Chemical and histological characterization of internodes of sugarcane and energy-cane hybrids throughout plant development

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ABSTRACT

Sugarcane and energy-cane are important *Saccharum spp*. hybrids that encompass desirable features for energy production. Their stem lignocellulose can be used as the raw material for the second-generation ethanol industry, contributing to accelerated fossil-fuel replacement. However, knowledge on the internode composition and saccharification of these hybrids is still needed to optimize their uses, especially in energy-cane. In this work, the agronomical, chemical, and histological internode features and saccharification potential were analyzed in sugarcane and energy-cane hybrids across four critical developmental stages (tillering, grand growth, early ripening and late ripening) and several internode positions along the stem. Except for galactose content that was higher in the sugarcane hybrid compared to the energy-cane, cell-wall composition was fairly similar in both types of cane across the growing cycle. Among main cell-wall components, maximum crystalline cellulose and matrix polysaccharides contents were observed at tillering and late ripening, respectively. Lignin concentrated towards upper internode positions and advanced developmental stages. Saccharification potential augmented towards apical internodes and early developmental stages, and negatively correlated to lignin and xylose contents. The energy-cane hybrid showed lower number of vascular bundles and metaxylem diameter and its lignin deposition was more extended towards the parenchymal tissue, when compared to the commercial sugarcane throughout the growth cycle. Taken together, these findings demonstrate that developmental stage and internode position largely affect cell-wall composition and its recalcitrance to enzymatic degradation. The use of sugarcane/energy-cane lignocellulose from early developmental stages could be beneficial for the second generation ethanol production. Novel management practices for that usage should consider economic, social and environmental sustainability standards under a sugar/ethanol production scheme.

Keywords: Sugarcane – Energy-cane - Cell-wall - Lignin - Vascular bundles – Second generation ethanol

Abbreviations

1G	First generation
2G	Second generation
ABSL	Acetyl bromide soluble lignin
AIR	Alcohol-insoluble residue
ANOVA	Analysis of variance
CC	Crystalline cellulose
DM	Dry matter
DW	Dry weight
FW	Fresh weight
GDD	Growing degree days
INTA	Instituto Nacional de Tecnología Agropecuaria
IP	Internode position
MP	Matrix polysaccharides
TVD	Top visible dewlap
TFA	Trifluoracetic acid
VB	Vascular bundles

1. Introduction

Lignocellulosic biomass from energy crops is a renewable energy resource, capable of significantly contributing to the reduction of greenhouse gasses emissions and to the replacement of fossil-fuels [1, 2, 3]. Among different energy crops, *Saccharum spp.* hybrids stand out for its highly efficient production of biomass together with other important desirable features, such as a highly positive carbon balance and a long harvest period [4, 5, 6]. The lignocellulose of two biotypes of *Saccharum spp.* hybrids, *i.e.* commercial sugarcane and energy-cane, can be used for energy purposes [7, 8].

Commercial sugarcane and energy-cane differ in origin and agronomic features. Sugarcane has been improved through traditional breeding methods to achieve outstanding sucrose concentration levels in the stems. This originates from the prevalence of *S. officinarum*'s genes (the high-sugar domesticated species) in their genetic background [9]. By contrast, energy-cane is a new biotype of *Saccharum* producing high biomass yield and stem fiber, showing a prevalence of *S. spontaneum* (resilient wild species) genes in their genome [10, 11, 12]. Based on its stem composition, energy-cane is commonly classified in type I, a multipurpose type of cane with 17% fiber and 13% sucrose, and type II, an energy dedicated cane with 30% fiber and 5% sucrose [13]. The stem lignocellulose -known as bagasse - of sugarcane and energy-cane is a suitable raw material for the 2G ethanol industry [6, 8, 14].

The sugarcane/energy-cane vegetative development, under commercial conditions, begins with the sprouting of axillary buds from underground cane stem pieces [15]. The crop enters a tillering stage, consisting in the appearance of new shoots from the base of the ones previously established, followed by a grand growth period, where rapid vegetative growth and stem elongation occur. The ripening stage involves slow vegetative growth and progressive sucrose accumulation in the parenchyma cells of the stem [16]. Under subtropical conditions of cultivation, the sugarcane harvest period begins early at the ripening stage to mitigate winter frost damage and extends to later ripening stages [17].

The sugarcane stem is constituted by a series of intercalated cylindrical nodes and internodes. Stem internodes become fully elongated when the internode achieves four positions below the internode carrying the first fully expanded leaf [18]. The cell division

and cell expansion of the internode enable the stem to increase in size, both radially and longitudinally [19]. The vascular bundles differ in size, shape and spacing, as well as in number and distribution among different varieties of sugarcane. A vascular bundle contains the xylem, encompassing protoxylem cells flanked by two metaxylem elements, the phloem and parenchyma cells, linking both tissues. The bundles are surrounded by a sheath of fiber cells with thick sclerenchyma walls [19, 20, 21].

In recent years, knowledge on the cell-wall composition of energy crops has become critical due to its impact on the efficiency of the 2G ethanol production process. Plant cell-walls are a complex array of biopolymers that includes cellulose, hemicellulosic polysaccharides and lignin [22]. These three major components represent 38–43%, 25–32% and 17–24%, respectively, of the cell-wall composition in mature sugarcane stems [23]. However, wide genetic variability has been reported for cell-wall components in sugarcane breeding populations [24, 25] and *Saccharum spp.* germplasm of diverse origin [26].

Cell-wall polymers form a network that hinders polysaccharide hydrolysis and consequently reducing the cellulosic conversion for 2G ethanol production. To reduce this recalcitrance, pretreatments are necessary to achieve significant fermentable sugar yields, increasing the cost of the process on an industrial scale [8, 27, 28, 29]. There is wide consensus in the scientific literature attributing a central role to lignin in biomass recalcitrance [30, 31, 32, 33]. Lignin, formed by an overly complex array of phenylpropanoid polymers, decreases enzyme access to fermentable polysaccharides and restricts their activity by unproductive associations with enzymes [34].

