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Cell Wall Composition Impacts Structural Characteristics of the Stems and thereby Biomass Yield

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Abstract

Maize stalks support leaves and reproductive structures, functionally support water and nutrient transport; besides their anatomical and biochemical characteristics have been described as a plant defense against stress, also impacting economically important applications. In this study, we evaluated agronomical and stem description traits in a subset of maize inbred lines that showed variability for cell wall composition in the internodes. Overall, a great proportion of lignin subunit G and a low concentration of *p*-coumaric acid and lignin subunit S is beneficial for greater rind puncture resistance and taller plants, with greater biomass yield. Also, the greater the proportions of subunit H, the longest the internode. By last, the lower the total hemicellulose content the greater the rind puncture resistance. Our results confirmed the effect of the cell wall on agronomic and stalk traits which would be useful in applied breeding programs focused on biomass yield improvement.

Keywords: *Zea mays*, cell wall, stem characteristics, biomass, maize, plant architecture

Introduction

The structure and function of the plant cell wall are controlled by how each of its components interacts within the cell wall. This strong assembly, apart from providing structural support and rigidity to the cell, determining its size and shape, also provides resistance to abiotic and biotic stresses and communication among cells [1,2]. Furthermore, the framework constituted by the cell wall is closely related to the growth and fitness of the plant and is expected to determine the functional characteristics of the stem, which are closely related to yield. [3,4].

From a breeder point of view, the first goal of the crop improvement is, on the one hand, to obtain increased grain yield, considered as the potential of the grain production and increased biomass yield expressed as tons of biomass produced per hectare [5,6]. In maize, increases in maize grain have been accompanied by increases in biomass, which indicates that breeding for biomass yield would not compromise grain yield [7]. Furthermore, increases in biomass or stover yield have been also a target trait for biofuel production and forage digestibility [8,9].

Because cell walls constitute more than 50% of the dry biomass weight; improvement of biomass relies largely on the cell wall components and anatomical arrangement of the stems, conditioning also plant height [9–12]. However, increases in plant height must have to deal with stem lodging losses. Stem lodging, caused by the bending or breaking of the stalk, is greatly impacted by stalk strength and stem morphological traits, therefore it could be said that maize stem strength impacts both grain yield and silage quality [10,13,14].

Research on cell wall composition and its influence in basic and applied aspects of maize stem strength would be important steps in maize breeding and improvement [10,15].

58 Overall, in the current study, we evaluated agronomical and stem description traits in a
59 subset of maize inbred lines that showed variability for cell wall composition, being the
60 main goal to identify cell wall components that can be used in applied breeding
61 programs.

Materials and Methods

Plant Material and experimental design

A set of 20 inbred lines was tested through two consecutive years (2016, 2017) in Pontevedra (Spain, 42° 24′ 22.3″ N, 8° 38′ 28.16″ W, 20 m above sea level). The set of inbred lines evaluated can be subdivided into three subsets: (i) inbreed lines included in previous evaluations for resistance to *Sesamia nonagrioides* or *Ostrinia nubilalis*, (ii) inbreds used in hybrids combinations for bioenergy and for silage, (iii) inbreds that perform wll in hybrid combinations. A complete and detailed description of the inbreds evaluated could be found in [16].

In both trials, the set was evaluated following a random block design with three repetitions. In 2017, the set was reduced to nineteen because there was not enough stock for the inbred line PB130. The experimental plots consisted of three rows, with 15 double-kernel hills each, with a total surface of 0.14 m^2 per plot, with a final density of ~ 70,000 plants ha^{-1} after thinning. The trials were maintained with local agronomical practices.

Agronomic Traits

Biomass yield. Seventy days after silking, considering it as days from planting until half of the plants in the plot showed visible silks; plots were harvested. Two to ten plants without ears from each plot were collected, weighed and chopped from which a stover sample was collected (sample fresh weight) for estimating the percentage of stover dry matter. For that, the fresh stover was pre-dried (35 °C) in a forced air-drying chamber, dried on a stove (60 °C), and again weighed after a week (sample dry weight).

Determination of Biomass yield in Mg ha^{-1} was done as it follows:

$$Biomass\ yield\ (\frac{Mg}{ha}) = \frac{weigth\ of\ fresh\ sample\ (g) * sample\ dry\ weight\ (g)}{Surfarce\ (m2) * sample\ fresh\ weight(g)\ x\ 100}$$

The surface was calculated as the number of plants per plot multiplied by the space between rows (0.80 m) and the space between plants (0.18 m). Following this equation biomass yield corresponds to the maximum yield.

