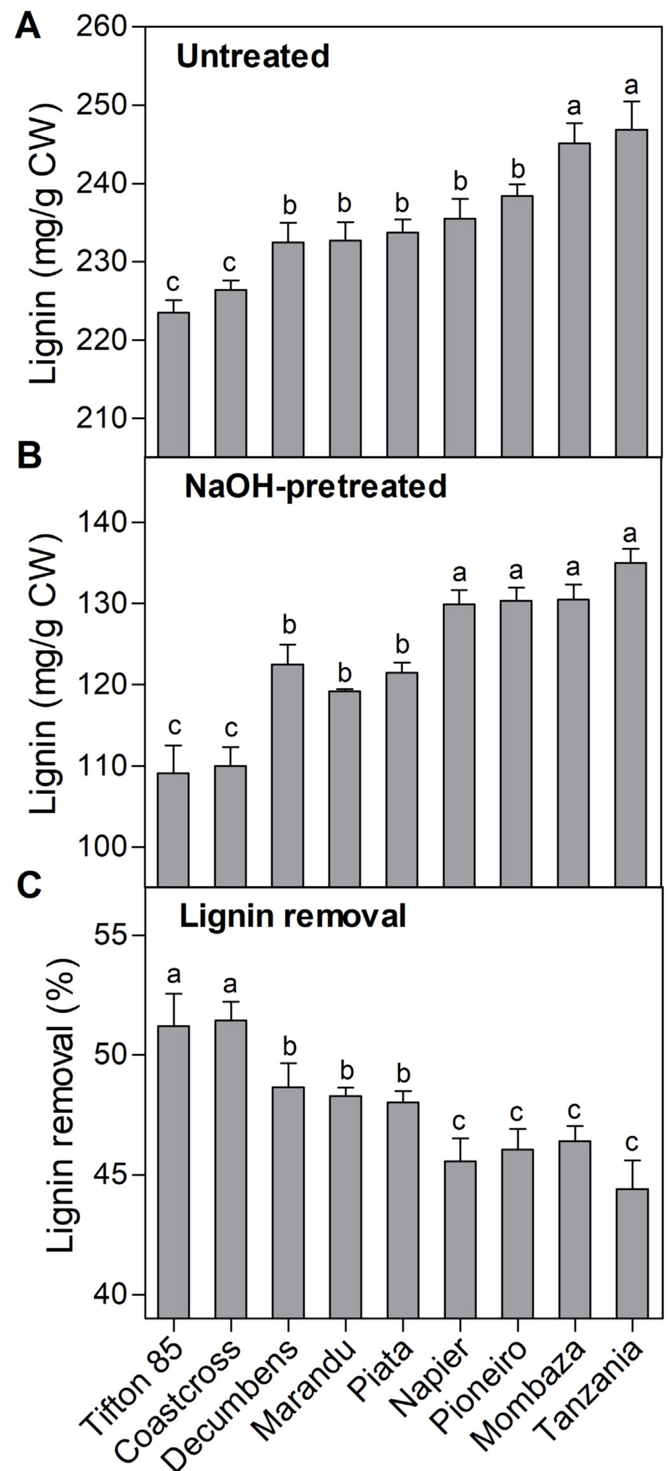

Fig. 2. Profiles of lignin content of forage grasses. A) Total lignin of untreated samples, B) NaOH-pretreated samples, and C) lignin removal. Mean values ± SEM ($n = 5$) marked with different letters are significantly different ($P \leq 0.05$, Scott-Knott test).

in Tifton 85–246.87 mg/g CW in Tanzania and was organized in three distinct groups according to its content: the lowest lignin content, Tifton 85 < Coastcross; the intermediate lignin content, Decumbens < Marandu < Piata < Napier < Pioneiro; and the highest lignin content, Mombaza < Tanzania. These findings were consistent with a previous study indicating that *P. maximum* (Mombaza and Tanzania) has higher lignin content, in comparison with *U. brizantha* and *P. purpureum* [10]. Lignin content is highly variable, not only between species, but also between tissues and cell types, cell wall layer, and between different developmental stage and stress conditions [49]. The advantage of discovering grasses with the lower lignin content is that there are now more diversity of plants to engineer their cell walls for biorefining applications [22].