In the past few years, several publications have focused on the internode composition of modern sugarcane and their ancestral species, owing to the increasing interest in the energetic applications of the crop [16, 35, 36, 37, 38]. Deepening the knowledge of the composition of sugarcane and energy-cane internodes during plant development would provide information to optimize its cultivation for sustainable bioenergy uses, particularly in energy-cane as many aspects about their physiology, chemical composition, and agronomic features remain unknown. In addition, better understanding of the effect of lignin and the monosaccharide array of cell-wall matrix polysaccharides (MP) on cell-wall recalcitrance is necessary to optimize pretreatments for sugarcane and energy-cane use in the 2G ethanol industrial process [39].

Here, novel approach that combines plant developmental stages (temporal profiles) and internode positions (spatial profiles) was used to characterize the agronomic, chemical and anatomical features of internodes, along with saccharification of the lignocellulose in sugarcane and energy-cane. Our findings provide a new insight on internode cell-wall compositional changes across sugarcane/energy-cane development and its enzymatic digestibility. We show the different anatomical features and tissue lignification patterns of the contrasting hybrids studied and provide information that can help to optimize their cultivation for energy purposes.

2. Materials and methods

2.1. Experimental design and sample collection

Two contrasting *Saccharum spp*. hybrids were used in this experiment: LCP 85-384, the main commercial sugarcane cultivar planted in Argentina [40], hereafter referred to as LCP384; and INTA 05-3116, an energy-cane hybrid developed by the Sugarcane Breeding Program of Instituto Nacional de Tecnología Agropecuaria (INTA), Argentina, hereafter referred to as INTA3116. Second-ratoon sugarcane stems (regrowth after second harvest) were randomly collected from a field experiment carried out at Estación Experimental Famaillá of INTA (27°03'S, 65°25'W, 363 masl, Tucumán province, Argentina). Five stems from each genotype were cut at ground level at each of the following developmental stages: tillering (December), grand growth (February), early ripening (May) and late ripening (August). These developmental stages were chosen for being critical for determining agronomic performance across the sugarcane cropping cycle [19].

Internode position (IP) was defined as the internode number counted from ground level; therefore, internodes retain their position during plant development allowing easier interpretation of internode compositional changes. The number of internodes sampled increased with plant development, thus IP1 was collected at tillering, IP1 and IP5 at grand growth, IP1, IP5, IP10 and IP15 at early ripening and IP1, IP5, IP10, IP15 and IP20 at late ripening. Additionally, the number of internodes below top visible dewlap (TVD) leaf was counted at each stage to consider the internode physiological age in the data analysis. Internodes from three out of five collected stems (biological replicates) were used to determine agronomic, chemical and saccharification performances, whereas internodes from the remaining two stems were used for histological analysis.

2.2. Meteorological and phenotypic data

Maximum and minimum daily temperatures (°C), and daily rainfall (mm) recorded at the Meteorological Station of INTA Famaillá (http://siga2.inta.gov.ar/#/data) were used to calculate cumulative rainfall and heat units requirement for internode development. The latter was expressed in growing degree days (GDD), according to the following formula [41, 42]:

$$GDD\ (ºC\ d^{-1}) = ((T\ max + T\ min)/2) - Base\ temperature$$

where Base temperature = 18 ºC.

2.3. Agronomic traits measurement

Length, diameter and fresh weight (FW) were measured in the internodes that were used for chemical analysis. Thereafter, juice extracted from these internodes were used for determining brix percentage using a digital brixometer. To determine dry weight (DW) and dry matter (DM) content, internodes were placed in a forced air oven at 60 ºC until reaching constant weight, and stored at room temperature until chemical analysis. Brix, FW, weight after juice extraction and DW were used to estimate fiber content as described by Fernandes [43]. At early and late ripening stages, the reminder portion of the stems was used to determine brix and pol (apparent sucrose content) in juice and calculate juice purity and pol in cane as described by Acreche *et al.* [44].

2.4. Cell-wall preparation

Alcohol-insoluble residue (AIR) was prepared from dried milled samples following the protocol described by de Souza *et al.* [45]. Falcon tubes with 1 g of sample were filled with 50 mL of 80% (v/v) ethanol and placed in distilled water at 80 ºC for 20 min (tubes were manually shaken several times), a centrifuge was used to separate phases and the supernatant was discarded. After performing six successive ethanol extractions, the AIR obtained was washed with 50 mL of distilled water and oven-dried at 40 ºC.

2.5. Compositional analysis

Dried AIR samples were used to study the internode cell-wall chemical composition as described in previous work [46, 47]. The measurements were conducted by triplicate (technical replicates) for each of the cell-wall components.

2.5.1. Lignin

Acetyl bromide soluble lignin (ABSL) was determined following the protocol described by Fukushima and Hatfield [48]. Four milligrams of powdered sample were weighted into 2 mL tubes and 250 µL of acetyl bromide solution (25% v/v acetyl bromide/75% glacial acetic acid) were added. Samples were heated at 50 °C for 2 h, heated for an extra hour with intermittent vortexing and were cooled at room temperature. The liquid was transferred to a 5 mL flask and 1 mL 2 M NaOH was added. Then, 175 µL of 0.5 M hydroxylamine HCl was added, and the flask was filled up to 5 mL with glacial acetic acid and mixed. The absorbance of the samples diluted 1:10 in glacial acetic acid was measured at 280 nm and the ABSL content was determined as follows:

$$\text{ABSL (\%)} = \{\text{absorbance}/ (\text{coefficient} \times \text{pathlength})\} \times \{(\text{total volume} \times 100\%)/\text{biomass weight})\}$$

The coefficient used in the formula was 17.75 (grasses).

2.5.2. Matrix polysaccharides content and monosaccharide analysis

Four mg of dried AIR samples were weighted in a 2 mL capped tube and hydrolyzed by adding 0.5 mL of 2 M Trifluoracetic acid (TFA). Vials were flushed with dry argon, mixed and heated at 100 ˚C for 4 h, vortexing every hour. TFA was evaporated by using a centrifugal evaporator with fume extraction. To the TFA pellets, 500 µL of propan 2-ol were added, mixed and evaporated (twice). Samples were resuspended in 200 µL of deionised water and mixed. The supernatant was placed in a new tube and filtered with 0.45 µm PTFE filters. The monosaccharide profile of non-cellulosic polysaccharides was analyzed using a high-performance anion-exchange chromatography on a CarboPac PA-20 column with pulsed amperometric detection [49]. An equimolar mixture of monosaccharide standards (external calibration), also previously treated with 2 M TFA, was used for quantification of individual monosaccharides.