Stem Lodging. Calculated at harvest as the sum of broken plants (split underneath the main ear) divided by the total number of plants in the plot, was calculated. Stem lodging is expressed in percentage.

Stem descriptions traits

Rind puncture resistance, the total number of internodes, and internode diameter were recorded 55 days after flowering, the rest of the stem description traits 70 days after flowering. A more detailed description of the methodology can be found in López-Malvar et al. 16

Briefly, plant height was calculated as the mean of plant height (in cm) measured from the base of the plant until the flag leaf, of five plants per plot; internode length was calculated as the total number of internodes divided by the height of the plant; in five plants, rind puncture resistance was the measured of the maximum force required to puncture the rind (in kg/section) on one side of the stalk using an Accuforce Cadet Force Gauge (Ametek, Mansfield and Green Division, Largo, FL); from the same five internodes, using an electronic caliper, the diameter was recorded, in millimeterss.

Biochemical Traits

104 The complete characterization of the cell wall was performed in the second internode
105 below the main ear from five plants per plot, collected 55 days after silking. The complete
106 description of the methodology can be found in López-Malvar et al. [16]

107 Briefly, cellulose was quantified in crude cell walls by the Updegraff method [18];
108 hemicellulose composition was determined using high-performance anion exchange
109 chromatography (HPAEC) (Carbopac PA-10; Dionex, Camberley, Surrey, UK) as
110 described previously by Jones et al. (2003), it included the quantification of glucose,
111 galactose, fucose, arabinose, rhamnose, xylose, mannose, arabinose:xylose ratio
112 glucuronic and galacturonic acid (the sum of all of them would be considered further on
113 as total hemicellulose content)**;** total lignin content was determined by Klason Lignin
114 protocol [21]; subunit composition was determined by thioacidolysis followed by Gas
115 chromatography–mass spectrometry (GC–MS)[22]; cell wall-bound hydroxycinnamates
116 quantification was performed by High-Performance Liquid Chromatography (HPLC)
117 following the protocol described in Santiago and col. [23]

118 **Statistical Analysis**

119 **Contrast analysis**

120 The SAS mixed model procedure (PROC MIXED) of the SAS program (version 9.4) [25]
121 was used for the individual and combined analyses of variance for each trait. Using the
122 combined data for the analysis across years, the best linear unbiased estimators (BLUES)
123 for each inbred line was calculated. We considered as fixed effects inbred lines and as
124 random effects years, replication within years, and lines × year. We used Fisher's
125 protected least significant difference (LSD) for means comparison.

126 After that analysis, inbred lines were qualitatively classified, according to their BLUEs,
127 in high, intermediate, and low groups for agronomic and stem traits (Table 1); high and
128 low groups differing $p<0.05$. With the qualitative dataset, mean comparisons for groups
129 with contrasting values were performed to look for differences in cell wall composition.

130 Table 1. Inbred lines under study were qualitatively classified according to the BLUES
131 for biomass yield and stem description traits evaluated in 2016 and 2017.

Inbred	Plant Height (cm)	Internode Length (cm)	Internode Diameter (mm)	Rind Puncture Resistance (kg/section)	Stover Yield (Mg/ha)
A509	Low	Intermediate	Intermediate	Low	Low
A632	High	Low	Low	Intermediate	High
A654	Low	Low	Intermediate	Intermediate	Intermediate
C103	High	High	Intermediate	High	High
CO348	Low	Low	Intermediate	High	Intermediate
CO384	High	Low	Intermediate	Intermediate	High
CO442	High	Low	Intermediate	Low	High
CO444	Intermediate	Low	Low	Intermediate	Intermediate
EC212	Intermediate	Low	Intermediate	High	Intermediate
EP105	High	Intermediate	Intermediate	Intermediate	Intermediate
EP125	High	High	Intermediate	Low	Low
EP17	High	Low	High	High	High
EP42	Intermediate	Low	Low	Intermediate	Intermediate
EP47	High	High	High	Intermediate	High
EP53	Low	High	Intermediate	High	Low
EP86	High	Intermediate	Low	Low	Low
F473	Low	Low	Low	Intermediate	Low
PB130	Low	Low	High	Intermediate	Intermediate

W182B	Low	Low	High	Low	Low
W64A	Intermediate	Low	High	Intermediate	Intermediate

132

133 **Multiple linear regression analysis**

134 For understanding the relationship between agronomic, stem description traits, and cell
135 wall components we performed a multiple linear regression model using the BLUEs. For
136 this analysis, we used, in SAS [25], the stepwise method following the PROC REG
137 procedure. Variables with a significance value less than 0.15 were not selected to take
138 part in the regression model. We considered as dependent variables agronomic and stem
139 description traits; as independent variables, we considered cell wall components.