To identify the relationship between the cultivars of forage grasses showing a range of compositional traits and sugar yields, we pretreated the biomasses with NaOH. Lignin content of NaOH-pretreated samples ranged from 109.97 to 135.00 mg/g CW (Fig. 2B). Notably, lignin profiles from both NaOH-pretreated and untreated samples were organized in three very closely groups by Scott-Knott test. To assess the effect of initial lignin content on pretreatment, we also calculated the percentage of lignin removal (Fig. 2C). Pretreatment removed 45–51% of lignin content from forage grasses, with a negative correlation between lignin content of untreated samples and lignin removal (Pearson correlation = −0.81, $P = 0.0079$). These findings suggested that biomass with lower lignin content, when submitted to pretreatment, presented greater lignin removal, due to lignification being a limiting

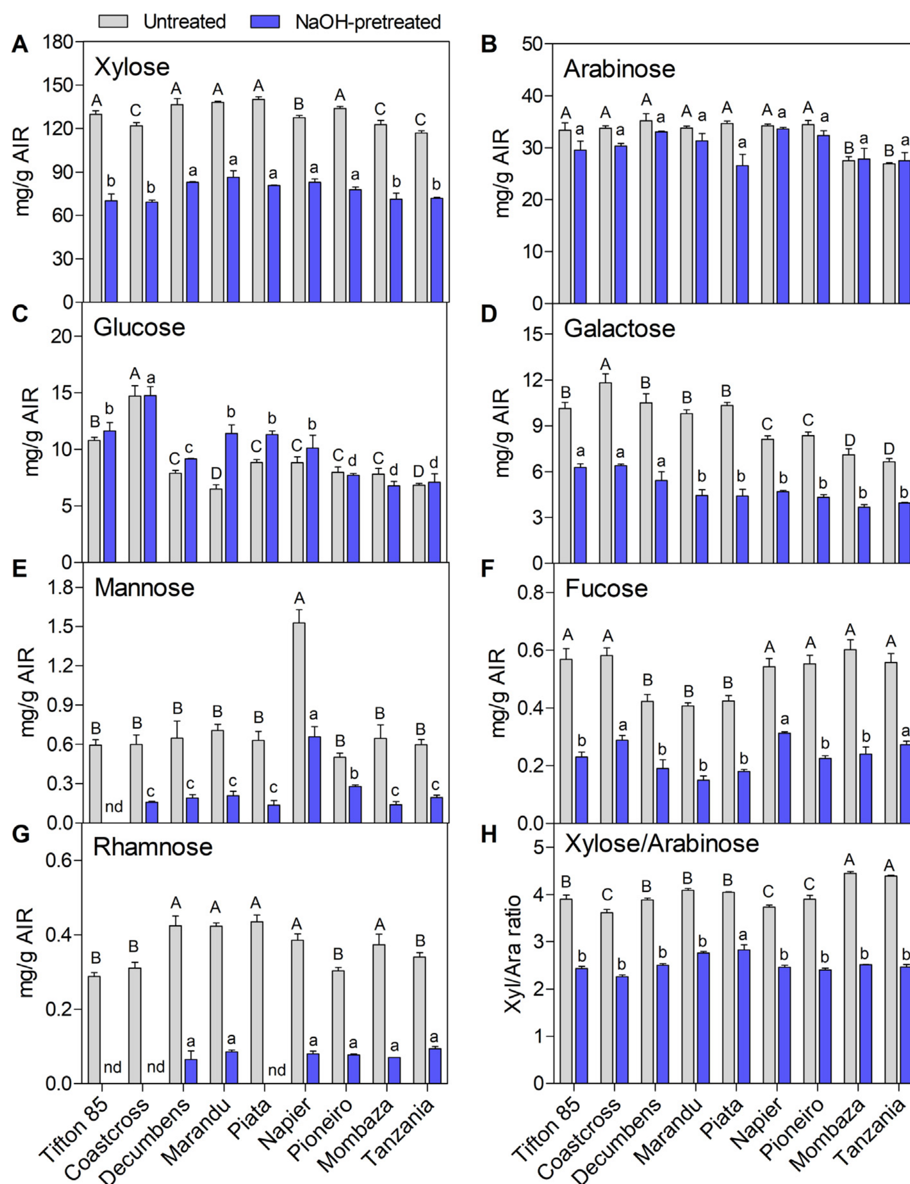

Fig. 3. Non-cellulosic monosaccharide profiles of forage samples ranked by their lignin content. Mean values ± SEM ($n = 3–4$). Uppercase letters indicate significant differences between untreated samples; lowercase letters indicate significant differences between pretreated samples ($P \leq 0.05$, Scott-Knott test). nd, not detected.

factor for the pretreatment efficiency. Alkali has been applied to the deacetylation of hemicellulose, partial removal of lignin, dissolution of low molar mass hemicelluloses, and fiber swelling, making lignocellulose more accessible to saccharification enzymes [25,50].

