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DYONI MATIAS DE OLIVEIRA

INFLUÊNCIA DOS FENILPROPANOIDES SOBRE A DIGESTIBILIDADE DA BIOMASSA LIGNOCELULÓSICA DE GRAMÍNEAS: IMPACTOS SOBRE A PRODUÇÃO DE ETANOL CELULÓSICO

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ATA DE DEFESA DA DISSERTAÇÃO DE MESTRADO DO ALUNO DYONI MATIAS DE OLIVEIRA

Aos vinte e seis dias do mês de fevereiro de dois mil e dezesseis, sexta-feira, realizou-se no Bloco G-56, sala 201, no campus universitário, a sessão pública de defesa da dissertação intitulada: "Influência dos fenilpropanóides sobre a digestibilidade da biomassa lignocelulósica de gramíneas: impactos sobre a produção de etanol celulósico", apresentado pelo aluno **Dyoni Matias de Oliveira,** licenciado em Ciências Biológicas, pelo Universidade Estadual de Maringá, que concluiu os créditos exigidos para obtenção do grau de "Mestre em Ciências Biológicas". Os trabalhos foram instalados às 9h07, pelo Prof. Dr. Wanderley Dantas dos Santos, Presidente da Banca Examinadora, constituído pelas seguintes professores: Dr. Marcos Silveira Buckeridge e Dr. Osvaldo Ferrarese Filho como membros. A Banca Examinadora, tendo se decidido a aceitar a dissertação, passou à argüição pública do candidato. Encerrados os trabalhos de argüição às 12h07 horas, os examinadores deram parecer final, considerando a dissertação APROVADO. Proclamado o resultado pelo Presidente da Banca Examinadora, foram encerrados os trabalhos e lavrada a presente ata que vai assinada pelos membros da Banca Examinadora.

Maringá, 26 de fevereiro de 2016

Prof. Dr. Wanderley Dantas dos Santos

Prof. Dr. Marcos Silveira Buckeridge

Prof. Dr. Osvaldo Ferrarese Filho

BIOGRAFIA

Dyoni Matias de Oliveira nasceu em 20 de novembro de 1989 em Marialva/PR. Possui licenciatura em Ciências Biológicas pela Universidade Estadual de Maringá (2013). Atualmente é aluno de Pós-Graduação em Ciências Biológicas (área de concentração em Biologia Celular e Molecular) pela Universidade Estadual de Maringá, onde atua principalmente em bioquímica vegetal com ênfase no metabolismo de lignina, arquitetura da parede celular e produção de enzimas para a hidrólise da biomassa lignocelulósica.

À MINHA DOCE THATI.

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Meus sinceros agradecimentos.

"Clouds are not spheres, mountains are not cones, coastlines are not circles, and bark is not smooth, nor does lightning travel in a straight line".

Benoit B. Mandelbrot (1983)

APRESENTAÇÃO

Esta dissertação é composta de dois artigos científicos. Em consonância com as regras do Programa de Pós-Graduação em Ciências Biológicas, os artigos foram redigidos de acordo com as normas dos respectivos periódicos, divergindo apenas na posição das figuras e tabelas que foram dispostas ao longo do texto para facilitar a leitura e compreensão dos trabalhos. O primeiro artigo é uma revisão bibliográfica intitulada "*Ferulic acid: a key component in grass lignocellulose recalcitrance to hydrolysis*", que descreve os recentes avanços biotecnológicos na compreensão do ácido ferúlico como componente na arquitetura e fisiologia da parede celular de gramíneas. Assim como a descrição detalhada do mecanismo de feruloilação de arabinoxilanos via BAHD acil-CoA transferases, a relação entre o ácido ferúlico e a recalcitrância da parede celular à hidrólise enzimática e a aplicação de ferramentas biotecnológicas para a melhoria da sacarificação. O segundo artigo intitulado "*Lignin plays a key role in determining biomass recalcitrance in forage grasses*" descreve a correlação entre a composição fenólica e lignina de nove gramíneas forrageiras com a hidrólise enzimática antes e após pré-tratamento alcalino suave, utilizado para remover parcialmente a lignina e melhorar a sacarificação das biomassas.

1. Dyoni M. de Oliveira, Aline Finger-Teixeira, Thatiane R. Mota, Victor H. Salvador, Flávia C. Moreira-Vilar, Hugo B. C. Molinari, Rowan A. C. Mitchell, Rogério Marchiosi, Osvaldo Ferrarese-Filho, Wanderley D. dos Santos (2015) Ferulic acid: a key component in grass lignocellulose recalcitrance to hydrolysis. *Plant Biotechnology Journal*, 13, 1224-1232.
JCR: 6,84 - Qualis A1.

2. Dyoni M. Oliveira, Thatiane R. Mota, Adriana Grandis, Gutierrez R. de Morais, Rosymar C. de Lucas, Maria L. T. M. Polizeli, Rogério Marchiosi, Marcos S. Buckeridge, Osvaldo Ferrarese-Filho, Wanderley D. dos Santos (2020) Lignin plays a key role in determining biomass recalcitrance in forage grasses. *Renewable Energy,* 147:2206-2217
JCR: 5,43 - Qualis A1.

RESUMO GERAL

INTRODUÇÃO E OBJETIVOS – A biomassa lignocelulósica desponta como uma fonte abundante e sustentável de energia capaz de substituir o petróleo na produção de combustíveis líquidos em um horizonte razoável de desenvolvimento tecnológico. Neste contexto, a conversão enzimática dos polissacarídeos em açúcares fermentescíveis se destaca como uma tecnologia em fase avançada, já em uso em algumas usinas espalhadas ao redor do mundo. Além da produção de biocombustíveis, a biomassa lignocelulósica já é empregada há milhares de anos na produção de papel, celulose e na nutrição animal. As gramíneas são as principais *commodities* agrícolas mundiais e a principal fonte de biomassa para a produção de bioetanol e apresentam altos níveis de ácido ferúlico na parede celular. Assim, iniciamos a dissertação realizando uma revisão bibliográfica sobre a importância do ácido ferúlico na recalcitrância da parede celular à hidrólise (Oliveira *et al.,* 2015). Diversos trabalhos reportam que a redução no conteúdo de ácido ferúlico esterificado à parede celular promove a sacarificação, em muitos casos, sem alterações no teor de lignina. Entretanto, a recalcitrância à digestão enzimática é um fenômeno multifatorial e o conteúdo de lignina influi fortemente sobre a digestibilidade. Assim, a fim de avaliar o impacto dos diferentes fenilpropanoides sobre a digestibilidade das gramíneas, selecionamos nove gramíneas forrageiras com alta produtividade e potencial para a produção de bioetanol, determinamos o teor de lignina, ácidos hidroxicinâmicos: ácidos ferúlico e *p*-cumárico; aldeídos fenólicos: vanilina e p-hidroxibenzaldeído; e correlacionamos com a digestibilidade da biomassa lignocelulósica.

MÉTODOS – Nove gramíneas forrageiras foram avaliadas neste estudo: dois cultivares de *Cynodon dactylon* (cv. Tifton 85 e Coastcross), um cultivar de *Urochloa decumbens* (formalmente *Brachiaria decumbens* cv. Basilisk), dois cultivares de *U. brizantha* (formalmente *Brachiaria brizantha* cv. Marandu e Piatã), dois cultivares de *Megathyrsus maximus* (formalmente *Panicum maximum* cv. Mombaça e Tanzânia) e dois cultivares de *Pennisetum purpureum* (cv. Napier and Pioneiro). Hastes com folhas foram coletadas, secadas a 60 °C por 48 h e pulverizadas em moinho de bolas. A biomassa seca foi submetida a extração com etanol 80% (*v/v*) e os níveis de compostos aromáticos e proteínas foram determinados do sobrenadante. O resíduo insolúvel em álcool (AIR) foi utilizado nos procedimentos seguintes. A sacarificação foi determinada aplicando o extrato liofilizado de *Aspergillus niveus* contendo atividades xilanase e celulase. Fenólicos ligados a parede celular foram extraídos por

saponificação com NaOH e analisados por cromatografia líquida de alta eficiência (CLAE). O AIR foi submetido ao pré-tratamento com NaOH 0,25 M a 130 °C por 40 min. O teor de lignina foi determinado e a caracterização qualitativa da lignocelulose foi avaliada antes e depois do pré-tratamento por espectroscopia de FTIR e RAMAN. As análises de correlações foram realizadas para obter a significância ($P \leq 0,05$), intensidade e direção das interrelações entre as características das biomassas utilizando o coeficiente de correlação de Pearson. Desta forma, painéis correlacionando as características da parede celular e a liberação de açúcares foram organizados para identificar os principais fatores limitantes para a hidrólise enzimática.

RESULTADOS E DISCUSSÃO – O conteúdo de lignina foi negativa e fortemente correlacionada à sacarificação enzimática nos diferentes cultivares avaliados. O conteúdo de ácido ferúlico foi inversamente correlacionado ao conteúdo de lignina nas diferentes forrageiras, sugerindo uma relação recíproca entre estes componentes na arquitetura da parede celular tipo II. Os conteúdos de ácido ferúlico e vanilina foram positivamente correlacionados com a digestibilidade, enquanto os conteúdos de ácido *p*-cumárico e *p*-hidroxibenzaldeído não apresentaram correlação com a digestibilidade. O papel destes aldeídos fenólicos na parede celular não é conhecido. Entretanto, seus conteúdos não se correlacionaram positivamente nem com o teor de lignina nem com os dos ácidos hidroxicinâmicos. Isto sugere fortemente que estes aldeídos não são produtos de degradação destes fenilpropanoides. Os ácidos ferúlico e *p*-cumárico apresentaram correlação negativa com a lignina e correlação positiva com a digestibilidade, mostrando que não foram limitantes para a digestibilidade. Ao contrário, a lignina apresentou forte correlação negativa com a digestibilidade. O pré-tratamento removeu aproximadamente metade da lignina das amostras, aumentando fortemente a sacarificação. As análises por FTIR confirmaram a redução de lignina e indicaram que o pré-tratamento removeu hemicelulose e aumentou a celulose amorfa.