Results

Inbred lines differed significantly for biomass yield and stem description traits. There were no significant differences for stem lodging, so it was not included nor in the contrast analysis, nor the multiple linear regression (Supplementary Table 1).

Contrast analysis

Significant differences between high and low contrast groups for every trait are shown in Table 2. Values for non-significant traits in the contrast analysis are included in Supplementary Table 2. Inbred lines presenting the greatest biomass yield showed the lower concentration of PCA, low proportion of lignin subunits S and H, low S:G ratio, and on the opposite greater proportion of subunit G.

In the same way, but attending to the plant height, the taller plants presented lower proportions of lignin subunit S and S:G ratio, and higher proportions of lignin subunit G, however, the H subunit showed the opposite trend for biomass yield.

Regarding to internode description traits, the greater proportion of subunit H, the longest the internode, in accordance with plant height results. Contrast groups for internode diameter did not differ for any cell wall trait. Finally, inbred lines showing the greater resistance to puncture were the ones showing the greatest cellulose content and the greatest proportions of subunit G; and the lowest concentrations of cell wall-bound hydroxycinnamates (namely PCA and diferulates), lowest total hemicellulose content (galactose, glucuronic and galacturonic acid, arabinose, xylose, and mannose) and lowest proportions of lignin subunit H and S:G ratio (Table 2).

Table 2. Contrast analysis of inbred lines attending to contrasting values of biomass yield and agronomic stem description traits. Only cell wall components that significantly differ among groups are included.

	Classification Group			
Cell Wall Component	**High**	**Intermediate**	**Low**	**LSD**
Biomass yield (Mg/ha)				
PCA (mg/g)	11.54	12.77	13.94	0.907
S subunit (%)	55.28	57.83	57.83	0.982
S:G ratio	1.317	1.44	1.479	0.052
G subunit (%)	42.17	39.27	39.27	0.851
H subunit (%)	2.548	1.95	2.910	0.052
Plant height (cm)				
S subunit (%)	55.65	57.26	58.88	0.98
S:G ratio	1.34	1.42	1.52	0.052
G subunit (%)	41.64	40.75	38.85	0.89
H subunit (%)	2.72	2.28	2.28	0.38
Internode length (cm)				
H subunit (%)	2.95	2.26	2.26	0.44
Rind puncture resistance (kg/section)				
PCA (mg/g)	11.38	12.70	13.90	0.99
DFA 8-5-l (mg/g)	0.046	0.060	0.062	0.008
DFA 8-5-b (mg/g)	0.088	0.105	0.105	0.156

DFA 5-5 (mg/g)	0.067	0.087	0.086	0.012
DFAT (mg/g)	0.274	0.338	0.326	0.045
Cellulose (mg/g)	441.63	441.63	382.11	34.324
Galactose (mg/g)	4.855	5.600	8.285	2.926
Galacturonic acid (mg/g)	6.734	9.230	10.166	2.422
Glucuronic acid (mg/g)	2.472	2.748	3.936	1.935
Arabinose (mg/g)	8.002	9.218	12.248	3.016
Mannose (mg/g)	2.429	2.586	3.699	0.80
Xylose (mg/g)	20.87	24.21	24.75	3.051
H Subunit (%)	2.248	2.356	2.703	0.398
G subunit (%)	41.15	40.73	39.77	1.041
S:G ratio	1.39	1.40	1.45	0.06

LSD: Least Square Distance ($P \leq 0.05$)

PCA: *p*-coumaric acid; DFA 8-5-l: Diferulic acid 8-5-Linear; DFA 8- o-4: Diferulic acid 8-O-4; DFA 8-5: Diferulic acid 8-5; DFA85b: Diferulic acid 8-5-Benzofuran; DFAT: Total diferulic acids

* some missing data for individual traits and inbreds could interfere in the final ratio calculations of the groups

165

166 **Multiple Linear Regression**

167 We found that a greater proportion of lignin subunit G and greater Total Hemicellulose
168 Content (mainly galactose) increase biomass yield; on the opposite, greater Galacturonic
169 acid and arabinose:xylose ratio decrease biomass yield (Table 3).