3.4. Monosaccharide profile

Untreated grasses showed similar monosaccharide profiles, composed mainly of xylose, arabinose, glucose and galactose, with

Table 3
Assignment of the main bands of spectroscopic analyses. F and R in parenthesis are bands identified and confirmed by FTIR and Raman, respectively, according to the Bekiaris et al. (2015) and Lupoi et al. (2015).

Vibration (cm^{-1})	Assignment	Biomass constituent
1735	Unconjugated C=O stretching	Xylan (F)
1633	C—C stretch of coniferaldehyde and sinapaldehyde	Lignin (F/R)
1600	Lignin aromatic skeletal vibrations	Lignin (R)
1510	Aryl ring stretch, asymmetric	Lignin (F)
1378	Symmetric C-H deformation and phenolic OH	Crystalline cellulose (F); lignin (R)
1272	Ring deformation, C—O stretching	Lignin (R)
1250	C—O stretching in lignin and xylan	Xylan (F)
1160	C-O-C asymmetric stretching	Crystalline cellulose (F)
1095	C—C and C—O stretching	Crystalline cellulose (R)
1053	C—C and C—O stretching	Crystalline cellulose (F/R)
898	C—O—C stretching	Amorphous cellulose (F/R)

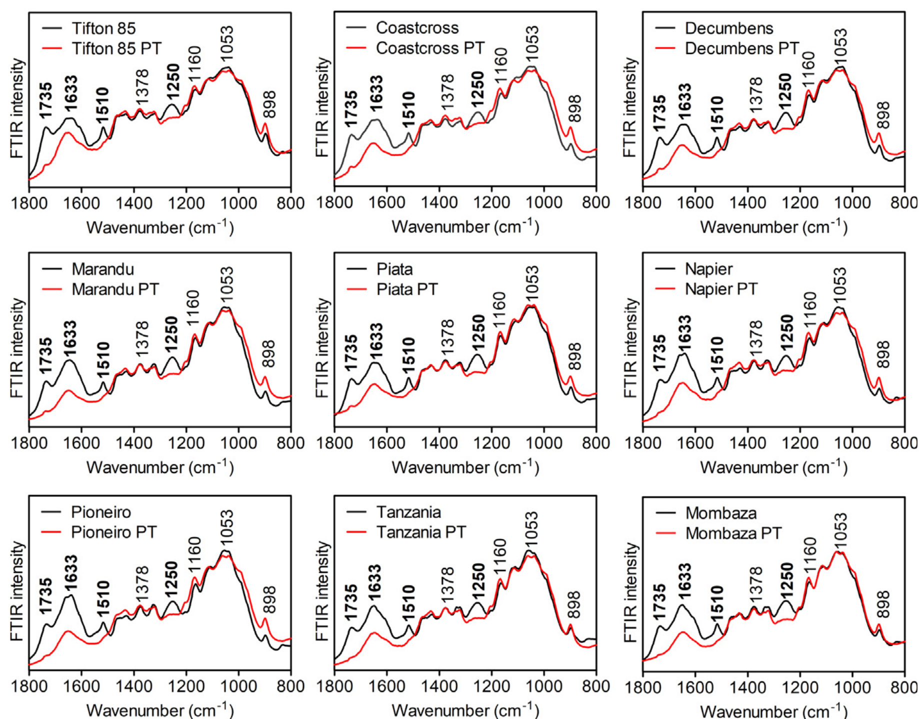

Fig. 4. FTIR spectra of untreated and NaOH-pretreated (PT) forage grasses. Differences between untreated and pretreated biomasses are presented in bold. Band at 1735 cm^{-1} corresponds to ester-groups of xylan; 1633, 1600, and 1510 cm^{-1} correspond to lignin; 1250 cm^{-1} corresponds to xylan; 1378, 1095, 1060, 1053, and 898 cm^{-1} correspond to cellulose.