CONCLUSÕES – O conteúdo de lignina foi o principal fator limitante para a sacarificação dos polissacarídeos da parede celular. A correlação fraca entre os aldeídos fenólicos e os hidroxicinamatos e suas correlações com a lignina sugere que vanilina e *p*-hidroxibenzaldeído podem ser componentes legítimos da parede celular tipo II. O pré-tratamento alcalino brando removeu cerca de metade da lignina aumentando em até duas vezes a digestibilidade em 72 h. Os cultivares de *Cynodon dactylon,* Tifton-85 e Coastcross, apresentaram os menores conteúdos de lignina e os maiores potenciais para a produção de açúcares fermentescíveis entre os cultivares de gramíneas forrageiras avaliados.

PALAVRAS-CHAVES: Pré-tratamento alcalino; Biocombustíveis; Hidrólise enzimática; Ácido ferúlico; Análises por FTIR; Ácido *p*-cumárico.

GENERAL ABSTRACT

INTRODUCTION AND AIMS – The lignocellulosic biomass has emerged as an abundant and sustainable source of energy that can replace petroleum in the production of liquid fuels in a reasonable horizon of technological development. In this context, the enzymatic conversion of polysaccharides into fermentable sugars stands out as an advanced technology, already in use in some plants scattered around the world. Besides the production of biofuels, lignocellulosic biomass is already used for thousands of years in the production of paper, pulp and animal nutrition. Grasses are the major agricultural commodities and the main source of biomass for production of bioethanol and present high levels of ferulic acid in the cell wall. Therefore, we started our project conducting a literature review on the importance of ferulic acid in the recalcitrance of cell wall hydrolysis (Oliveira *et al.,* 2015). Several studies report that reduction in content of ferulic acid esterified to cell wall promotes saccharification, in many cases, without altering lignin content. However, the recalcitrance to enzymatic digestion is a multifactorial phenomenon and lignin content impacts strongly on the digestibility. Thus, in order to assess the impact of the different phenylpropanoids on the digestibility of grass lignocellulose, we selected nine cultivars of forage grass with high productivity, we determined the content of lignin, phenylpropanoids: ferulic acid and *p*-coumaric acid; and phenolic aldehydes: vanillin and *p*-hydroxybenzaldehyde; and correlated them with the digestibility of lignocellulosic biomass.

METHODS – Nine grasses were evaluated in this study: two cultivars of *Cynodon dactylon* (Tifton 85 and Coastcross), one cultivar of *Urochloa decumbens* (formerly *Brachiaria decumbens*; cv. Basilisk), two cultivars of *U. brizantha* (formally *B. brizantha*; cv. Marandu and Piata), two cultivars *Megathyrsus maximus* (formally *Panicum maximum* cv. Mombaza and Tanzania) and two cultivars of *Pennisetum purpureum* (cv. Napier and Pioneiro). Stems with leaves were collected, dried at 60 °C for 48 h and pulverized in mill ball. Dry biomass was submitted to extraction with 80% ethanol (*v/v*) and the levels of aromatic compounds and proteins were determined in the supernatant. The alcohol insoluble residue (AIR) was used for the following procedures. Saccharification was measured applying lyophilized extract from *Aspergillus niveus* containing xylanase and cellulase activities. Cell wall-phenolic compounds were extracted by saponification with NaOH and analyzed by high-performance liquid chromatography (HPLC). The biomass was pretreated with 0.25 M NaOH at 130 °C for 40 min.

The lignin content was determined and the qualitative characterization of lignocellulose was evaluated before and after pretreatment by FTIR spectroscopy. Correlation analyzes were performed to identify the significance ($P \leq 0.05$), intensity and direction of the interrelationships between the characteristics of biomass using the Pearson correlation coefficient. Thus, panels correlating cell wall properties and sugar yield were organized to identify the main limiting factors for enzymatic hydrolysis.

RESULTS AND DISCUSSION – The lignin content was negative and strongly correlated to enzymatic saccharification in the different cultivars evaluated. Ferulic acid content was inversely correlated with the lignin content in different forage lignocellulose materials, suggesting a reciprocal relationship between these components in type II cell walls. Ferulic acid and vanillin contents were positively correlated with digestibility, while *p*-coumaric acid and *p*-hydroxybenzaldehyde did not present correlation with digestibility. The role of these phenolic aldehydes in the cell wall it is not known. However, their content is correlated positively neither with lignin nor with hydroxycinnamic acids content. It strongly suggests that these aldehydes are not degradation products of these phenylpropanoids. Ferulic and *p*-coumaric acids showed negative correlation with lignin and positive correlation with digestibility, indicating that they were not limiting to digestibility. Instead, the lignin showed a strong negative correlation with digestibility. Pretreatment removed nearly half of lignin, strongly increasing the saccharification. FTIR analysis confirmed lignin reduction and indicated that pretreatment removed hemicellulose and increased amorphous cellulose content.

CONCLUSIONS - Lignin content is the main limiting factor for saccharification of cell wall polysaccharides. The weak correlations among phenolic aldehydes and hydroxycinnamates and their correlations with lignin suggest that vanillin and *p*-hydroxybenzaldehyde can be proper type II cell wall components. Mild alkaline pretreatment removed about half of lignin improving up to twice the digestibility in 72 h. *Cynodon dactylon* cultivars, Tifton 85 and Coastcross, presented the lowest lignin content and the highest potential to production of fermentable sugars among the assessed forage grass cultivars.

KEYWORS: Alkaline pretreatment; Biofuels; Enzymatic hydrolysis; Ferulic acid; FTIR analysis; *p*-Coumaric acid.

Chapter 1. Review article

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Ferulic acid: a key component in grass lignocellulose recalcitrance to hydrolysis

Dyoni Matias de Oliveira[1], Aline Finger-Teixeira[1], Thatiane Rodrigues Mota[1], Victor Hugo Salvador[1], Flávia Carolina Moreira-Vilar[1], Hugo Bruno Correa Molinari[2], Rowan Andrew Craig Mitchell[3], Rogério Marchiosi[1], Osvaldo Ferrarese-Filho[1], Wanderley Dantas dos Santos[1]*

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Summary

In the near future, grasses must provide most of the biomass for the production of renewable fuels. However, grass cell walls are characterized by a large quantity of hydroxycinnamic acids such as ferulic and *p*-coumaric acids, which are thought to reduce the biomass saccharification. Ferulic acid (FA) binds to lignin, polysaccharides and structural proteins of grass cell walls cross-linking these components. A controlled reduction of FA level or of FA cross-linkages in plants of industrial interest can improve the production of cellulosic ethanol. Here, we review the biosynthesis and roles of FA in cell wall architecture and in grass biomass recalcitrance to enzyme hydrolysis.

Keywords: cellulosic ethanol, saccharification, lignocellulose, type II cell wall, phenylpropanoid pathway, *BAHD acyl-CoA transferases.*

Introduction

The worldwide enrichment occurred after industrial revolution was fueled with coal and petroleum (dos Santos *et al.*, 2011). But, as a finite resource, fossil fuels can not sustain technological development indefinitely. New technologies that allow us to ensure a continuous economic development are needed (Escobar *et al.*, 2009). As the most abundant renewable raw material available (Gnansounou, 2010), plant biomass has a great potential to replace oil in a reasonable economic and technological development horizon (Buaban *et al.*, 2010; Poovaiah *et al.*, 2014). Grasses residues including switchgrass, miscanthus, sugarcane and maize are potential sources of lignocellulose for the production of bioenergy (Burton and Fincher, 2012; Torres *et al.*, 2014).

To date, almost all ethanol produced in the world comes from food carbohydrates, such as sucrose and starch (Balat, 2011). After extraction of soluble carbohydrates, the residual biomass is underused as solid fuel to produce electricity (Goldemberg *et al.*, 2008) or as cattle feed (Rocha *et al.*, 2012). However, crop residues such as sugarcane bagasse are mainly constituted by polysaccharides. Once hydrolyzed, monosaccharides can be partially fermented and, thus, contribute for increase of ethanol production (Buckeridge *et al.*, 2010), without competing with food production (Alvira *et al.*, 2010).

To produce cellulosic ethanol at competitive costs, several challenges need to be overcome. It is necessary to develop efficient enzymatic cocktails to understand how the enzymes act together, how to cope with catalytic inhibitors and also drastically reduce catalysts costs (Jørgensen *et al.*, 2007). This task is not trivial as recalcitrance of lignocellulose is the result of hundred million of years of co-evolution between plants and predators (Cheynier *et al.*, 2013; Malinovsky *et al.*, 2014). Among the adaptations against biological attack, we highlight the presence of hydroxycinnamates in primary and secondary cell walls of grasses and other commelinoids (dos Santos *et al.*, 2006; Hatfield *et al.*, 1999).