2.5.3. Crystalline cellulose (CC)

TFA pellets obtained as described in the previous section, were washed with 1.5 mL of water and then three times with 1.5 mL of acetone before drying the samples at room temperature overnight. Complete hydrolysis was performed by adding 90 µL of 72% (w/v) sulfuric acid. Thereafter, 1.89 mL of water was added and heated at 120 ºC for 4 h. The colorimetric Anthrone assay, with a glucose standard curve, was used to determine glucose content in the supernatant.

2.6. Enzymatic saccharification analysis

Saccharification potential of AIR samples was assessed using an automated robotic platform, following the protocol described by Gomez *et al.* [50]. Saccharification measurement of each AIR sample was conducted by triplicate. Firstly, samples were placed into 96-well plates using a custom-made robotic platform (Labman Automation, Stokes- ley, North Yorkshire, UK). Samples were subjected to an alkaline pretreatment at 90 ˚C for 30 min, followed by enzymatic hydrolysis using an enzyme cocktail of Celluclast and Novozyme 188 (Novozymes Enzymes) in a 4:1 ratio and released reducing sugars were quantified.

2.7. Histological analysis

Stem sections of approximately 2 cm were manually cut at the internode midpoint in each sampling of the study. Sections were fixed in a FAA (formol -alcohol -acetic acid) solution for 48 h and preserved in alcohol 70%. Hand sections were stained using phloroglucinol-HCl (Wiesner reagent) for 5 to 10 min. This reagent stains bright red those regions containing lignin, varying the intensity with the lignification [51]. The stained sections were rinsed in distilled water, mounted and examined with bright-fields under an optical transmission microscope (Leica DMRXP). The images were captured with an image size of 1704 x 2272 pixels, using a digital camera (Canon - PowerShot S45 - 4.0 mega pixels).

Software ImageJ V. 1.53 (open-source license, https:/imagej.nih.gov) was used to determine the length and width of vascular bundles (VB), and the metaxylems diameter. From each of the two replicates, 20 to 40 images of VB in the central pith were taken into consideration. Additionally, the number of VB in the pith was measured considering the observed area as the unit of study.

2.8. Statistical analysis

Data analysis was performed using R software v 2.5.2 [52] through an interface with Infostat software [53]. Analysis of variance (ANOVA) with a general linear mixed model approach [54] was used to analyze the effects of genotype, IP and developmental stage on the traits studied. Because of biological restrictions (the full set of internodes were not available at all developmental stages) two independent statistical analyses were used. The first analysis included the effects of genotype, IP and genotype:IP interaction

and was partitioned by developmental stages. The second analysis included the effects of genotype, developmental stage and genotype:developmental stage interaction and was partitioned by IP. A random effect of multiple vascular bundle measurements was incorporated for anatomical data analysis. Akaike Information Criterion and Bayesian Information Criterion indexes were used to select the best-fit model for the two statistical analyses, and Fisher's LSD test was used as a post-hoc mean comparison test. VarIdent function (nlme package) was used for modeling heteroscedasticity [55].

Pearson correlation coefficients were calculated to investigate the association of saccharification potential with other traits of interest in the experiment. Coefficients were calculated using "cor" and "cor.mtest" functions (corrplot R package) [56] and were considered significant for p values <0.05 (*).

3. Results

To provide further insights into the potential for using sugarcane/energy-cane for bioenergy applications, physiological and agronomic features, major cell-wall polymers, non-crystalline monosaccharide composition, and saccharification and histochemical analyses were performed across the temporal and spatial profiles of stem internodes throughout plant development. For this purpose, genotype, internode position (IP), stage, genotype:IP interaction, and genotype:developmental stage interaction effects were assessed for each of the traits studied.

3.1. Internode physiologic and agronomic features

A schematic illustration of the experimental design used for the internode characterization, and the accumulation of growing degree days (GDD) together with daily rainfall data of the growing season, are shown in Fig. 1a and Fig. 1b, respectively. The growing season of the sugarcane crop extended for 317 d with concentrated rainfall and favorable temperatures for growth and development of the plant (Fig. 1b). Cumulative GDD recorded at each sampling date were 409 ºC d^{-1} (tillering), 908 ºC d^{-1} (grand growth), 1106 ºC d^{-1} (early ripening) and 1124 ºC d^{-1} (late ripening) (Fig. 1a). The cumulative rainfall for the period was 1623 mm.

Stem length growth increased with GDD accumulation, but it was faster in INTA3116 (12.1 cm/ºC d^{-1}) than in LCP384 (10.1 cm/ºC d^{-1}) (Table 1) resulting in higher plant height in the case of INTA3116. At tillering, LCP384 accounted for one more

internode than INTA3116, which was the highest internode difference recorded in the experiment (Table 1). Therefore, it can be assumed a closely similar physiological age for internodes collected at the same IP within each developmental stage. Grand growth, a sugarcane plant developmental stage characterized by active stem growth and elongation, was accompanied by the fastest number of internode appearance and stem height in both hybrids. At late ripening, both hybrids developed the same number of internodes below the top visible dewlap (TVD) leaf (Table 1) with an average thermal requirement of 73.7 ºC d-1 per internode.

The internodes of LCP384 were thicker than those of INTA3116 at all stages of development (Table 1). Internodes dry weight (DW) levels in each hybrid at grand growth, and early and late ripening were three times higher than at tillering (Table 1). The internode spatial profiles for length revealed not only that IP5 was the longest and heaviest in both hybrids, but also that internode length diminished towards the stem tip. In addition, dry matter (DM) and brix concentrations in both hybrids continuously decreased from basal to tip internodes. Fiber also decreased towards upper IPs in INTA3116, but in LCP384 depended on the plant developmental stage considered.