small amounts of mannose, fucose and rhamnose (Fig. 3). Neutral monosaccharides released from hemicellulose and pectin fractions of untreated forage grasses showed high levels of xylose (116.94 mg/g AIR in Tanzania to 140.15 mg/g AIR in Piata), followed by arabinose, similar levels of glucose and galactose, and low levels of mannose, fucose and rhamnose (<1.5 mg/g AIR). The amount of xylose from Piata (140.15 mg/g AIR) and Marandu (138.06 mg/g AIR) was slightly higher in comparison with other forage grasses. Rhamnose (Fig. 3G), typically found in pectins, was lower than 0.43 mg/g AIR in all forage grasses, similar to contents previously

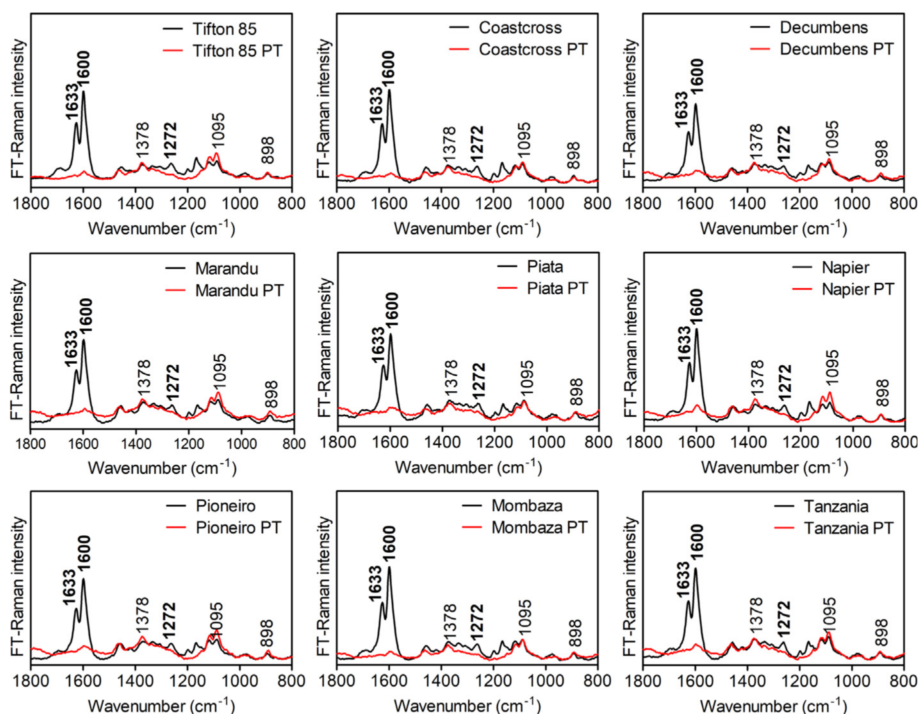

Fig. 5. Raman spectra of untreated and NaOH-pretreated (PT) forage grasses. Differences between untreated and pretreated biomasses are presented in bold. Bands at 1633, 1600, and 1272 correspond to lignin, 1095 and 898 cm^{-1} correspond to cellulose.

reported by Lima et al. (2014). These findings agree with the features of hemicelluloses of type II cell wall of grasses, with high levels of xylose and arabinose in xylans and AXs [15,16,29,51].

Next, we also evaluated the monosaccharide profile after alkaline pretreatment (Fig. 3 blue bars). The pretreatment drastically reduced xylose, galactose, mannose, fucose and rhamnose levels, indicating the effective removal of monosaccharides from hemicellulose and pectin fractions. In contrast, arabinose content was barely affected by pretreatment (Fig. 3B), suggesting that AX domains with different arabinose substitutions were distinctly affected by alkaline pretreatment. In *Miscanthus*, the degree of arabinose substitution of AXs is the main factor that positively affects biomass saccharification upon NaOH and H_2SO_4 pretreatments [52]. Genetic engineering of xylan biosynthesis to tailor its structure has been proposed as an approach for improving the production of biofuels and biorenewables [20].

Arabinosyl substitutions of grass xylans can vary from ratios of 2:1 xylose:arabinose (Xyl:Ara) to levels of 30:1 depending on the tissue and maturity of the specific grass evaluated [40]. In this study, we observed the Xyl:Ara ratio between 3.6:1 to 4.5:1 in untreated forage grasses (Fig. 3H), with a reduction of Xyl:Ara ratios (2.8:1 to 2.3:1) after alkaline pretreatment. Xylan branches dictate the strength of the covalent interactions among wall polysaccharides, mainly the binding of cellulose to xylan, influencing the structural properties of the wall [40].