Produced in the phenylpropanoid pathway, ferulic acid (FA) is able to couple oxidatively with other FA and derivatives (Buanafina, 2009), lignin and structural proteins from cell wall (Carpita *et al.*, 2001). However, in contrast with the alcohol function of monolignols, hydroxycinnamic acids have a carboxylic group at the end of their propenyl group, which provides the ability to esterify hemicelluloses (Figure 1). Thus, FA is covalently linked to the lignin and proteins by ether bonds and with polysaccharides by ester bonds, acting as a universal connector between cell wall polymers. It performs a key roles in cessation of cell growth,

anchoring lignin in cell wall polysaccharides restricting the accessibility of plant pathogens (Lygin *et al.*, 2011).

The revolutionary potential of cellulosic ethanol for civilization justifies *per se* the importance of overcoming cell wall recalcitrance (dos Santos *et al.*, 2011). However, the relevance of this knowledge can reach far beyond the fuel industry with benefits which comes from plant physiology and wood science to paper industry and cattle feed. Herein, we reviewed the role of FA in type II primary and secondary cell wall architecture and properties highlighting its relevance in recalcitrance to enzyme hydrolysis.

Composition and architecture of cell wall

Lignocellulosic biomass is composed mainly by cellulose, hemicellulose, lignin, pectin, proteins and aromatic compounds. Nevertheless, there are relevant differences in the proportion of these constituents among plant species and tissues (Carpita *et al.*, 2001). Overall, such components account for over 90% of the total dry weight. Specific kinds of polysaccharides and the monomeric composition vary in different plant groups (Boerjan *et al.*, 2003; Buckeridge *et al.*, 2010). The main component of cell wall is cellulose, a homopolysaccharide consisting of a long and linear chain of β(1→4) linked glucose units. Each cellulose molecule is tightly bound to other molecules by means of multiple hydrogen bonds producing insoluble, rigid and crystalline microfibrils (Carpita and McCann, 2000). These cellulose fibres are impeded to collapse with each other by hemicelluloses. The richer topology of these frequently branched hemicelluloses allows them to link only occasionally with cellulose microfibrils. Intermittent pattern of free and bound regions from hemicellulose with cellulose results in the cross-linking of microfibrils (Carpita, 1996). The network of cellulose and hemicellulose molecules is still embedded in a matrix of pectin (Scheller and Ulvskov, 2010). These very complex heteropolysaccharides are branched with acidic sugars as glucuronic and galacturonic acids. They adsorb high amounts of water forming a gel which is involved in cell-cell adhesion, pore sizing, pH control and cation trapping (Buckeridge *et al.*, 2010).

Primary cell walls are classified in types I and II. The majority of dicots plants share a similar type I cell wall composition and architecture. Type I is found in dicots noncommelinoid angiosperms (e.g. aroids, alismatids and liliods) and gymnosperms. The main hemicellulose of type I cell walls is xyloglucan, which is composed by a glucose backbone branched with xylose which in turn may be substituted with galactose and fucose (Carpita and Gibeaut, 1993).

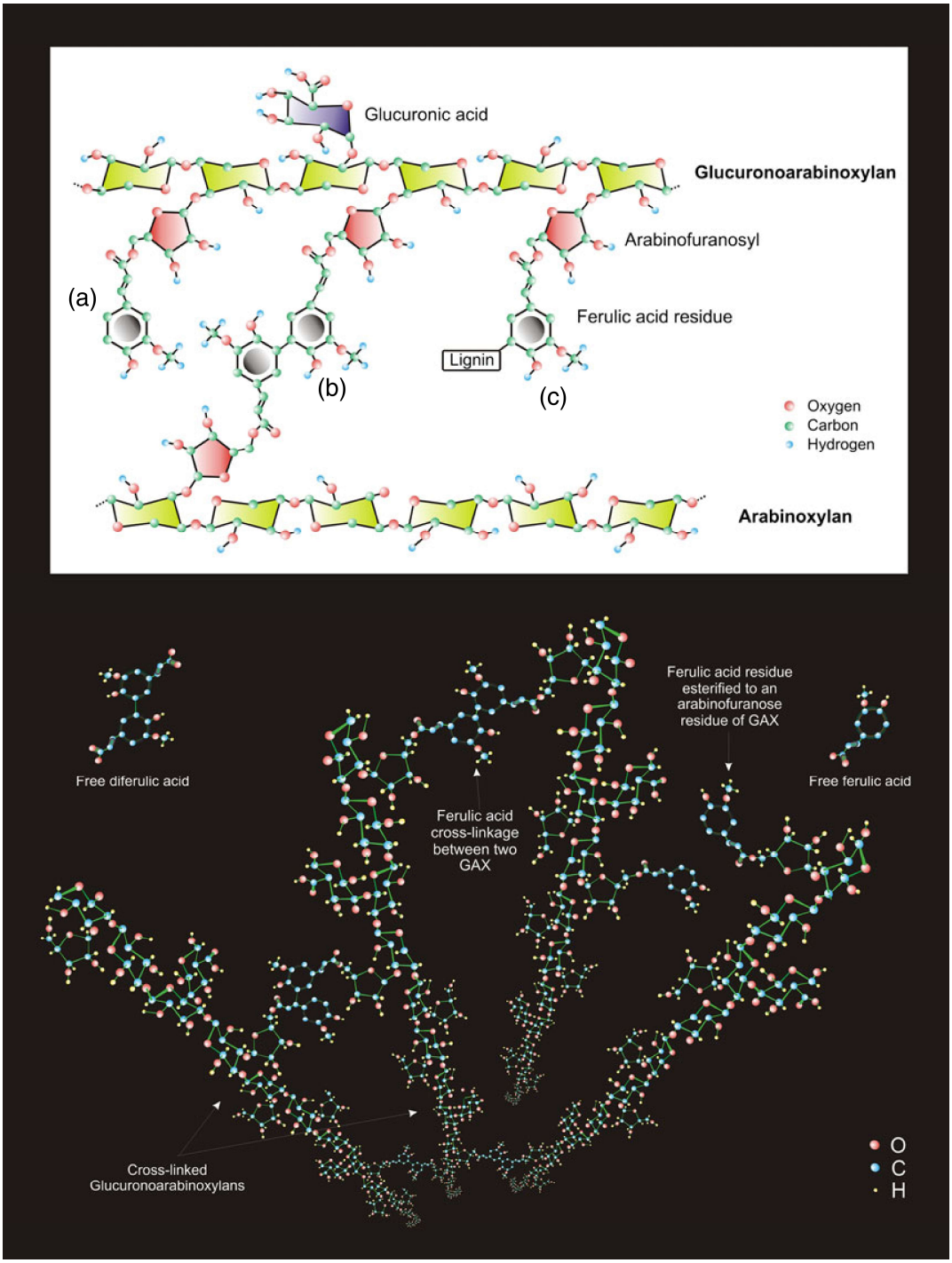


Figure 1. Above (white background): Feruloylated glucuronoarabinoxylan (FA-GAX). (a) ferulic acid residue esterified to the arabinofuranosyl residue of GAX; (b) diferulic acid cross linking two FA-GAX; (c) ferulic acid residue anchoring lignin to the GAX. Below (black background): Perspective view of the cross-linkages among vicinal FA-GAX.

In commelinoid monocotyledons, a group of angiosperms including grass, palms, bromeliads and ginger have a type II cell wall, which contrasts with type I cell wall of other angiosperms (Carpita and Gibeaut, 1993; Vogel, 2008). A relevant characteristic of type II cell

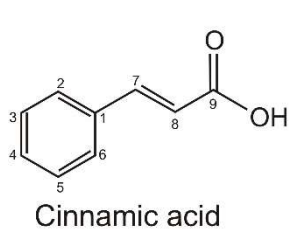
walls is the abundance of glucuronoarabinoxylan (GAX). It is composed by a core chain of xylan branched with arabinose and glucuronic acid (Dodd and Cann, 2009). The arabinose residues can be further esterified with feruloyl and *p*-coumaroyl residues (Figure 1). Produced in the phenylpropanoid pathway together with monolignols, feruloyl esterified to polysaccharides can oxidatively polymerize to produce dehydrodimer and oligomers that cross-link vicinal GAX. In commelinoids, FA branched GAX (FA-GAX) is abundant in primary cell walls. This polysaccharide is believed to occur only in these species and performs a set of distinguished structural and physiological roles in their cell walls (Carpita *et al.*, 2001). In addition, Poales type II cell walls contains significant quantities of mixed-linkage glucans (β-glucans) present exclusively in this order (de Souza *et al.*, 2013; Smith and Harris, 1999).

In spite of being a rich source of energy itself, cell wall is a frontline cell defence against microorganisms (Grabber *et al.*, 1998a). To get access to the energetic content of lignocellulosic biomass, microorganisms must make use of catalytic tools to break the lignin-carbohydrate complex and cellulose crystallinity (Wyman, 1994). Only then, polysaccharides become available to hydrolysis and monosaccharide fermentation. Lignin is one of the hardiest cell wall barriers to overcome. Beyond being hard to digest, lignin fractions adsorb hydrolytic proteins reducing the access of enzymes to the polysaccharides (Huang *et al.*, 2011). In mature cells, lignin forms a highly hydrophobic matrix of C-C and C-O-C linked phenylpropanoids, mainly coniferyl, sinapyl and *p*-coumaroyl alcohols. Respective residues in lignin are dubbed guaiacyl (G), syringyl (S) and *p*-hydroxyphenyl (H) units. The amount of lignin as well as its monomeric composition is ontogenetic-tissue-species dependent. Lignin is deposited in secondary cell walls of schlerenchymatic tissues in fibre and vessels. It confers high hydrophobicity and mechanical resistance to cell walls required by xylem vessels to perform the capillary transport of water and for fibres support the massive habit of trees (Pedersen *et al.*, 2005; Zobiole *et al.*, 2010). In lignified secondary cell walls, the FA ester linked to GAX are nucleation sites for lignin polymerization through ether bounds, anchoring lignin to polysaccharide moiety (Carpita *et al.*, 2001; Renger and Seinhart, 2000).