Internode length and Fresh Weight (FW) hit maximum levels at grand growth that declined towards the final developmental stages of both hybrids (Table 1). Conversely, the temporal profiles of internode brix, fiber, and DM exhibited average concentrations that steadily increased as plants of both genotypes grew and developed (Table 1), hence their respective accumulation rates responded to the carbon partitioning strategies of each hybrid type. At late ripening, the sugarcane cultivar accounted for 19% higher brix content than the energy-cane hybrid, whereas fiber and DM contents were 121% and 23% higher, respectively, in the energy-cane hybrid (Table 1).

3.2. Lignin

The distribution of lignin across the internode spatial profiles for the two hybrids, showed lower accumulation of this component in top internodes than in the basal ones (Table 2, Fig. 2a), in accordance with the fact that lignification of the wall is still underway in young internodes (Fig. 3). Moreover, the magnitude of this difference increased towards the end of the growing cycle *e. g.* the upper internode at grand growth, early ripening, and late ripening accounted for 2.9%, 4.3% and 11.0% lesser lignin

content, respectively, than the average lignin content of their remaining stem internodes. Lignin content differed between genotypes only at grand growth, where LCP384 exhibited superior lignin values than INTA3116 (Table 2).

The analysis of the internode temporal profiles for lignin showed that maximum contents of this complex polymer were accomplished at late ripening (Table S1). This lignification pattern is consistent with the overall lignin accumulation observed in the stem internodes throughout plant stem development, as average lignin content increased 76% in LCP384 and 62% in INTA3116 from tillering to late ripening (Table 2, Fig. 2a).

3.3. Polysaccharides content

Genotype and genotype:IP interaction had no effect on crystalline cellulose (CC) content across the internode spatial profiles studied; however, IP effect was significant (Table 2). At grand growth, lower CC was observed in IP5 than in IP1, yet at late ripening, lower CC was observed in IP10 than in the rest of the stem internodes (Table 2, Fig. 2b). On average, tillering accounted for the highest CC content in LCP384 (43.3% AIR) and in INTA3116 (38.9% AIR), while the subsequent plant developmental stages registered average CC contents within the range of 26.7-27.5% alcohol insoluble residue (AIR) in LCP384 and 27.4-32.3% AIR in INTA3116 (Table 2). In addition, no significant CC variations were observed across the internode temporal profiles, except for IP10 that showed a CC reduction from early to late ripening in both hybrids (Table S1).

Similar to CC content, genotype and genotype:IP interaction had no effect on the matrix polysaccharides (MP) content across the internode spatial profiles, but a significant effect of IP was observed (Table 2). At grand growth, greater MP content was observed in IP1 (83.8 mg g^{-1} AIR) than in IP5 (61.0 mg g^{-1} AIR); conversely, at early ripening, MP content gradually decreased down the stem from IP15 (79.3 mg g^{-1} AIR) to IP1 (60.4 mg g^{-1} AIR) (Table 2, Fig. 2c). At late ripening, MP content showed no differences among IPs. Analysis of the internode temporal profiles showed that the highest MP concentrations were achieved at late ripening for all the IPs studied (Table S1).

3.4. Monosaccharide composition

The monosaccharide composition of non-crystalline polysaccharides was assessed throughout spatial and temporal profiles as performed for major cell-wall

components. Despite the effects studied had a remarkable impact on the individual monosaccharides amounts (Tables 1 and S1) the ranking of monosaccharides (from the most to the least abundant) had no significant changes (Fig. 3). Xylose was the prevailing monosaccharide in the non-cellulosic fraction (46.1%) showing more than twofold the amount of the following two most abundant monosaccharides, glucose (20.6%) and arabinose (19.0%). Galactose (7.1%), galacturonic acid (3.8%), rhamnose (1.6%), mannose (0.9%), glucuronic acid (0.7%) and fucose (0.1%) were found to a lesser extent (Fig. 3). On average, the sum of pentoses was 50.9 mg g^{-1} AIR and 23.6 mg g^{-1} AIR of hexoses (excluding uronic acids); therefore, the monosaccharide pentoses:hexoses ratio was 2.2:1.

Significant genotype and IP effects, and lack of genotype:IP interaction significant effects were observed along the internode spatial profiles for monosaccharides (Table 2). LCP384 showed higher galactose than INTA3116 in all the internodes analyzed. In addition, the sugarcane cultivar exhibited higher fucose at early ripening, and higher galacturonic and glucuronic acids at late ripening as compared to the energy-cane hybrid (Table 2). At grand growth, all monosaccharides (except for fucose and mannose) were more abundant in IP1 than in IP5. Conversely, at early ripening all monosaccharide contents (except for fucose and glucuronic acid) increased towards the stem apex. Identical trend was detected for fucose, galactose, glucose and sugar acids contents at late ripening (Table 2).

Except for the internode temporal profiles for glucuronic acid that decreased towards the end of the season, internode temporal profiles for remaining monosaccharides showed no tendency across plant developmental stages (Table S1).

3.5. Saccharification potential

Saccharification potential analysis across the internode spatial profiles revealed significant differences between genotypes. At tillering, 26% higher reducing sugars were found in INTA3116 than in LCP384, whereas at grand growth (considering the average value of the internodes studied at this stage) the internode saccharification was 18% superior in LCP384 than in INTA3116. At early ripening, genotype:IP interaction was statistically significant for saccharification potential because it was greater in LCP384 IP15 than in the rest of the IPs of both hybrids. Finally, saccharification potential exhibited no differences between genotypes at late ripening (Table 2, Fig. 2d). In addition,

the temporal profiles for saccharification potential showed an increase from bottom to top IPs in the two hybrids studied *e.g.,* at early ripening of LCP384 saccharification potential increased 95% from IP1 to IP15, and at late ripening of INTA3116 this trait increased 76% from IP1 to IP20 (Table 2, Fig. 2d).

Analysis of internode temporal profiles for saccharification potential revealed that LCP384 and INTA3116 differed in their potential for reducing sugars release at IP5, IP10, and IP15 (Table S1, Fig. 2d). In addition, saccharification decreased from early to late plant developmental stages in both hybrids *e.g.,* saccharification potential of IP1 in INTA3116 decreased 41% from tillering to late ripening, and IP5 of LCP384 decreased 68% from grand growth to late ripening (Table S1).