We found that 34% of the variance for plant height was affected lignin subunit S, with a negative effect. In the case of internode length and internode diameter, no variable met the 0.15 significance level to be included in the model (Table 3). Rind puncture resistance was mainly affected by Galacturonic acid concentration and Arabinose:Xylose Ratio, negatively, and positively by Glucose reporting 53 % of the variation for rind puncture resistance(Table 3).

Table 3. Multiple linear regression model (using stepwise selection) of biomass yield and stem description traits on cell wall composition of a set of inbred lines evaluated in 2016 and 2017.

Stepwise Selection		
Biomass yield (Mg/ha)	**R^2 Partial**	**R^2**
Subunit G (%)	0.31	0.31
Arabinose:Xylose Ratio	0.14	0.46
Total Hemicellulose (mg/g)	0.09	0.55
Galacturonic Acid (mg/g)	0.07	0.62
Galactose (mg/g)	0.07	0.69
Model	Biomass yield: -16.11997 + 0.48330*G -5.68752* ARA:XYL +0.55854*Galactose -0.38920*Galacturonic Acid +0.03730*Total Hemicellulose	
Plant height (cm)	**R^2 Partial**	**R^2**

Subunit S (%)		0.34	0.34
Model	Plant height= 469.3563-5. 72250*S		
Rind puncture resistance (kg/section)		**R^2 Partial**	**R^2**
Galacturonic acid (mg/g)		0.36	0.36
Arabinose:Xylose Ratio (mg/g)		0.11	0.47
Glucose (mg/g)		0.07	0.53
Model	Rind puncture resistance: 3.83618 + 0.01978*Glucose - 0.15986*Galacturonic Acid -0.58579*Arabinose:Xylose Ratio		
R^2: Total % of the variance explained by the model; R^2 partial: % of the variance explained by each trait.			

179

Discussion

Our results confirm that biomass, stem strength, and other stem features such as plant height or internode length rest on the organization and composition of the stem cell walls. Secondary cell wall formation, characterized by lignin deposition, seems to play a central role in maize stem characteristics.

Contrast analysis

In the contrast analysis, we noted that it is not the total lignin content, but the lignin subunit composition the trait that most influences differencet groups of lines classified and in high and low groups for plant biomass and stem architecture; the lignin with higher proportions of subunit G in detriment of subunit S, is valuable for both increased biomass yield, plant height and rind puncture resistance (Table 2). The composition and proportion of the subunits highly influence the molecular structure of lignin. It affects the degree of crosslinking with the polysaccharides and also the branching of the polymer, affecting, as it has been demonstrated, economically important processes such as biofuel production and digestibility [16,26]. In addition, some other related phenylpropanoids also contributed to biomass yield and anatomical traits of the stems. We have shown that a great concentration of PCA in the cell wall is unfavorable for increasing biomass yield and rind puncture resistance. Most PCA is bound to S units in lignin, esterified to the γ -position of phenylpropanoid sidechains [27]. The PCA acylation influences the bonding mode of S lignin units and on the spatial organization of lignin, and by consequence also on the way that lignin and polysaccharides interact [28]. In this sense, S-type lignin presents a more linear structure [29] with almost no branching and with a lesser degree of polymerization; lignin G is more condensed than lignin S [30]. In