Ferulic acid function, biosynthesis and cell wall esterification

Ferulic acid [(E)-3-(4-hydroxy-3-methoxyphenyl) prop-2-enoic acid] (Figure 2) was first isolated from *Ferula foetida* (devil's dung) in 1866 (Fazary & Ju, 2007) being more abundant in tissues as epidermis, xylem and sclerenchyma. Since its early discovery, FA has

also been reported to exhibit a wide range of important biological (dos Santos *et al.*, 2008a) and therapeutic properties (Paiva *et al.*, 2013). These include anti-inflammatory, anti-bacterial, antidiabetic, anticarcinogenic, antiaging, and neuroprotective effects, which can be attributed to its antioxidant capacity (dos Santos *et al*., 2008b; Paiva *et al*., 2013). FA is highly abundant in the cell walls of all commelinoid orders: Arecales (palms), Commenlinales (water hyacinth), Poales (grasses) and Zingiberales (banana, ginger) (Smith & Harris, 2000). It is present in much smaller quantities in cell walls of most (probably all) dicots such as Caryophyllales (cactus) (Hartley & Harris, 1981), Solanales (tomato) (Keller *et al.*, 1996), Brassicaceae (*Arabidopsis*) (Chen *et al.*, 1998) and Apiales (Parsley and devil's dung) (Parr *et al.*, 1997). In the Chenopodioideae and other dicots, FA is found to be ester-linked to pectin (Ishii, 1997).



Substituents			
C3	C4	C5	
H	OH	H	***p*-coumaric acid**
OH	OH	H	caffeic acid
OCH_3	OH	H	**ferulic acid**
OCH_3	OH	OCH_3	sinapic acid

Figure 2. Chemical structure of cinnamic acid and substituents.

Although type II cell walls only are found in few orders clustered as commelinoid, these includes all cereal crops and perennial grasses (Carpita & McCann, 2000) responding for most of the biomass produced in agriculture (dos Santos *et al.*, 2011). In type II cell walls, GAX intermittently interact with cellulose through hydrogen linkages cross-linking microfibrils (Lee *et al.*, 2008) which contributes with up to 40% of dry weight (Scheller & Ulvskov, 2010). Xyloglucans, pectins and structural proteins are minor compounds while FA and *p*-coumaric acid abound. Ferulic acid is ester linked to *O*-5 position of arabinose residue of GAX (Ishii, 1997).

More recent researches on the phenylpropanoid pathway have shown that the traditional view of lignin biosynthesis is incorrect (Humphreys and Chapple, 2002). Although the hydroxylation and methylation reactions of the metabolic pathway were long thought to occur at the level of the free hydroxycinnamic acids, it now seems clear that the enzymes catalysing phenylpropanoid 3-hydroxylation and 3-*O*-methylation reactions use shikimate and coenzyme A (CoA) conjugates as substrates (Franke *et al.*, 2002a, 2002b; Guo *et al.*, 2001; Parvathi *et al.*, 2001; Schoch *et al.*, 2001; Sullivan and Zarnowski, 2010; Zhong *et al.*, 1998). Similarly, it is not completely elucidated how FA is synthesized in plants. Although a supposed free acid

pathway has been suggested (Buanafina, 2009), the following evidences indicates that the synthesis of FA from *p*-coumaric acid seems unlikely *in vivo* (Figure 3): 1) The enzyme *p*-coumaroyl shikimate/quinate 3-hydroxylase (C3'H) does not directly hydroxylate *p*-coumaric acid to caffeic acid, but rather act on *p*-coumaroyl ester derivatives (Schoch *et al.*, 2006). The preferred substrate for C3H appears to be *p*-coumaroyl-shikimate, although it can hydroxylate other *p*-coumaroyl esters (Franke *et al.*, 2002; Schoch *et al.*, 2001). 2) The 3-*O*-methyltransferase (COMT) has a clear preference for 5-hydroxyferulic acid than for caffeic acid (Louie *et al.*, 2010). In addition, down-regulation of COMT activity in transgenic alfalfa, maize and poplar plants produces significant effect on S lignin, but little effect on G lignin (see Louie *et al.*, 2010 and references therein). Thus, the current knowledge suggests that the mainstream phenylpropanoid is better described as illustrated in Figure 3. L-Phenylalanine is deaminated by phenylalanine ammonia-lyase (PAL) to produce *t*-cinnamic acid. This step is followed by hydroxylation of the aromatic ring, catalysed by cinnamate 4-hydroxylase (C4H), to give *p*-coumaric acid, the first phenolic compound produced in the pathway. The next step is the activation of the acid to a thioester via 4-coumarate:CoA ligase (4CL) to yield *p*-coumaroyl-CoA. This compound is transesterified to shikimate or quinate by action of *p*-hydroxycinnamoyl CoA:quinate/shikimate *p*-hydroxycinnamoyltransferase (HCT). The ester is further hydroxylated in the C3 to produce caffeoyl-shikimate/quinate ester by *p*-coumaroyl shikimate/quinate 3-hydroxylase (C3´H). Caffeoyl-shikimate/quinate is transesterified back with CoA by HCT and *O*-methylated in the hydroxyl group in C3 by caffeoyl-CoA *O*-methyltransferase (CCoAOMT) to produce feruloyl-CoA, the activated form of FA (Chen *et al.* 2000, Zhong *et al.* 1998; 2000).

Despite the potential relevance of CCoAOMT to feruloylation in grasses there is no information about the effects of CCoAOMT down-regulation to feruloylation, lignification or digestibility in grasses. An additional pathway to produce FA was revealed by Nair and collaborators in 2004. The synthesis of monolignols uses FA-CoA as intermediate to produce coniferaldehyde in a reduction catalysed by cinnamoyl-CoA reductase (CCR). The aldehyde can be oxidized directly to FA by action of coniferyl aldehyde dehydrogenase (CALDH). The free form produced by CALDH can be directly exported to the cell wall where FA acts as a strong antioxidant and an UV protector. However, in order to be esterified to GAX, the free form of FA must be first activated to its active form FA-CoA (Guo *et al.*, 2001; Schmitt *et al.*, 1991). The enzyme 4CL has been demonstrated to be responsible for catalysing the esterification of exogenous free FA to CoA *in vivo*. In soybean, the 4CL reaction has been

suggested as entrance point into the pathway for exogenously supplied FA, which contributed for monolignol biosynthesis (G lignin) and esterification of FA residues into cell wall polysaccharides (dos Santos *et al.*, 2008b). At this time, the possibility that a portion of the FA synthesized in grasses is also generated by other enzyme pathways cannot be excluded. At least in specific situations, it has been shown that thioesterases as HCT break ester linkages between hydroxycinnamic acids and shikimate, quinate or CoA (Hoffmann *et al.*, 2003).

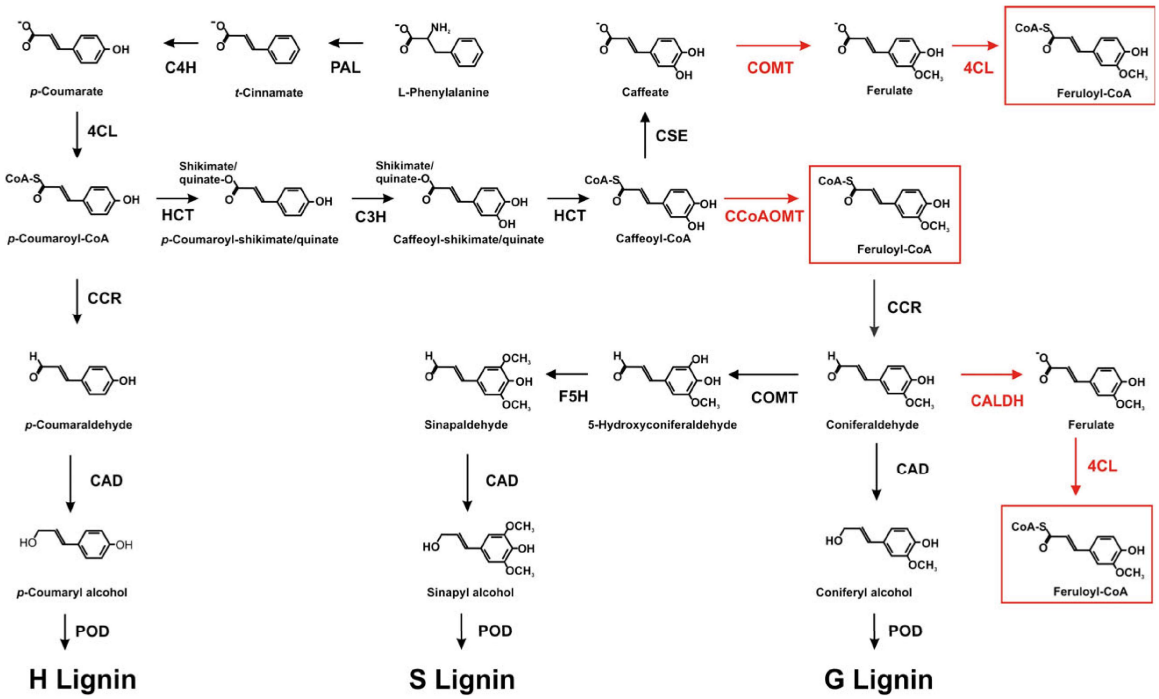


Figure 3. Imbrications between biosynthesis of monolignols and ferulic acid (as well as its coenzyme A-activated derivative). Red arrows draw attention to enzymatic steps that produce ferulic acid (black squares) and feruloyl-CoA (red squares). PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, p-coumarate:CoA ligase; HCT, p-hydroxycinnamoyl-CoA:shikimate/quinate p-hydroxycinnamoyl transferase; C30H, p-coumaroyl shikimate/quinate 3-hydroxylase; CCoAOMT, caffeoyl-CoA 3-O-methyltransferase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase; F5H, ferulate 5-hydroxylase; COMT, caffeic acid 3-O-methyltransferase; CALDH, coniferaldehyde dehydrogenase; POD, peroxidase. H, p-hydroxyphenyl; G, guaiacyl; S, syringyl.