3.6. Histological analysis

Two regions were recognized in cross sections of sugarcane internodes: the central pith, and the ring in the periphery of the stem, adjacent to the epidermis (Fig. 4). In the pith, vascular bundles (VB) were in low density and scattered with an organized distribution, separated by storage parenchyma cells. Conversely, VB were in greater concentration and tightly packed in the ring and served to strengthen the stem. Detection of lignin with phloroglucinol/HCl reagent allowed lignin accumulation visualization in the VB sclerenchyma of both hybrids. This was displayed as a sheath of thick-walled fibrous cells around the bundle with early lignin deposition in the cell-wall of both hybrids that increased as the internodes aged, both spatially and temporally. Sclerenchyma sheath shapes differed in both genotypes, being rhomboidal in INTA3116 and oval in LCP384 (Fig. 4).

Overall, spatial profiles of internode VB lengths were similar across plant growth and development. However, the longest internode VB were exhibited in LCP384 IP1 at grand growth and INTA3116 IP1 at early ripening (Fig. 5a, Table S2). Internode VB widths differed between hybrids, primarily at grand growth where LCP384 internode VB were wider than INTA3116´s. Width VB genotype differences concentrated in the IPs close to the stem apex, both at early and late ripening, revealing wider VB in LCP384 (Fig. 5b, Table S2). Although IP metaxylem diameter values for each hybrid were stable at tillering and early ripening. LCP384´s internode metaxylem diameter presented higher values than in the energy-cane hybrid. At late ripening instead, upper LCP384 IPs exhibited higher values for metaxylem diameter than bottom internodes, contrasting with

values in INTA3116 IPs that were similar along the same spatial profile (Table S3). The number of VB in both hybrids was stable across the internode spatial profile of each developmental stage. Nevertheless, except for tillering, a greater number of LCP384 VB were measured than in INTA3116 of the remaining plant developmental stages (Fig. 5c, Table S2).

Except for IP1 and IP5, the temporal profiles of VB length for the remaining IPs studied unveiled no variation between genotypes across plant growth and development (Table S3). Alike the temporal profiles of internode VB width, temporal profiles of internode metaxylem diameter and number of VB differed between genotypes (Table S3).

3.7. Correlation analysis

Pearson correlation coefficients were calculated to investigate associations between cell-wall components, saccharification potential and number of VB (Fig. 6, Table S4). CC negatively correlated with mannose content (r= -0.56) whereas MP content positively correlated with all monosaccharides excluding fucose, mannose and uronic acids, showing the strongest correlation with arabinose content (r= 0.97). Lignin content positively correlated with xylose (r= 0.41) and negatively correlated with fucose (r= -0.65), galacturonic (r= -0.53) and glucuronic acids (r= -0.58) (Fig. 6).

The correlation analysis also revealed that saccharification potential positively correlated with glucuronic acid (r= 0.34) and number of VB, and negatively correlated with lignin (r= -0.46) and xylose (r= -0.38) contents (Fig. 6). The number of VB also negatively correlated with lignin (r= -0.39), MP (r= -0.27), arabinose (r= -0.37) and xylose (r= -0.53) and positively correlated with glucuronic acid (r= 0.54).

4. Discussion

In the present study, we investigated the internode chemical composition of sugarcane and energy-cane hybrids throughout the temporal and spatial profiles of plant development. Saccharification potential values were correlated with cell-wall compounds and number of VB to study the presence of recalcitrant factors for cellulosic ethanol production. We also provided a detailed description of agronomic and histological traits with special focus on lignin distribution.

The internode expansion across the profiles studied in sugarcane and energy-cane was highly dependent on temperature and water supply, as shown by Lingle [42]. A small variation (up to one internode) in the internode appearance rate was observed between genotypes across the growing cycle. Lingle and Tew [57] also observed internode appearance rate oscillations in sugarcane cultivars, registering differences up to 7 internodes between genotypes from grand growth to ripening. Rapid reduction of internode elongation rates, accompanied with increasing sucrose accumulation, was observed at the onset of the dry and cold winter season of the sugarcane cropping region of Argentina. Our results demonstrated that the internode number is practically defined by the beginning of this season in both hybrids.

Opposite strategies for internode C-partitioning were found in sugarcane and energy-cane. During plant growth and development, LPC384 prioritized sucrose storage, whereas INTA3116 synthesized mainly lignocellulosic fibers. The high fiber levels combined with low moisture content in INTA3116 internodes suggest that energy-cane stems are an attractive raw material for a wide range of lignocellulose energetic uses [29]. Like most of the energy-cane cultivars [38, 58, 59, 60] INTA3116 is closely related to the wild high-fiber ancestor *S. spontaneum* [25]. Not only the influence of this ancestral species was evident in internode agronomic and compositional features, but also it was at the histological level. INTA3116 showed lower number of VB and metaxylem vessel diameter than the sugarcane cultivar, which is likely to be inherited from *S. spontaneum* [36]. Moreover, histochemical results revealed that lignin deposition in INTA3116 extended to parenchymal cell-walls, and this lignin distribution pattern was previously reported for *S. spontaneum* and *S. Robustum* [36, 37].

Developmental stage and IP effects had a remarkable impact on lignin concentration, showing very similar patterns in both hybrids. Lignin increased towards upper IPs and advanced developmental stages in both sugarcane and energy-cane hybrids, in accordance with previous reports [35, 38, 61]. Differential lignin content on an AIR basis was solely detected between genotypes at grand growth. Nevertheless, INTA3116 accounted for an early deposition of lignin in the parenchymal cells, even though LCP384 unveiled a higher number of VB, where lignification is mainly concentrated. The more intense phloroglucinol staining in INTA3116 internodal tissue, compared to LCP384, was likely due to INTA3116's higher overall fiber content (twice LCP384's amount) rather than a difference in lignin content.

Cell-wall polysaccharides content was similar between sugarcane and energy-cane hybrids, being most of the significant compositional changes a consequence of stage and IP effects. CC showed the smallest variation among main cell-wall components, probably due to the fact that most of the internodes analyzed were elongated, and consequently their cellulose accumulation finalized [16]. Maximum MP contents were registered at late ripening in all the IPs studied. Lingle and Thomson [16] observations suggested higher hemicellulosic polysaccharides at grand growth than at ripening, whereas cellulose and lignin content increased towards the mature stage. It should be noted that different methods were used for hemicellulosic quantification as well as different IPs were considered in the analyses.