203 our case, S-type lignin, is detrimental for increases in biomass yield, plant height, and
204 rind puncture resistance.
205 The network formed by the fibres within the cell wall (cellulose-lignin-hemicellulose) is
206 believed to define the functional properties of the stems [31]. We found that increases in
207 cellulose would favor greater rind puncture resistance, therefore stalk strength, while
208 greater concentrations of total hemicellulose content would be disadvantageous for rind
209 puncture resistance. Increases in rind puncture resistance and formation of the cortex
210 tissue have been closely related with cellulose and lignin deposition, serving as
211 structural support to the cell wall [32]. Moreover, cellulose compositional features, such as
212 crystallinity, have been related to stalk lodging and stalk strength, which could be
213 associated with rind puncture resistance, as previously mentioned [15]. It has been also
214 proved the positive association between the quantity of cellulose amorphous regions and
215 the arabinose-substitution of xylans; also the negative effect that increasing levels of
216 arabinose have in cellulose crystallinity has been demonstrated [33,34]. In the contrast
217 analysis, the group of inbred lines presenting the higher rind puncture resistance present
218 a reduced arabinose:xylose ratio and thus a reduction in the arabinose content. In a cell
219 wall presenting a low concentration of arabinose, the hemicellulose and cellulose chains
220 tend to interact through of hydrogen bonds, which would contribute more crystalline
221 cellulose, which is more uniform, ordered, and hard; which could indicate a greater
222 resistance to puncture. Contrary to cellulose, hemicelluloses are not chemically uniform.
223 Xylan containing β-(1,4)-linked xylose residues, is one of the most complex heteroxylans
224 in the fibre of maize [35]. Based on Appeldoorn et al. [36] and Van Eylen et al. [37] a reduced
225 incidence of uronic acid, acetic acid, and arabinose side groups in
226 glucuronoarabinoxylans would drive changes in the properties of the cell wall. Contrast

analysis showed that the presence of more glucuronic acid and galacturonic acid may contribute to a less strengthen stalk, less resistant to puncture.

Finally, the mechanical resistance granted by DFAs would make us think in a cell wall with greater strength and higher tissue toughness, would also present a greater resistance to the penetrometer, however, regarding our contrast analysis, the group of inbred lines showing the greatest rind puncture resistance showed the lowest concentrations of diferulates. But our results are in accordance with the ones obtained by Manga-Robles et al.[15] in a previous study. They observed a significantly higher level in diferulic individual dimers in inbred lines showing low rind penetrometer strength. Attending to the plasticity of the cell wall we may argue that some of the other components of the cell wall have a more significant part in the strengthening and support like, for this panel of inbred lines, great cellulose content or lignin presenting low S:G ratio, which would increase rind puncture resistance.

Multiple linear Regression

Mainly, the results obtained in the multiple linear regression analysis support the ones obtained in the contrast analysis. Again, the influence of lignin subunit composition and how PCA acetylation of lignin subunit affected the final lignin structure, showed significant effects on biomass yield and plant height. Lignin with a greater proportion of subunit G may be beneficial for greater biomass yield, and lignin presenting lower proportions of subunit S would produce taller plants.

We have already mentioned how the fibre proportion of the cell wall takes part in determining stem anatomical characteristics, according to our results the structural support granted by Total Hemicellulose content produces greater biomass yields.

250 Besides, we found that reduced arabinose:xylose ratio, and lower concentrations of
251 galacturonic acid decrease both Rind Puncture Resistance (accordance with contrast
252 analysis) and Biomass Yield. As previously explained for the contrast analysis results,
253 the influence of matrix polysaccharides (total hemicellulose content) has been confirmed
254 to affect rind puncture resistance; and in the same way, could affect biomass yield. It has
255 been demonstrated the negative relationship between arabinose content and cellulose
256 crystallinity. The intra and intermolecular hydrogen bridges within the cellulose have
257 as a result a crystalline configuration that gives cellulose mechanical solidity, which may
258 be beneficial for biomass increases [38].
259
260 In this representative material, S-type lignin accompanied by increases in *p*-coumaric
261 acid would be in detriment of biomass yield, plant height, and rind puncture resistance,
262 whereas, cell walls richer in cellulose and with a lower proportion of total hemicellulose,
263 would be beneficial for stalk strength (Figure 1). These results prove that cell wall
264 composition clearly influences structural characteristics of the maize stems and thereby
265 can be useful to improve maize biomass yield.
266