3.5. Chemical fingerprints

FTIR and Raman spectroscopies were used to further probe the chemical fingerprints of untreated and NaOH-pretreated biomasses [53,54] (Table 3). The FTIR and Raman spectra of untreated samples were very similar for all biomasses (Figs. 4 and 5, and Table 3); however, those of NaOH-pretreated samples showed chemical alterations in the lignocellulose. The most marked differences between untreated and pretreated biomasses, identified by FTIR, concerned reductions in absorption bands for lignin structure (1633 and 1510 cm^{-1}), and ester-linked feruloyl and *p*-coumaroyl groups between AX and lignin (1735 cm^{-1}) (Fig. 4 and Table 3). Overall, the findings agreed with reductions in bands identified by Raman spectroscopy for lignin (1633, 1600 and 1272 cm^{-1}) (Fig. 5). Lignin assignments decreased considerably in intensity after pretreatment, and this was supported by the results obtained with the acetyl bromide method (Fig. 2B and C). FTIR also revealed that alkaline pretreatment partially removed the AX fraction, as indicated by the reduction of the band at 1250 cm^{-1} and xylose content (Fig. 3A). FTIR and Raman bands at 1378, 1160, 1095 and 1053 cm^{-1}, which are assigned to crystalline cellulose, did not display significant alterations after alkaline pretreatment. Although, the band at 898 cm^{-1} attributed to amorphous cellulose was slightly intensified after pretreatment.

The FTIR and Raman results confirmed that NaOH effectively deconstructed the lignocellulosic materials, removing lignin together with AX, but not the cellulose fraction. Such chemical alterations in the lignocellulosic materials have generally been considered advantageous for improving biomass saccharification [19,24,29].

3.6. Enzymatic saccharification and correlation between cell wall properties

The screening of forage grasses for saccharification potential was performed to identify grasses suitable for ethanol production. First, we measured the reducing sugars released by enzymatic hydrolysis from AIR without any further pretreatment, to avoid any interference by other compounds in the extractives or differences in biomass recalcitrance between forage grasses. Second, we evaluated the production of reducing sugars at 2 h (Fig. 6A) and 72 h (Fig. 6A) of enzymatic hydrolysis of untreated and NaOH-pretreated samples, using a xylanase-rich extract of *A. fumigatus* var. *niveus* (Table 2). The characterization of enzymatic activities revealed that xylanase (88.69 U/mg protein) was the main enzyme in the extract followed by β-glucosidase (10.71 U/mg protein), arabinanase (4.85 U/mg protein), cellulase (2.24 U/mg protein), and mannanase (1.36 U/mg protein). Arabinofuranosidase and β-xylosidase showed low specific activities (<0.64 U/mg protein), with no pectinase and laccase activities.

Reducing sugars released by the action of the enzyme extract at 72 h of hydrolysis from untreated biomasses ranged from 187.15 mg/AIR in Mombaza to 256.89 mg/g AIR in Coastcross. Tifton 85 and Coastcross (both *C. dactylon* cultivars), clustered in the group with the lowest lignin content (Fig. 2B), showed higher enzymatic saccharification in comparison to the other grasses at 2 h and 72 h of saccharification (Fig. 6A and B). Alkaline pretreatment strongly enhanced lignocellulose saccharification by 240–320% at 2 h, and by 65–110% at 72 h of enzymatic hydrolysis (Fig. 6A and B). In