The relative amount of monosaccharides found in LCP384 and INTA3116 internodes is consistent with previous studies on sugarcane and other grass species [39, 47, 62]. IP and developmental stage predominantly affected the monosaccharide composition while a minor impact was observed for genotype effect. An exception was the galactose content that was higher in LCP384 than in INTA3116, and statistically significant for all the internodes analyzed. Mason *et al.* [63] reported a negative correlation between galactose and glucose release during enzymatic digestion in different types of sugarcane tissue, however, in the present study this association was not observed. Monosaccharide variability was also reported in four *Saccharum* species [37].

The degree of association between internode anatomical and compositional features with saccharification values was assessed using a correlation analysis. The results suggest that lignin and xylose depositions contribute to increase internode cell-wall recalcitrance during sugarcane and energy-cane development. This is consistent with other findings [62, 63, 64, 65] and confirms, in addition, that not only lignin content but also monosaccharides arrangement in the cell-wall provide important information to understand biomass recalcitrance to cellulosic biofuels [22, 45]. Although these findings are relevant for understanding internode recalcitrance across sugarcane/energy-cane growing, some clear differences in saccharification values were not associated with any cell-wall or histological trait. For instance, we observed different saccharification potential between hybrids at three plant developmental stages (tillering, grand growth, and early ripening) despite both showing roughly similar cell-wall composition. Probably, the recalcitrant effect of cell-wall components such as MP content, arabinose and galactose [20, 63, 66] was not detected because it was diluted in the presence of multiple

recalcitrant factors in the cell-wall. In fact, the moderate intensity of the correlations observed supports the idea of multiple recalcitrant factors controlling cell-wall recalcitrance during internode development. Lignocellulose properties not measured in this study *e.g.,* porosity, cellulose crystallinity, and protection of cellulose by hemicelluloses [67] might have also affected reducing sugars release in sugarcane/energy-cane internodes.

Altogether, chemical and histological data put forward for consideration that lignification across internode development in sugarcane and energy-cane is mainly a consequence of lignin accumulation around VB rather than an effect of the VB number. This pattern is in accordance with the lignification process along maize stems [68]. In fact, our results showed a negative correlation between VB number and lignin. This could be explained by the fact that lignin concentrated towards the ripening developmental stage where decreasing VB number per unit area was observed due to a predominant increase of non-vascular internodal tissue growth. In addition, no correlation was detected between VB number and saccharification levels. This lack of association was unexpected since vascular tissue is considered highly resistant to microbial degradation in plants [69]. Previous studies on sugarcane reported a detrimental effect of VB on the saccharification levels of internode ring tissue when compared to the pith tissue [20] and in nodal tissue compared to internodal tissue [70]. However, the present study was mainly based on fully elongated internodes, where the magnitude of the VB number variations can be small, as observed in other grass species [71]. In addition, VB counting was performed only in the pith region of the internode, thus, other effects of VB number variation on lignin content and saccharification potential in the ring region could be plausible.

Overall, saccharification data suggested that sugarcane/energy-cane lignocellulosic biomass from developmental stages preceding the regular harvesting season for sugar production in Argentina is more suitable for cellulosic biofuels production due to its lower recalcitrance. Delaying harvest dates has been associated with increasing recalcitrance levels in other bioenergy crops such as *Miscanthus x giganteus* [72]. The energy-cane hybrid stood out for its elevated saccharification at tillering. In addition, both hybrids showed higher saccharification at grand growth than in the following developmental stages, especially the sugarcane hybrid, which registered the maximum values of the entire experiment. Taking advantage of this less recalcitrant biomass could be possible by adopting new strategies for sugarcane/energy-cane

cultivation. Particularly, the implementation of multiple harvest systems could allow the utilization of low recalcitrant lignocellulose together with an extension of the period of lignocellulosic biomass supply. In the case of sugarcane, the earlier harvest could be utilized for 2G (second generation) ethanol production and the latest for combined sugar or 1G (first generation) ethanol + 2G ethanol production from bagasse. As for energy-cane, since sucrose extraction is inefficient considering its high fiber content, lignocellulosic feedstock from two harvests per year could supply 2G ethanol industries.

Despite the above-mentioned advantages of the utilization of sugarcane/energy-cane lignocellulosic biomass in its early developmental stages as a potential benefit for cellulosic ethanol efficiency, a holistic approach should take other relevant aspects into account. Our results underscore the fact that internode DM and sucrose concentrations from early developmental stages are remarkably lower than in the ripening stages, thus limited biomass production is expected. Also, under subtropical conditions in Argentina, achieving adequate biomass yields after a first harvest would be restricted by the short sugarcane/energy-cane cropping cycle. One study of an energy-cane crop management with two harvest dates in one cropping cycle reported the appearance of negative collateral effects such as high levels of nutrient removal and low biomass yield [73]. Still, more research is still needed to adequate this alternative for sugarcane and energy-cane while integrating economic, social and environmental sustainability standards under a convenient sugar/1G + 2G ethanol production scheme.

5. Conclusions

Altogether, agronomic and histological data highlighted contrasting features of the sugarcane and energy-cane hybrids and the influence of *S. spontaneum* ancestor in energy-cane. The internode cell-wall composition underwent significant changes during the sugarcane/energy-cane growing cycle, which affected the recalcitrance to enzymatic degradation. The spatial and temporal profiles designed for the internode characterization exhibited roughly similar lignin, cellulose and MP concentrations in both sugarcane and energy-cane hybrids, but differences in galactose content and saccharification potential. In addition, the findings demonstrated that biomass recalcitrance increased towards advanced developmental stages and apical IPs and was associated with raising deposition of lignin and xylose. Potential usage of sugarcane/energy-cane internodes from early developmental stages for cellulosic biofuels conversion could be advantageous due to

their low recalcitrant levels. Still, its implementation would require novel adaptations of current management practices in order to satisfy and fulfill appropriate economic and environmental standards. Our findings provide valuable information that could be applied to optimize sugarcane and energy-cane utilization in novel annual schemes of 2G + 1G ethanol production from sugarcane and 2G + 2G ethanol production from energy-cane.