There are little evidences about the mechanism by which FA is esterified into GAX. The simplest mechanism proposed is that GAX may be synthesized in the Golgi apparatus and then feruloylated by feruloyl transferases using FA-CoA transported from cytosol (Buanafina, 2009). However, a more complex mechanism is also possible. The reasoning behind the second mechanism is putative involvement of *BAHD acyl-CoA transferase* genes in feruloylation of glucuronoarabinoxylans (GAX) as proposed by Mitchell *et al.* (2007), based on a bioinformatics approach. Expressed sequence tags (ESTs) from grasses and dicots were

compared to identify clades where genes were much more highly expressed in grasses than their homologs in dicots. One of the clades showing the greatest bias was within the *BAHD acyl-CoA transferase* gene family (carrying the PFAM PF02458 domain). (St-Pierre & Luca, 2000) named the *BAHD acyl-CoA transferase* family after the first four characterized members: 1) benzylalcohol *O*-acetyl transferase from *Clarkia breweri* (**B**EAT); 2) anthocyanin *O*-hydroxycinnamoyl transferases from *Petunia*, *Senecio*, *Gentiana*, *Perilla*, and *Lavandula (***A**HCTs); 3) anthranilate *N*-hydroxycinnamoyl/ benzoyltransferase from *Dianthus caryophyllus* (**H**CBT) and; 4) deacetylvindoline 4-*O*-acetyltransferase from *Catharanthus roseus (***D**AT) (Mitchell *et al*., 2007; Tuominen *et al*., 2011).

Experimental support for the involvement in GAX feruloylation was obtained from transgenic rice where the candidate genes were suppressed by RNAi, resulting in significant decreases in cell wall ferulate (Piston *et al.*, 2010). In another study, rice lines where one *BAHD* gene in the clade was up-regulated increased the amount of *p*-coumaroyl ester-linked to GAX (Bartley *et al.*, 2013). Taken together, these studies provide a strong circumstantial support for the hypothesis that *BAHD* genes are responsible for GAX feruloylation, although definitive evidence is still lacking. As *BAHD* proteins can be localized in the cytosol, another mechanism has been suggested to explain feruloylation as shown in Figure 4 (Molinari *et al.*, 2013). The precursor of GAX UDP-arabinose would be feruloylated to form FA-Ara-UDP which then enters into the Golgi apparatus. After, a glycosyl transferase introduces the FA-Ara into the nascent GAX chains (Figure 4). However, a rice *xax1* mutant had been identified to be unable to produce a specific Ara substitution in xylan. The mutant presented an unexpected decrease in feruloyl and coumaroyl esters content. No decrease in feruloyl esters would be expected if the substrate for feruloylation were UDP-feruloyl-arabinose (Chiniquy *et al.* 2013). The last steps in both mechanisms occur in the Golgi apparatus, and the feruloylated glucuronoarabinoxylan (FA-GAX) is exported to apoplast by exocytosis (Buanafina, 2009; Lenucci *et al*., 2009). The involvement of esterification models involving transport of FA from RE pathway has been partially discarded. [^{14}C]Ferulated polysaccharides were observed both in protoplasm and apoplast when wheat roots were incubated with *trans*-[U-^{14}C]cinnamic acid or L-[1-^{14}C]arabinose. The appearance of [^{14}C]ferulated polysaccharides occurred even in the presence of the lactone antibiotic brefeldin A, which is used to suppress the transport between RE and Golgi apparatus (Mastrangelo *et al.*, 2009).

It is well established that oxidative coupling of polysaccharides through ester-linked feruloyl groups might occur both within the protoplast in the Golgi apparatus (dos Santos *et al.,*

2008b; Mastrangelo *et al*., 2009; Umezawa, 2009), and after their secretion into the apoplast. This process is catalysed by multiple cell wall bounds and putative Golgi resident isoperoxidases, which use hydrogen peroxide as substrate in a mechanism similar to that of lignin polymerization (Fry, 1986; Hatfield *et al.*, 1999; Lindsay & Fry, 2008).

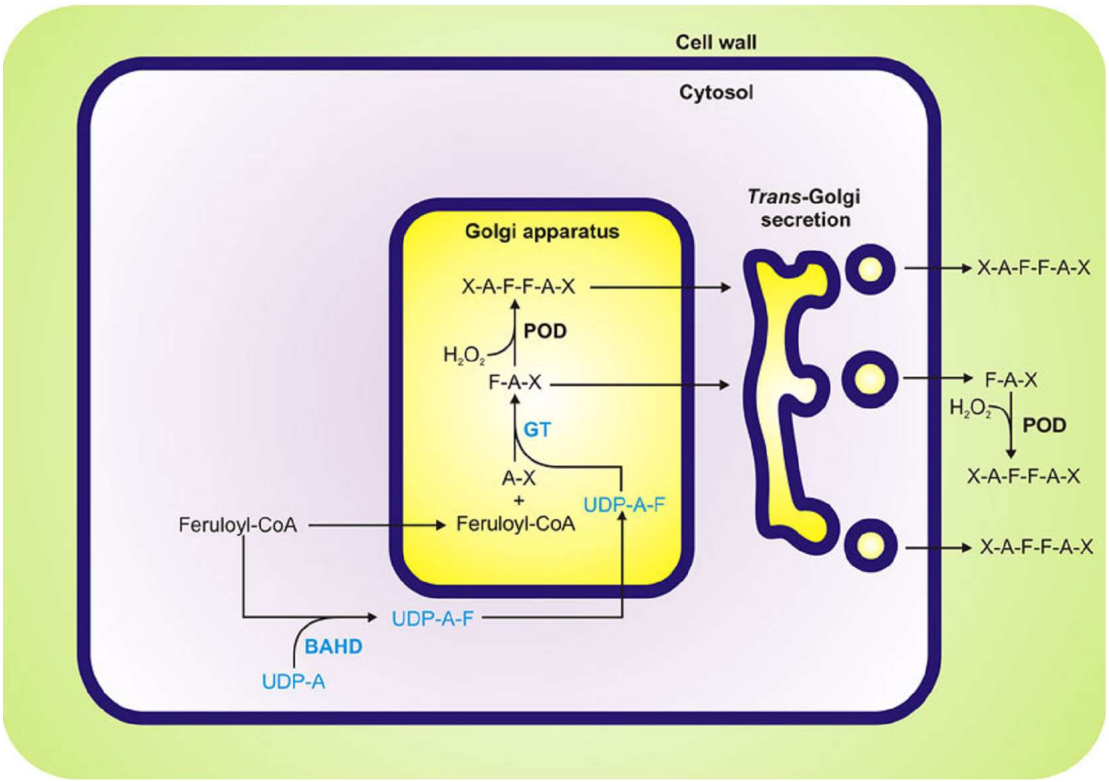


Figure 4. Blue text shows possible pathways for feruloylation based on cytosolic location of BAHD proteins implicated in feruloylation. A, arabinosyl; F, feruloyl group; F-A-X, feruloylated arabinoxylan; GT, putative glycosyl transferase; POD, peroxidase; UDP-A, UDP-arabinofuranose; X, xylan polymer. (Buanafina, 2009; dos Santos *et al.*, 2008a; Nair *et al.*, 2004; Umezawa, 2010).

The apoplastic polymerization among FA residues esterified to GAX has been related with cessation of cell wall growth and defence-related mechanisms. Ferulic acid residues couple when they are oxidatively activated by radicals produced by peroxidases or external oxidizing agents, such as chemical oxidants or UV radiation (dos Santos *et al*. 2008b; Mastrangelo *et al.*, 2009; Umezawa, 2009).

The level of FA in the cell wall is related to biomass recalcitrance

Cross-linking of grass cell wall components, specially through FA and dehydrodimer diferulate esterified to GAX, affects many cell wall properties, such as adherence, extensibility,

accessibility and biodegradability (Bunzel, 2010; Ralph *et al.*, 1998). Besides its relevance in plant physiology and crop protection, these properties are of great interest in food and agricultural chemistry, food technology and nutritional sciences (Barros-Rios *et al.*, 2012). An additional consequence of FA dehydrodimer cross-linkages of GAX is the reduction of digestibility of cell wall polysaccharides by polysaccharidases, a major limitation for conversion of biomass in ethanol (Chen & Dixon, 2007a; Grabber *et al.,* 2009; Jung *et al.*, 2013). Several *in vitro* enzyme digestion assays suggest that cell wall digestibility and FA level are negatively correlated (Grabber *et al*., 1998b; Grabber *et* al., 1998a; Iiyama *et al.*, 1994; Kamisaka *et al*., 1990). FA dehydrodimer cross-linkages can reinforce the cell wall against action of cellulases, xylanases, pectinases (Akin *et al*., 1993; Damásio *et al.*, 2012; Wojtaszek, 1997) and laccases from pathogens (Arora & Sharma, 2009; Sterjiades *et al.,* 1993). In sugarcane bagasse, approximately 50% of the FA content is ester-linked to hemicelluloses (Harris & Trethewey, 2010; Xu *et al.*, 2005), and all ether and dehydrodimer cross-links involve ester links that can be cleaved by alkalis and feruloyl esterases (Fazary & Ju, 2007; Harris & Trethewey, 2010).