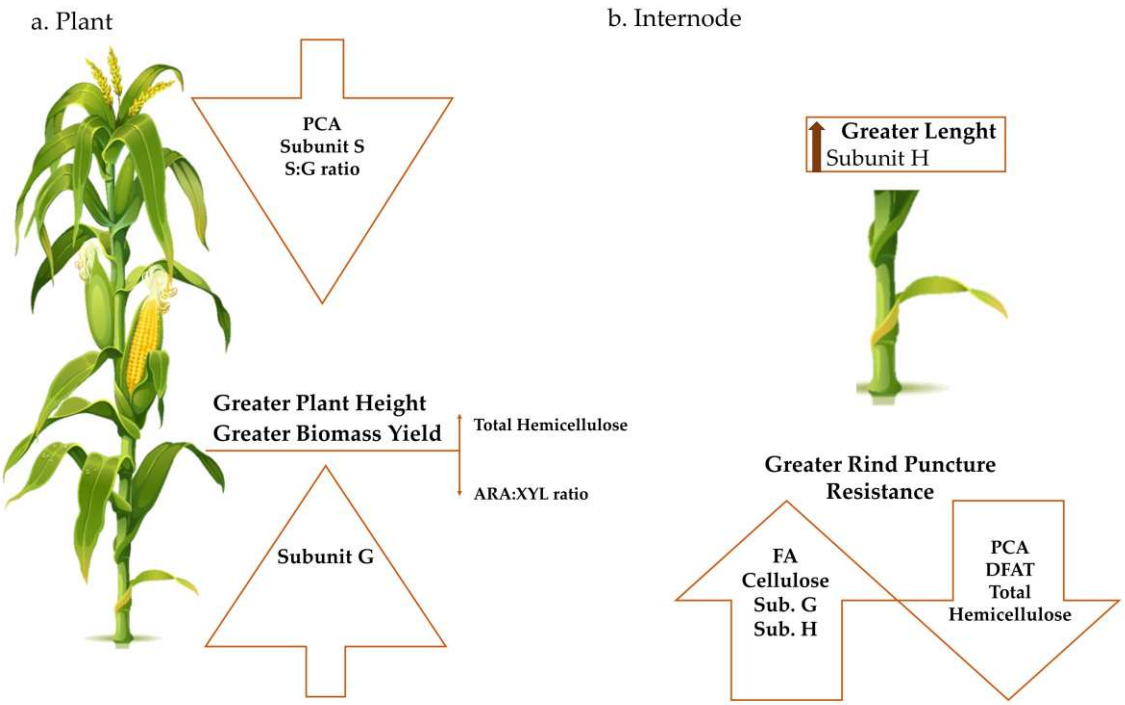

Figure 1. Graphical summary of the results obtained. a: Results concerning the whole plant; b: results concerning the second internode below the main ear.

List of Abbreviations

TFA: Trifluoroacetic acid; HPAEC: high-performance anion exchange chromatography; GC–MS: Gas chromatography–mass-spectrometry; BLUES: Best Linear Unbiased Estimators; FA: Ferulic acid; PCA: p-coumaric acid; DFA 8-5-l: Diferulic acid 8-5-Linear; DFA 8- o-4: Diferulic acid 8-O-4; DFA 8-5: Diferulic acid 8-5; DFA85b: Diferulic acid 8-5-Benzofuran; DFAT: Total diferulic acids; G: Subunit; GLUA: Glucuronic Acid

Supplementary material

279 Supplementary Table 1: Means of 20 inbred lines evaluated for agronomic and stem
280 description traits.
281 Supplementary Table 2. Contrast analysis of inbred lines attending to contrasting values
282 of biomass yield and agronomic stem description traits. Means for cell wall components
283 with **non-significant differences** among groups are included.

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297 **Authors' contributions**
298 RAM and RS conceived the study. RS, RAM, and AL, participated in the experimental
299 design, carried out the field trials, and participated in sample collection; AL carried out
300 biochemical determinations and statistical analysis; AL wrote the draft. JBR and LG

assisted on biochemical analysis, results and discussion. XCS contributed to the results discussion. All authors read and approved the final manuscript.

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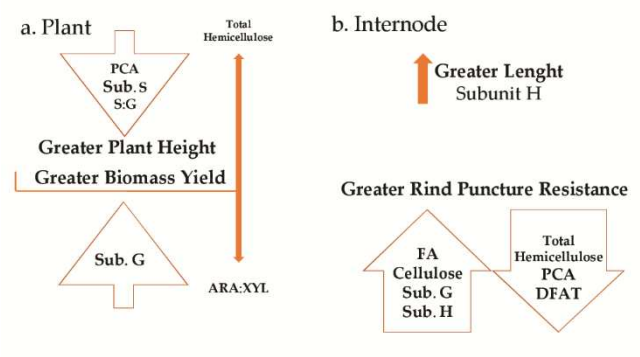

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