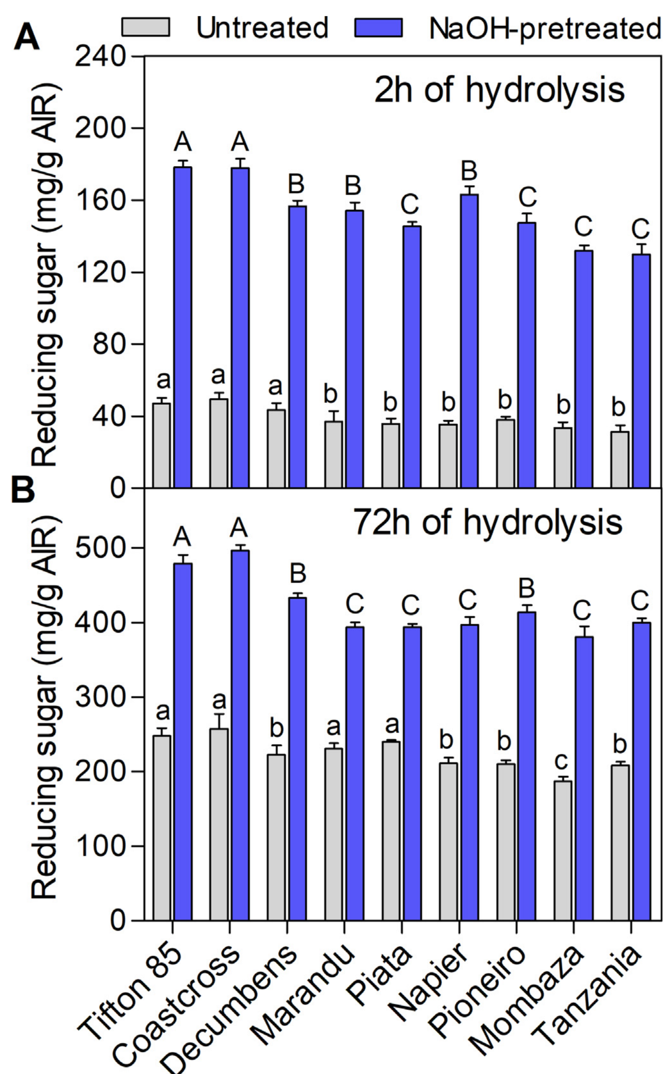

Fig. 6. Enzymatic saccharification of untreated and pretreated forage grasses at 2 h (A) and 72 h (B) of hydrolysis. The samples are ranked by their crescent lignin content. Mean values ± SEM ($n = 5$). Uppercase letters indicate significant differences between untreated samples; lowercase letters indicate significant differences between pretreated samples ($P \leq 0.05$, Scott-Knott test).

general, the effect of pretreatment on saccharification was consistent among the cultivars and with that reported for alkali-pretreated sugarcane [10,50].

The high sugar yields after enzymatic hydrolysis of pretreated biomass indicated that the holocellulose fraction was more accessible to enzymes. This occurred due to the partial removal of hemicellulose and lignin fractions, which otherwise would obstruct the access of hydrolases to polysaccharides and adsorb enzymes reducing their activity [23]. Additionally, alkaline pretreatment also removes acetyl, feruloyl, *p*-coumaroyl and uronic ester groups from hemicellulose, reducing the steric limitation that these compounds impose on hydrolytic enzymes [55].

We carried out a principal component analysis (PCA) to investigate the contribution of the thirteen different cell wall traits on saccharification at 72 h (Fig. 7). The PCA separated untreated grasses into three distinct groups (Fig. 7A) similarly to the groups separated by the Scott-Knott test, based on lignin content (Fig. 2A). Group A was represented by two cultivars of *P. maximum* (cv. Mombaza and Tanzania), with the highest lignin content (Fig. 2A) and the lowest FA (Fig. 1A), arabinose (Fig. 3B) and galactose contents (Fig. 3D). In contrast, group C was represented by two cultivars of *C. dactylon* (cv. Tifton 85 and Coastcross), with the lowest lignin and highest FA contents, though without correlations for arabinose and galactose. Other grasses were clustered in group B, which was bigger than the other two groups, and whose members presenting less fucose (Fig. 3F), VAN (Fig. 1B), and more rhamnose (Fig. 3G). Because Mombaza and Tanzania (group A) presented more lignin, the saccharification of them was lower than group C (Tifton 85 and Coastcross; Fig. 4A). Napier has been widely evaluated as a feedstock source for biomass saccharification and bioethanol production [11,12,26]. However, based on our PCA analysis, Tifton 85 and Coastcross were qualified as the best potential feedstock sources for the lignocellulosic ethanol production. PC1 and PC2 were significantly different between the cultivars (Table 4).

The analysis of the relationship among variables of biomass composition from untreated samples revealed a negative