Enhancement of enzymatic digestibility using feruloyl esterases

Synergy between cellulases, hemicellulases and accessory enzymes with carboxylic ester hydrolases potentiates the saccharification process. Among the carboxylic ester hydrolases, the feruloyl esterases deserves special attention (Wong, 2006). The term feruloyl esterase denotes a group with several close-related enzymes able to catalyse the cleavage of ester linkage between hydroxycinnamoyl substrates and polysaccharides. Due to their varied degree of substrate specificity, they can be named as feruloyl esterase, cinnamoyl hydrolases, *p*-coumaroyl esterases or hydroxycinnamoyl esterases (Koseki & Fushinobu, 2009).

It has been demonstrated that bacteria and fungi secrete feruloyl esterases to hydrolyse the ester bond between FA and polysaccharides (Topakas *et al*., 2007; Wong, 2006). Feruloyl esterases are able to release phenolic acids and its bio-products from biomass and are important tools for understanding structural differences and pattern of FA esterification in plant biomass (Zhang *et al.*, 2013; Wong *et al.*, 2013). Damásio *et al.* (2012) demonstrated that FA and *p*-coumaric acid can be enzymatically extracted from sugarcane bagasse by using a feruloyl esterase from *Aspergillus clavatus*. In pulp and paper process, application of feruloyl esterase contribute to water removal from pulp (Record *et al.*, 2003; Sigoillot *et al.*, 2008).

Several studies have demonstrated that feruloyl esterases show synergy with xylanases, cellulases, pectinases and accessory enzymes in the degradation of cell wall (Faulds *et al.*, 2006; Selig *et al.*, 2008; Wong *et al.*, 2013). Gottschalk *et al.* (2010) reported a synergic action between cellulase, xylanase, β-glucosidase and feruloyl esterase from *Trichoderma reesei* and *Aspergillus awamori* in the hydrolysis of sugarcane bagasse. The capacity of feruloyl esterase to hydrolyse ester bonds between carbohydrates residues and phenolic compounds is thought to enhance the access of hydrolases to the polysaccharides (Faulds *et al.*, 2006; Wong, 2006). This synergy reduces the amount of enzyme necessary to achieve saccharification and the bioethanol production costs from lignocellulose biomass (Tabka *et al.*, 2006).

Digestion enhancement by down-regulation of enzymes from phenylpropanoid pathway

Li *et al.* (2008) reported that different studies have obtained different findings on the impact of down-regulation of certain gene on lignin content and digestibility. Some studies suggest that down-regulation of genes early in the phenylpropanoid pathway, such as PAL, C4H, HCT and C3´H, reduced strongly lignin content and biomass (Chen and Dixon, 2007; Li *et al.*, 2008; Poovaiah *et al.*, 2014), while down-regulation of F5H or COMT reduced the lignin S/G ratio, but has a smaller effect on lignin (Li *et al.*, 2008) or digestibility (Reddy *et al.*, 2005). Chen and Dixon (2007) investigated six alfalfa lines independently down-regulated for C4H, HCT, C3´H, F5H, CCoAOMT or COMT. Alfalfa lines suppressed in C4H, HCT and C3´H showed the lowest lignin level (<50%) with enzymatic saccharification efficiencies that were almost double that the controls. On the other hand, lines suppressed in COMT, CCoAOMT and F5H produced little effect on lignin content and digestibility efficiencies. In contrast with data produced by Chen and Dixon (2007), in alfalfa, Zhong *et al.* (2000) found that poplar transgenic lines with up 80% reduction in CCoAOMT presented up to 40% reduction in lignin content.

Yet, down-regulation of COMT in maize resulted in biochemical alterations of lignin content and increased cell wall digestibility (Piquemal *et al.*, 2002). Suppression of COMT by RNA interference also decreased biomass recalcitrance up to 23%, without reducing the production of sugarcane biomass (Jung *et al.*, 2013). The down-regulation reduced up to 6% the lignin content; however, it was not able to reduce content of FA in the cell wall.

Down-regulation of enzymes from phenylpropanoid pathway may affect feruloylation, but they are known to produce effects on lignin content, digestibility and in plant growth and development (Boerjan *et al.*, 2003; Li *et al.*, 2008; Verma and Dwivedi, 2014). Jung and Phillips

(2010) have identified and demonstrated a putative mutation in maize seedling that reduce the content of ferulate ester and ether cross-linking in the cell wall and increase the biomass digestibility without affecting plant growth and yield.

The enzyme CCoAOMT catalyses the synthesis of FA-CoA, an intermediate of monolignols biosynthesis, but also, the active for required for polysaccharide feruloylation. The probable participation of CCoAOMT on feruloylation makes it an interesting target for downregulation in grasses to improve saccharification. Nair *et al.* (2004) identified a gene in *Arabidopsis thaliana* mutant that encodes a functional aldehyde dehydrogenase (ALDH). It oxidizes both sinapaldehyde and coniferaldehyde to sinapic acid and FA, respectively. The authors described other plant species with ability to synthesize FA from coniferaldehyde in dicots (*A. thaliana*, *Nicotiana tabacum* and *Raphanus sativus*), monocots (*Zea mays*), gymnosperm (*Pinus strobus*) and pteridophyte (*Ceratopteris richardii*). The 4CL has demonstrated to be active *in vivo* on exogenous ferulic acid, improving both polysaccharide feruloylation and G lignin (dos Santos *et al.* 2008a). So, 4CL is a plausible candidate to perform activation of FA to FA-CoA. If so, CALDH can be another interesting target to gene suppression in order to control feruloylation and biomass digestibility.

Conclusions

Herein, we reviewed the role of FA in the plant cell wall architecture and physiology. A better understanding of FA metabolism, its esterification to the cell wall polysaccharides and the controlled coupling mechanisms will contribute to our understanding on plant physiology, but also to develop new technologies to control the cell wall properties. Such know-how will impact in several areas, from improvement of cattle feed and organoleptic properties of vegetables to production of paper and cellulosic ethanol, a technology that promises to revolutionize the way civilization to obtain energy.

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Chapter 2. Research article

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

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Abstract

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.

Keywords: Bioenergy; Cell wall; Enzymatic saccharification; Ferulic acid; Lignocellulose

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.

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 of cellulose, 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% to 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.

Material and Methods

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 (23º 25' S, 51º 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.

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 (6,750×*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 ºC 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.

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 (2,180×*g* at 4 ºC 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.

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 4,000×*g* for 10 min. The supernatant was discarded and the solid fraction was washed three times with 80% (*v/v*) ethanol following the centrifugation (4,000×*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.

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 ºC 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 (1,400×*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.

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.

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} to 4000 cm^{-1}, and the laser power at the sample was 150 mW. A Nd:YAG laser was used for excitation at 1064 nm.

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.

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 2 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].

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.

Results and Discussion

Ethanol-soluble extractives

The levels of compounds in ethanol-soluble extractives, aromatic compounds and soluble proteins differed markedly among cultivars (Table 1). 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 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 to 31% (Table 1). 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].

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]

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 to 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].

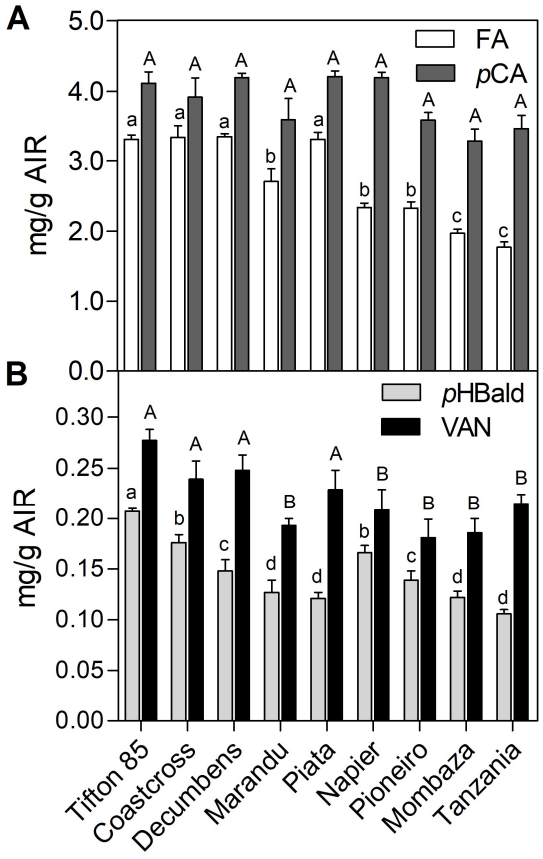

Figure 1. Profiles of cell wall-bound phenolics of forage grasses. A) Ferulic acid (FA) and *p*-coumaric acid (*p*CA), B) *p*-hydroxybenzaldehyde (*p*HBald) and vanillin (VAN). Mean values ± SEM (n = 5) marked with different letters are significantly different ($P \leq 0.05$, Scott-Knott test).

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].

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) in Tifton 85 to 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 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].