Supplementary material

Table S1. Least square means the main effects of internode temporal profiles (IP1, IP5, IP10, IP15 and IP20) for cell-wall components and saccharification potential across four plant developmental stages (tillering, grand growth, early and late ripening).

Table S2. Least square means the main effects of internode spatial profiles for histological features at tillering, grand growth, early and late ripening.

Table S3. Least square means the main effects of internode temporal profiles (IP1, IP5, IP10, IP15 and IP20) for histological features across four plant developmental stages (tillering, grand growth, early and late ripening).

Table S4. Pearson correlation coefficients between internode cell-wall components, saccharification potential and number of vascular bundles below the main diagonal; significance test for each pair of correlation coefficients, above the main diagonal. p-value with confidence level = 0.95.

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Competing interests

The authors declare that no competing interests exist.

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Tables

Table 1. Internode numbers and agronomic features of LCP384 (sugarcane cultivar) and INTA3116 (energy-cane) across four plant developmental stages. The values represent the mean of internodes collected at each developmental stage.

	Tillering		Grand growth		Early ripening		Late ripening	
	LCP384	**INTA3116**	**LCP384**	**INTA3116**	**LCP384**	**INTA3116**	**LCP384**	**INTA3116**
[a]Internode number	6±0	5±0	13.6±0.6	13±0	18.6±0.6	18.2±0.5	21±0	21±0
Stalk length (cm)	73.4±6.1	75.7±9.5	176±8.6	196±6.5	222.6±10.8	240.4±12.9	237±4.9	265.6±6.4
Internode length (cm)	89.7±13.8	79±25.2	109.3±19.6	121±31	104.6±23.4	120.8±23.8	91.5±31	106.9±31.3
Internode diameter (mm)	22.8±0.8	18.3±1.3	24.3±2.1	17.5±1.4	22.2±1.6	15.8±2.4	22.7±1.7	15.7±1.9
Internode FW (g)	35.5±6.5	21±7.9	53.5±9	30.4±4.6	43.9±12.5	27.2±8.6	39.9±15.3	22.9±9.9
Internode DW (g)	3.6±0.9	2.5±1	13.3±2.4	8.8±1.3	12.4±4.8	9.9±4.4	12.6±5	9.1±4.4
Internode brix (%FW)	5.6±0.7	5±0.5	16.2±2.2	8±1.6	16.8±3.4	9.8±2.5	20.4±1.1	14.2±1.2
Internode fiber (%FW)	4.6±0.6	6.7±0.9	8.8±1.3	21.2±2.2	10.8±1.3	25.1±4.3	11.1±1.1	24.6±3
Internode DM (%FW)	10.1±0.9	11.7±1.2	24.9±1.8	29.2±3.6	27.5±4.3	34.8±6.3	31.3±1.4	38.4±3.5
Pol in cane (%FW)	[b]nd	nd	nd	nd	13.1	8.3	15.7	11.6

[a]Internodes below fully expanded leaves, [b]nd= do data available.

Table 2. Least square means of main effects of internode spatial profiles for internode cell-wall components and saccharification potential at tillering, grand growth, early and late ripening.

	[a]Lignin	[a]CC	[b]MP	[b]Fuc	[b]Ara	[b]Rha	[b]Gal	[b]Glu	[b]Xyl	[b]Man	[b]GalA	[b]GluA	[c]SP
Tillering													
Genotype													
LCP384	17.2a	43.3a	[d]93.8	0.2a	17.2a	1.8a	**11.0a**	20.9a	33.1a	1.1a	7.5a	1.2a	**68.5b**
INTA3116	19.1a	38.9a	[d]79.5	0.1a	14.7a	1.3a	**6.4b**	16.4a	35.1a	1.3a	3.6a	0.6a	**86.3a**
ANOVA													
Genotype	ns	ns	-	ns	ns	ns	*	ns	ns	ns	ns	ns	*
Grand growth													
Genotype													
LCP384	27.1a	26.7a	74.7a	0.1a	13.2a	1.3a	**4.9a**	16.6a	34.2a	1.1a	2.8a	0.6a	**94.5a**
INTA3116	24.4b	27.4a	70.3a	0.1a	13.0a	1.2a	**3.5b**	15.2a	33.8a	1.1a	2.0a	0.5a	**79.8b**
IP													
1	**27.2a**	**35.3a**	**83.8a**	0.1a	**15.7a**	**1.7a**	**5.1a**	**18.2a**	**38.2a**	**1.8a**	2.5a	0.5a	83.3a
5	**24.3b**	**18.9b**	**61.0b**	0.1a	**10.5b**	**0.8b**	**3.3b**	**13.6b**	**29.8b**	**0.4b**	2.3a	0.6a	91.1a
ANOVA													
Genotype	*	ns	ns	ns	ns	ns	**	ns	ns	ns	ns	ns	*
IP	*	*	**	ns	**	**	**	*	*	**	ns	ns	ns
Genotype:IP	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Early ripening													
Genotype													
LCP384	29.3a	27.5a	70.9a	**0.1a**	12.8a	1.3a	**4.7a**	14.4a	33.1a	0.7a	0.2a	0.8a	**86.0a**
INTA3116	28.2a	32.3a	68.3a	**0.0b**	12.5a	0.9a	**3.6b**	14.4a	33.3a	0.5a	0.2a	0.6a	**69.7b**
IP													
1	**28.7b**	33.8a	**60.4d**	0.0a	**10.6d**	**0.9bc**	**3.4c**	**13.2b**	**29.1c**	**0.5ab**	**1.9c**	0.7a	73.1a
5	**29.9ab**	26.2a	**64.8c**	0.0a	**11.6c**	**0.8c**	**3.5c**	**13.0b**	**32.5b**	**0.5b**	**2.3c**	0.7a	75.8a
10	**31.0a**	31.8a	**73.9b**	0.0a	**13.8b**	**1.1b**	**4.5b**	**14.5b**	**35.5a**	**0.5ab**	**3.2b**	0.7a	79.3a
15	**25.5c**	27.8a	**79.3a**	0.1a	**14.7a**	**1.5a**	**5.3a**	**16.9a**	**35.5a**	**1.0a**	**3.7a**	0.8a	83.2a
ANOVA													
Genotype	ns	ns	ns	*	ns	ns	*	ns	ns	ns	ns	ns	**
IP	**	ns	**	ns	**	**	**	**	**	**	**	ns	ns
Genotype:IP	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*
Late ripening													
Genotype													
LCP384	30.2a	27.2a	88.3a	0.1a	17.8a	1.75a	**8.1a**	17.4a	38.7a	0.5a	**3.6a**	**0.5a**	73.6a
INTA3116	31.0a	30.0a	84.0a	0.1a	16.9a	1.25a	**5.83b**	16.8a	39.8a	0.6a	**2.51b**	**0.3b**	68.3a
IP													
1	**31.2a**	**34.6a**	84.6a	**0.0c**	16.3a	1.3a	**6.4b**	**16.4ab**	40.2a	**0.9a**	**2.8b**	**0.4bc**	**61.4b**
5	**33.8a**	**29.4a**	84.1a	**0.1bc**	16.8a	1.6a	**6.3b**	**16.0ab**	39.9a	**0.3b**	**2.8b**	**0.4ab**	**58.3b**
10	**36.2a**	**18.9b**	84.1a	**0.1b**	17.6a	1.4a	**5.9b**	**15.6b**	40.7a	**0.3b**	**2.3b**	**0.3c**	**62.8b**
15	**30.0a**	**33.8a**	88.9a	**0.1bc**	18.1a	1.5a	**7.3ab**	**18.7a**	38.9a	**0.6ab**	**3.3a**	**0.5a**	**82.2a**
20	**21.8b**	**26.4a**	89.2a	**0.1a**	17.9a	1.7a	**8.9a**	**18.7a**	36.8a	**0.5ab**	**4.0a**	**0.6a**	**90.1a**
ANOVA													
Genotype	ns	ns	ns	ns	ns	ns	**	ns	ns	ns	**	**	ns
IP	**	**	ns	**	ns	ns	*	**	ns	**	**	**	**
Genotype:IP	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