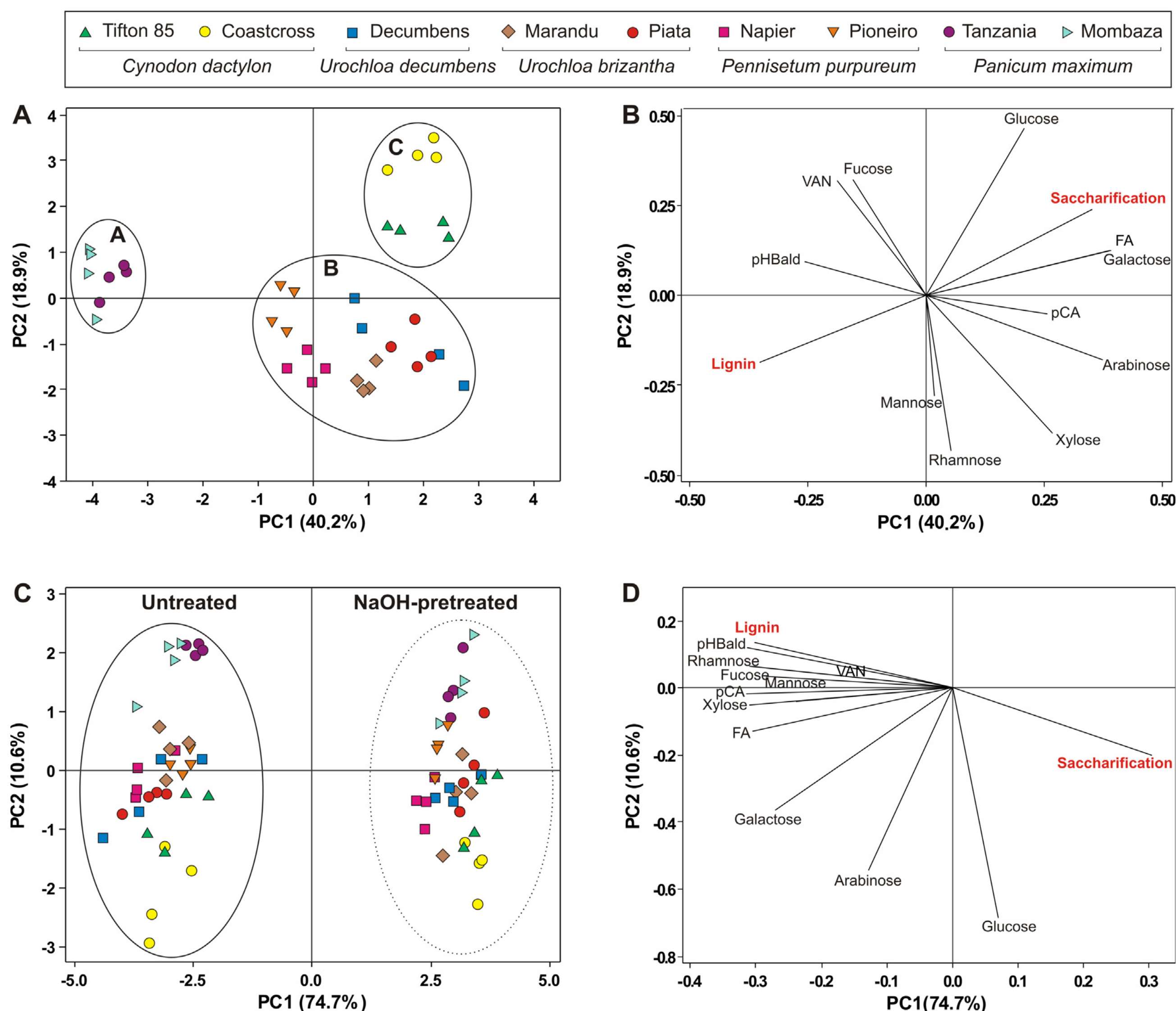

Fig. 7. Principal component analysis (PC1 and PC2) of thirteen different cell wall traits of forage grasses. A) Distribution of forage grasses for untreated biomasses in the plane defined by the first and second main components; B) relationship among variables of biomass composition; C) forage grasses distribution for untreated (solid circle) and NaOH-pretreated (dotted circle) in the plane defined by the first and second main components (PC1 and PC2); D) plot of the PC1 and PC2 loading vectors, describing the relationship among variables of plant composition and pretreatment used. Percentage values in parentheses (x- and y-axes) indicate the proportion of the variance explained by each axis. The vector values and statistical analyses are shown in Table 4.

Table 4
Eigenvalues and proportions of variance corresponding to each of the axes (PC1 and PC2) generated by the Principal component analysis (PCA) of forage grasses. The column Treatments corresponds the untreated and pretreated samples and column Cultivars corresponds the analysis by distribution of cultivars without pretreatment (Fig. 5). General Linear Model (GLM-analysis) was performed to test the significance of the synthetic variables for each principal component (PC) and expressed in F and *P* values. In bold are the main vectors to represent the PC and in bold/italic are significant differences in PC for forage grasses, treatment or interaction for both ($n = 4$).