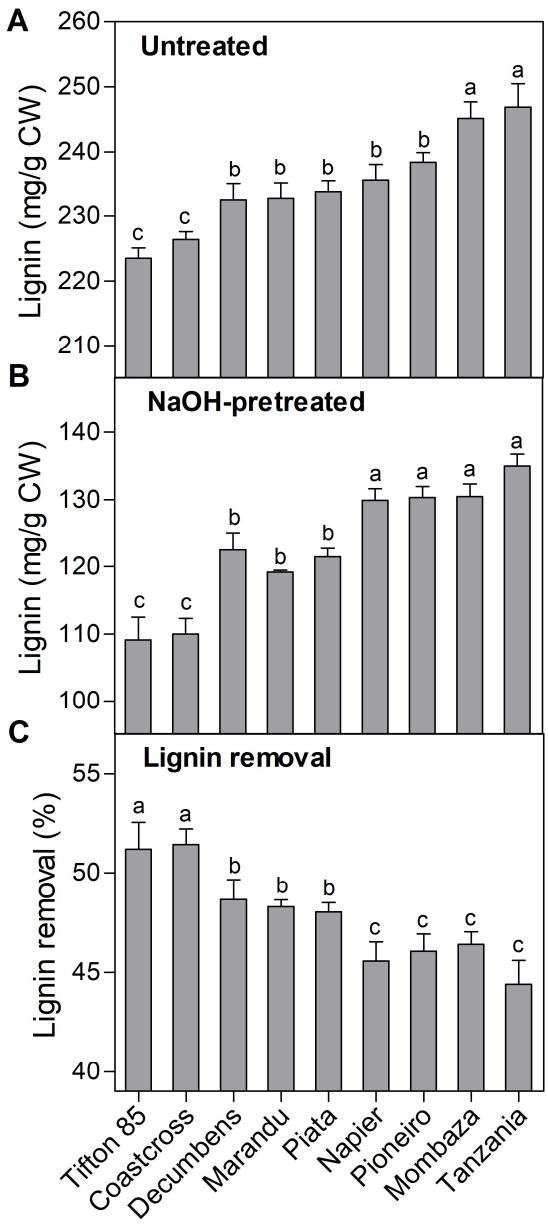

Figure 2. Profiles of lignin content of forage grasses. A) Total lignin of untreated samples, B) NaOH-pretreated samples, and C) lignin removal. Mean values ± SEM (n = 5) marked with different letters are significantly different ($P \leq 0.05$, Scott-Knott test).

Monosaccharide profile

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

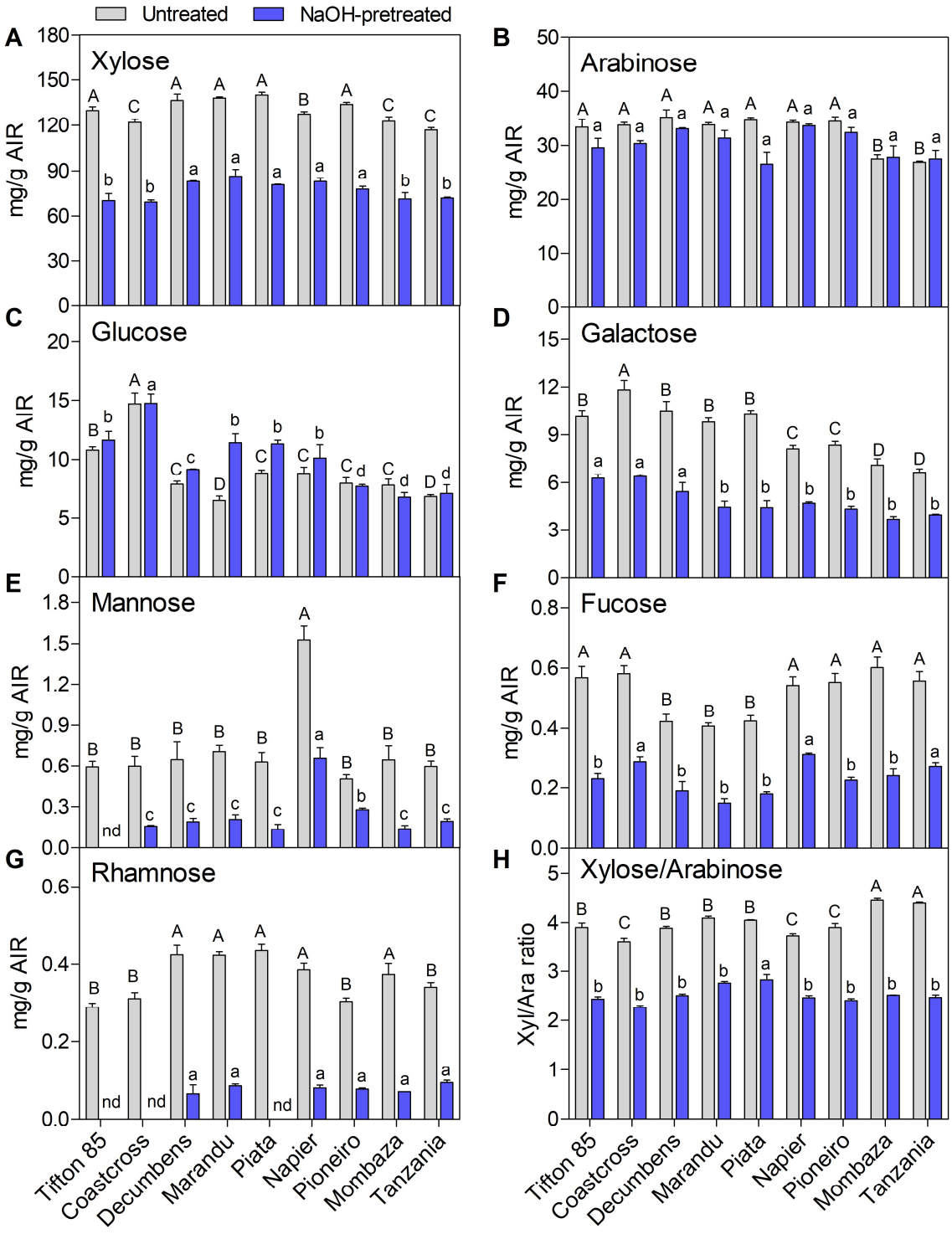

Figure 3. Non-cellulosic monosaccharide profiles of forage samples ranked by their lignin content. Mean values ± SEM (n = 3-4). Uppercase letters indicate significant differences between untreated samples; lowercase letters indicate significant differences between pretreated samples ($P \leq 0.05$, Scott-Knott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

Rhamnose (Fig. 3G), typically found in pectins, was lower than 0.43 mg/g AIR in all forage grasses, similar to contents previously 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].

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 (Fig. 4 and 5); 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). 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–C).

Table 3. Assignment of the main bands of spectroscopic analysis. 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/R)
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)

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].

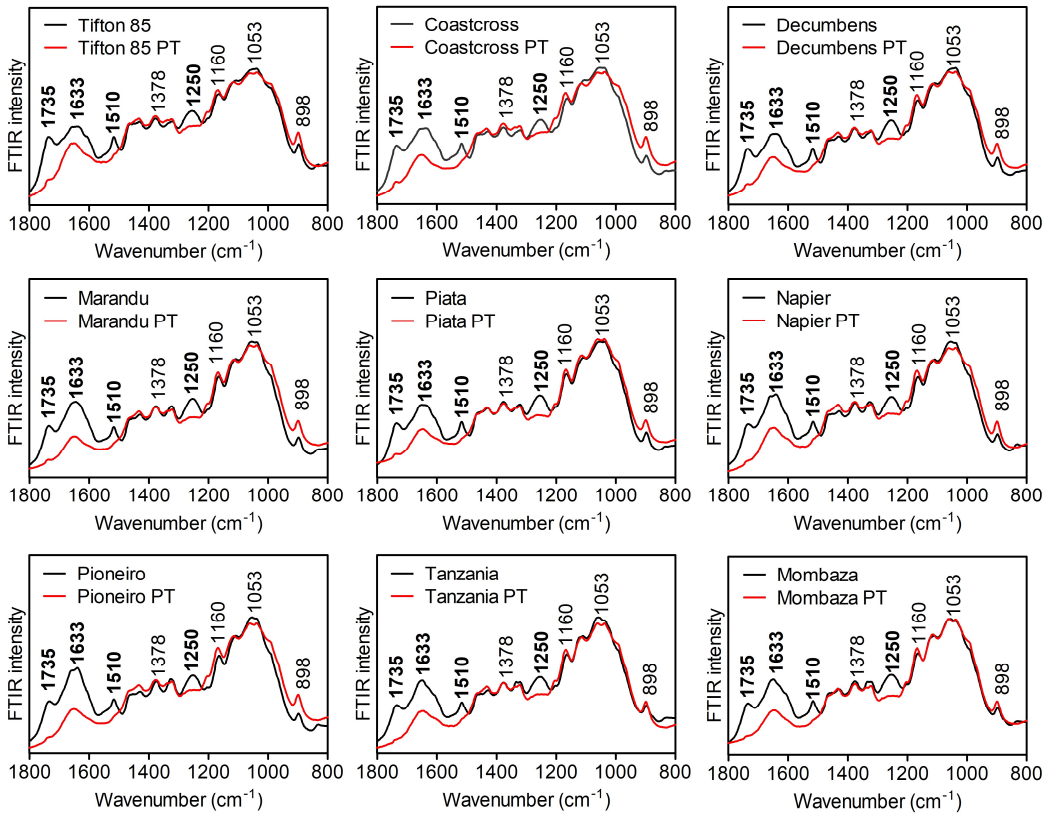

Figure 4. FTIR spectra of untreated and NaOH-pretreated (PT) forage grasses. Differences between untreated and pretreated biomasses are presented in bold. Band at 1735 cm^{-1} corresponds to ester-groups of xylan; 1633, 1600, and 1510 cm^{-1} correspond to lignin; 1250 cm^{-1} corresponds to xylan; 1378,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

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.