where IP= internode position, CC= crystalline cellulose, MP= matrix polysaccharides, Xyl= xylose, Glu= glucose, Ara= arabinose, Rha= rhamnose, Gal= galactose, Man=

mannose, Fuc= fucose, GluA= glucuronic acid, GalA= galacturonic acid, SP= saccharification potential.

[a] (% AIR), [b] (mg g^{-1} AIR), [c] (nmol mg^{-1} AIR h), [d] no replicates available.

Significant at * $P < 0.05$, ** $P < 0.01$. ns= non significant

Different letters within developmental stages indicate significant differences for Fisher's LSD test ($p < 0.05$).

Figure captions

Fig. 1. (a) Schematic illustration of the experimental design used for the sugarcane and energy-cane internode characterization. Internode composition was studied along a temporal profile that includes four critical developmental stages across the plant growing cycle. At each developmental stage, a spatial profile that involves several internode positions (IPs) from soil level was assessed. Created with BioRender.com **(b)** Meteorological and phenotypic experimental data. Plot lines show cumulative growing degree days (GDD) for sugarcane internode elongation (red) and daily rainfall for the 2018/2019 sugarcane growing season (blue). Arrows indicate GDD at sampling for each developmental stage.

Fig. 2. Lignin **(a)**, crystalline cellulose **(b)**, matrix polysaccharides **(c)** and saccharification potential **(d)** of LCP384 and INTA3116 internodes alcohol-insoluble residue (AIR) samples at four developmental stages (T=tillering, GG= grand growth, ER=early ripening and LR=late ripening) and different internode positions above soil level (1, 5, 10, 15 and 20).

Fig. 3. Monosaccharide composition of the internode matrix polysaccharides in LCP384 (sugarcane cultivar) **(a)** and INTA3116 (energy-cane) **(b)** at four developmental stages (T=tillering, GG= grand growth, ER=early ripening and LR=late ripening) and different internode positions (IP) above soil level (1, 5, 10, 15 and 20). Xyl= xylose, Glu= glucose, Ara= arabinose, Rha= rhamnose, Gal= galactose, Man= mannose, Fuc= fucose, GluA= glucuronic acid, GalA= galacturonic acid.

Fig. 4. Cross sections of different internode regions of sugarcane and energy-cane stained with acid phloroglucinol (red). Representative images of ring region of IP5 in INTA3116 **(a)** and LCP384 **(b)**; pith region at three developmental stages of IP1 in INTA3116 **(c, d, e)** and in LCP384 **(f, g, h)**. VB= vascular bundle, mx= metaxylem, px= protoxylem, ph= phloem, fp=fundamental parenchyma, e= epidermis, f= fibers. Scale bar 100 µm.

Fig. 5. Histological features of sugarcane and energy-cane internodes. Length **(a)**, width **(b)** and number of vascular bundles **(c)** of LCP384 and INTA3116 at four developmental stages (T=tillering, GG= grand growth, ER=early ripening and LR=late ripening) and different internode positions (IP) above soil level (1, 5, 10, 15 and 20).

Fig. 6. Pearson correlation coefficients for cell-wall components, saccharification potential and anatomical features, for all the internodes used in this study (n=24). Significance level: "***" p-value < 0.001; "**“p-value < 0.01; "*“p-value < 0.05. Cellulose (% AIR), lignin (% AIR), MP= matrix polysaccharides (% AIR), Xyl= xylose (mg g^{-1} AIR), Glu= glucose (mg g^{-1} AIR), Ara= arabinose (mg g^{-1} AIR), Rha= rhamnose (mg g^{-1} AIR), Gal= galactose (mg g^{-1} AIR), Man= mannose (mg g^{-1} AIR), Fuc= fucose (mg g^{-1} AIR), GluA= glucuronic acid (mg g^{-1} AIR), GalA= galacturonic acid (mg g^{-1} AIR), SP= saccharification potential (nmol mg^{-1} AIR h), Number.VB= number of vascular bundles in the observed area.