	TREATMENTS		CULTIVARS	
	PC1	PC2	PC1	PC2
Eigenvalue	97.071	13.812	52.202	24.519
Proportion	0.747	0.106	0.402	0.189
Variables	**PC1**	**PC2**	**PC1**	**PC2**
Lignin	**−0.315**	0.121	**−0.352**	−0.187
Ferulate	**−0.306**	−0.128	**0.392**	0.124
p-Coumarate	**−0.316**	−0.017	0.257	−0.052
p-OH-Benzaldehyde	**−0.303**	0.136	−0.258	0.093
Vanillin	**−0.309**	0.064	−0.189	**0.317**
Saccharification 72 h	**0.304**	−0.200	**0.350**	0.239
Xylose	**−0.312**	−0.051	0.267	−0.384
Arabinose	−0.128	**−0.542**	**0.374**	−0.18
Galactose	−0.271	**−0.363**	**0.388**	0.126
Glucose	0.07	**−0.686**	0.207	**0.466**
Fucose	−0.285	0.035	−0.154	**0.322**
Mannose	−0.24	−0.038	0.017	−0.278
Rhamnose	**−0.311**	0.064	0.052	**−0.433**
GLM- analysis	**F/P values**	**F/P values**	**F/P values**	**F/P values**
Forage grass	F = 4.05/***P* = 0.001**	F = 42.17/***P* = 0.000**	F = 113.35/***P* = 0.000**	F = 46.28/***P* = 0.000**
Treatment	F = 5305.2/***P* = 0.000**	F = 0.57/P = 0.453	–	–
Forage grass*Treatment	F = 3.65/***P* = 0.002**	F = 2.02/P = 0.062	–	–

correlation between lignin content and saccharification (Fig. 7B). Previous studies have shown the negative correlation between FA content and saccharification [16,20,56]. However, in this study, saccharification exhibited a positive correlation with FA and a negative correlation with lignin content. It was interesting to note that FA and galactose were positively correlated in forage grasses (Fig. 7B). A possible explanation for the inverse effects of FA and lignin on saccharification is the difference in their contents in cell walls; lignin amounts (223.48−46.87 mg/g) were 67 to 140-fold higher compared to FA amounts (1.77−3.36 mg/g). Therefore, our results suggested that lignin plays a hierarchical role, as the main factor in reducing saccharification, in comparison with other cell wall traits.

The variable distribution revealed that, after alkaline pretreatment, the lignocellulose composition was drastically altered (Fig. 7C). PC1 clearly separated forage grasses into two groups: untreated and NaOH-pretreated plants. The synthetic variables that corresponded to this PC1 were lignin, FA, *p*CA, *p*HBald, VAN, xylose, and rhamnose. All these variables were negatively correlated with saccharification, with high significance in PCA1 for lignin (PC1 = − 0.315) and *p*CA (PC1 = − 0.315) (Table 4). PC1 was significant for cultivars, pretreatment and interaction with both these components. In PC2, arabinose, galactose and fucose were the main components of importance (Fig. 7D and Table 4), although this distribution was significant only for forage grass cultivars. This analysis showed that lignin removal from lignocellulosic materials was very important for saccharification efficiency, leading to increased access to cell wall polysaccharides. However, it was important to note that other differences in biomass composition for each cultivar might also interfere with saccharification (PC2). The role of each cell wall constituent in biomass recalcitrance is not completely understood; however, it is thought to involve an intricate cross-linking of lignin, FA and carbohydrate complexes within the cell wall rendering polysaccharide inaccessible to degradation [16,44,57]. Taken together, cell wall composition, saccharification and PCA analysis suggested that predicting suitability model plants for lignocellulosic ethanol production could be strongly dependent on cultivars, species and pretreatment used. The elucidation of the influence of lignin in determining biomass recalcitrance in forage grasses can facilitate the genetic engineering of plants to exhibit reduced lignin content for improved biofuel production.

4. Conclusion

By comparing, nine untreated and alkali-pretreated forage grasses and thirteen cell wall parameters, this study demonstrated that lignin was a hierarchical factor determining biomass recalcitrance. We suggested that the degree of AX acylation with FA and *p*CA was relevant for saccharification only in plants with similar lignin contents. In addition, lignin can be used as a marker for the selection of grass cultivars suitable for ethanol production and animal feeding.

Declaration of competing interest

The authors have no conflict of interest.

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