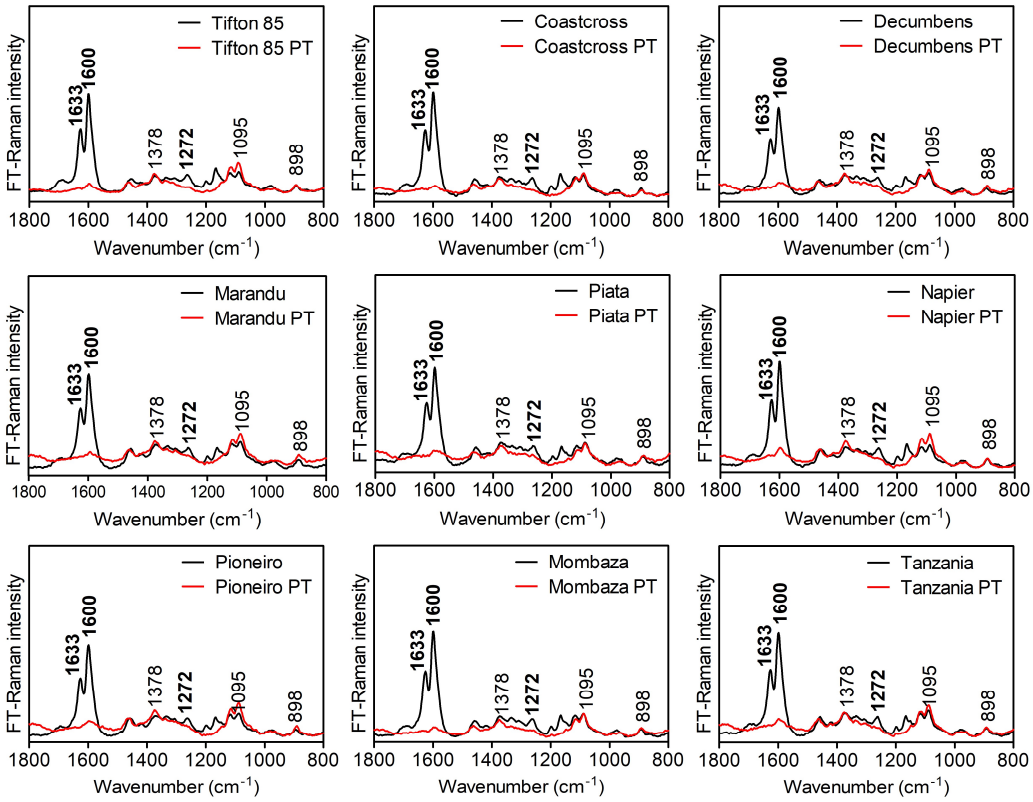

Figure 5. Raman spectra of untreated and NaOH-pretreated (PT) forage grasses. Differences between untreated and pretreated biomasses are presented in bold. Bands at 1633, 1600, and 1272 correspond to lignin, 1095 and 898 cm^{-1} correspond to cellulose.

Table 2. Enzymatic activities from *Aspergillus 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.

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

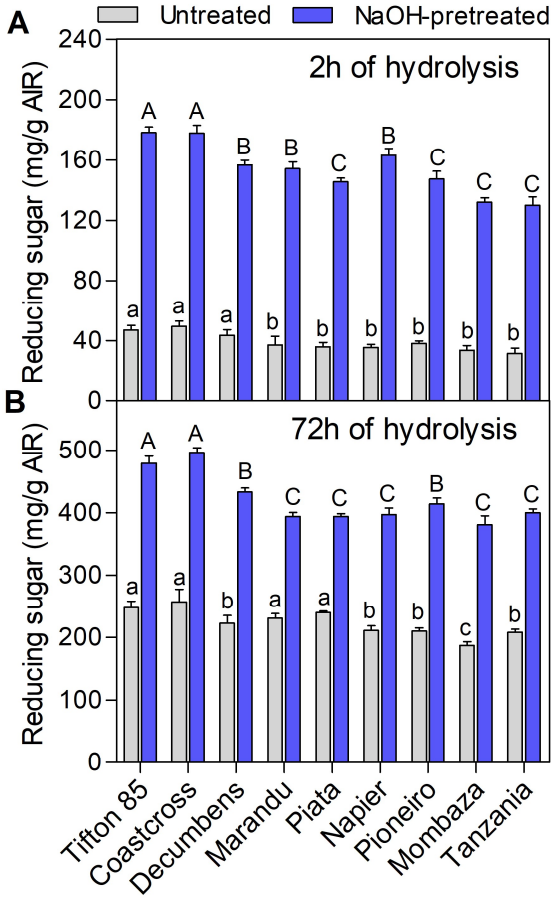

Figure 6. Enzymatic saccharification of untreated and pretreated forage grasses at 2 h (A) and 72 h (B) of hydrolysis. The samples are ranked by their crescent lignin content. Mean values ± SEM (n = 5). Uppercase letters indicate significant differences between untreated samples; lowercase letters indicate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

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 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.

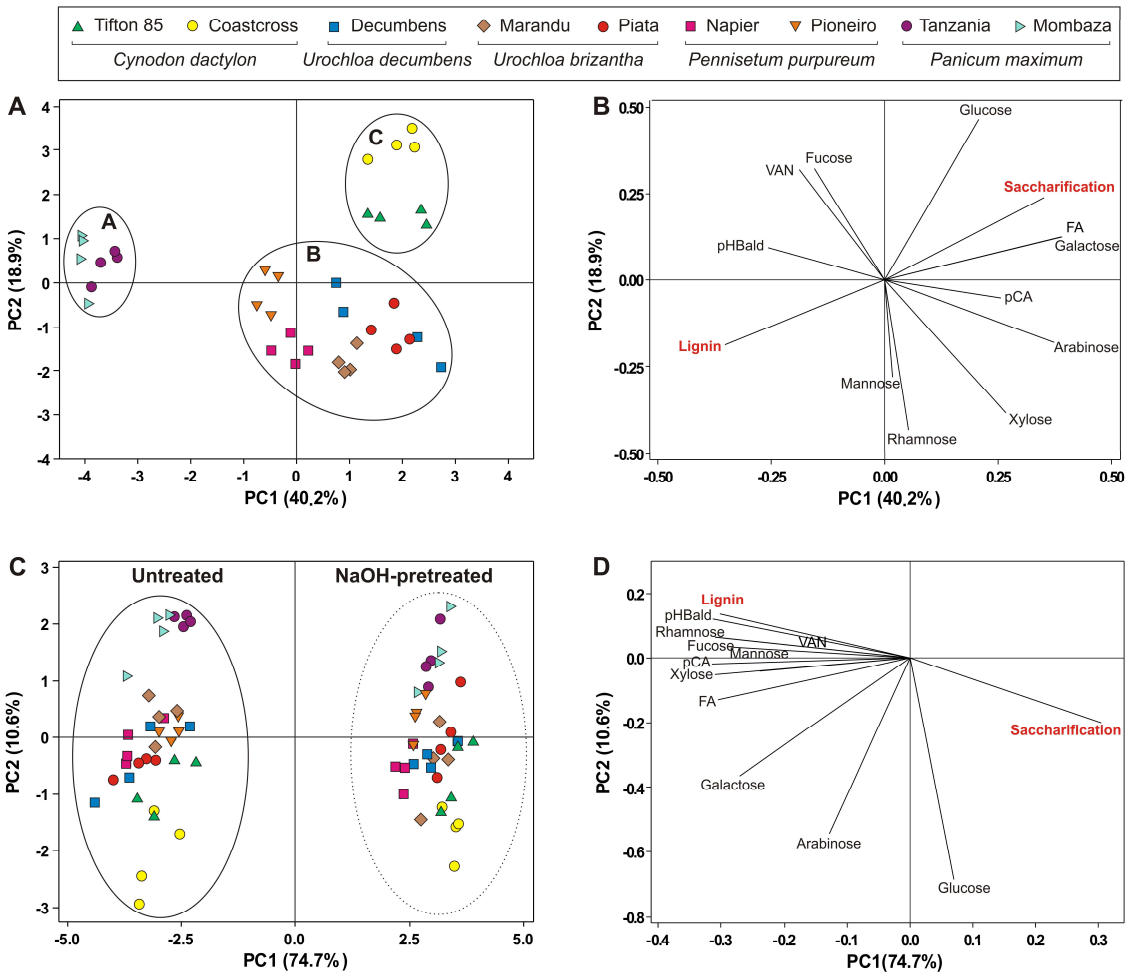

Figure 7. Principal component analysis (PC1 and PC2) of thirteen different cell wall traits of forage grasses. A) Distribution of forage grasses for untreated biomasses in the plane defined by the first and second main components; B) relationship among variables of biomass composition; C) forage grasses 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.

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).

Table 4. Eigenvalues and proportions of variance corresponding to each of the axes (PC1 and PC2) generated by the Principal components analysis (PCA) of the nine forage grasses. Column Treatments corresponds to untreated and pretreated plants and column Cultivars corresponds the analysis by distribution of cultivars without pretreatment (**Figure 5**). Values of the coefficients calculated for each of the variables measured along the experiment of monosaccharides, saccharification and lignin parameters. 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 bold/italic correspond to 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 72h	**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	-	-

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.

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.

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Competing interests

The authors have no conflict of interest.

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