Elucidating compositional factors of maize cell walls contributing to stalk strength and lodging resistance

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Abstract

Lodging is one of the causes of maize (*Zea mays* L.) production losses worldwide and, at least, the resistance to stalk lodging has been positively correlated with stalk strength. In order to elucidate the putative relationship between cell wall, stalk strength and lodging resistance, twelve maize inbreds varying in rind penetration strength and lodging resistance were characterized for cell wall composition and structure. Stepwise multiple regression indicates that H lignin subunits confer a greater stalk strength. Besides, the predictive model for lodging showed that a high ferulic acid content increases the resistance to lodging, whereas those of diferulates decrease it. These outcomes highlight that the strength and lodging susceptibility of maize stems may be conditioned by structural features of cell wall rather than by the net amount of cellulose, hemicelluloses and lignin. The results presented here provide biotechnological targets in breeding programs aimed at improving lodging in maize.

Keywords: *Zea mays*, cell wall, lignin, ferulic acid, dehydrodiferulate isomers, lodging resistance, rind penetration strength, stalk strength.

25 **Abbreviations**

AIR	Alcohol insoluble residue
CW	Cell wall
DFA	Diferulate
DFAT	Total Diferulates
FA	Ferulic acid
FTIR	Fourier Transformed Infrared
G	Guaiacyl
H	*p*-hydroxyphenyl
H-RPS	High rind penetration strength
L-RPS	Low rind penetration strength
LSD	Least significant difference
*p*CA	*p*-coumaric acid
PCA	Principal component analysis
R-lines	Resistant lines to lodging
RPS	Rind penetration strength
S	Syringyl
S-lines	Susceptible lines to lodging

26

1. Introduction

Maize (*Zea mays* L.) is one of the most important, essential and widespread crops in the world, providing multiple products used for several purposes such as human consumption, animal feeding, or feedstock for second generation biofuels [1]. During maize cultivation, lodging, has been identified as one of the most significant causes of yield reduction (up to 25%) worldwide [2]. High stalk lodging has been usually related to diverse environmental conditions, from biotic stresses such as corn borer insects (*Ostrinia nubilalis* (Hübner) and *Sesamia nonagrioides* (Lefèbvre) [Lepidoptera] in European conditions) or fungal pathogens (*Fusarium* sp.) to abiotic detrimental conditions such as strong winds or unbalanced plant nutrition [3–5].

Lodging resistance has been positively correlated with stalk strength in maize [6]. Several studies indicate that stalk strength, and consequently stalk lodging resistance, can be predicted by methods based on measuring the force needed to puncture the rind or rind penetration strength (RPS) [6,7]. Extending this logic further, stalk strength, measured as RPS, would be determined to some extent by the rind area, and thereby by secondary cell wall (CW) features [8]. In the same way, recently, quantitative trait loci and maize mutant analyses have revealed that stalk lodging is related to genes involved in secondary CW structure and composition [9].

Secondary CWs are macromolecular nanocomposites mainly consisting of lignin and cellulose, hemicelluloses (as main matrix polysaccharides) and minor amounts of structural proteins and enzymes [10]. Depending on the species and cellular types, the composition of lignin, matrix polysaccharides and proteins can differ [11–13].

Cellulose is a glucose homopolymer composed of β-(1,4)-glucan chains organized in microfibrils [10], and it is the main CW constituent reaching up to 50% of secondary CWs dry weight [11]. In secondary CWs, cellulose microfibrils are typically deposited in different orientations contributing to its featured layered shape. Their plain configuration together with the ability of β-(1,4)-glucan chains to form intra- and intermolecular bonds make cellulose a highly stable crystalline compound. Scattered through the crystalline cellulose, amorphous or non-crystalline regions have also been described [14]. Matrix polysaccharides are prone to interaction with cellulose leading to create these amorphous regions [10].

Hemicelluloses are polysaccharides mostly composed of a linear backbone of xylose, glucose or mannose, with short branches of arabinose, xylose, galactose, fucose or glucuronic acid [12]. In maize as in other grasses, xylans are the main hemicelluloses [15]. This xylan backbone is composed of a chain of (1,4)-linked β-xylose commonly substituted by arabinose and/or (methyl)glucuronic acid [16]. Poaceae xylans are characterized by the presence of hydroxycinnamates, principally ferulic acid (FA) and *p*-coumaric acid (*p*CA) esterified on the arabinose residues of arabinoxylan [17].

From a quantitative point of view, lignin is the second most important component of secondary CWs. Lignin is a complex phenolic heteropolymer constituted by three different monomers: *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units together with minor amounts of other phenolics such as FA, *p*CA and tricin [13,18]. Lignin monomers, also known as monolignols, are synthesized in the cytosol by the phenylpropanoids pathway and transported into the CW where they are subjected to oxidative polymerization by the action of peroxidases and laccases [18]. In the case of maize, lignin of mature stalk is composed mainly by G and S units with relative high contents in *p*CA [19].

It is widely accepted that cellulose-lignin-hemicellulose interaction is a key factor in supporting and reinforcing the secondary CW structure [9,16]. Therefore, these interactions are expected to determine the functional characteristics of the stalk. Due to its crystallinity, cellulose is defined as the scaffold around which other CW components are organized [11]. In particular, lignin is thought to be polymerized on secondary CWs coating cellulose microfibrils [20]. Arabinoxylans can interact with cellulose microfibrils, generally by H-bonding with their non-crystalline zones [21]. Beside this, arabinoxylans can cross-link themselves and with lignin through ferulate-bridges, predominantly diferulates (DFA), forming large hemicellulose-lignin complexes [22].

Recently, it has been proposed that cellulose and lignin constitute two highly hydrophobic domains with limited direct interaction [23]. According to this model, rigid and dehydrated xylans regions would bind cellulose microfibrils by H-bonding, whereas, well-hydrated xylans zones would connect lignin domains. Interestingly, it was proposed that xylan-lignin interaction relies essentially in electrostatic bonds between monolignols (particularly S units) and xylan polar groups [23].

89 The initial hypothesis underlying this research is that maize inbred lines presenting a
90 diverse range in rind penetration strength and/or lodging resistance will display
91 differences in the composition and/or structure of CWs. In previous studies, the cellulose
92 and lignin were considered to be the main components that affect stalk strength of maize
93 [24,25]. At present, a precise association between CW composition and lodging is not yet
94 defined [7,26] and refs. therein. Thus, the objective of the current research was to clarify the
95 putative relationship between stalk CW composition, rind penetration strength and
96 lodging. For this purpose, an in-depth characterization of CWs from stalks of maize
97 inbred lines differing in their RPS and lodging resistance has been performed.

98 Results provided here could be useful in order to develop new CW-based markers for
99 breeding programs aiming at improving the resistance of maize plants to lodging and/or
100 lodging-related causes.

2. Material and methods

2.1. Plant materials

Maize inbred lines (B73, H84, Mo20W, B14A, PB55, EP104, H106W, B93, EP126, EA2024, B84, EP2008-20) were provided by Misión Biológica de Galicia-CSIC, Pontevedra (Spain). Those inbreds were selected from their bank germplasm because they showed contrasting values for rind penetration strength (RPS) in previous evaluations (data not shown): higher (H-RPS) or lower (L-RPS) than 15 Kg/cm^2. In the same way, inbreds were considered resistant to lodging (R-lines) with lodging values < 10% and susceptible (S-lines) with lodging values ≥ 10%.

Maize inbred lines were cultivated at the Mas Badia-IRTA Centre (La Tallada d´Empordà, Girona) and Misión Biológica de Galicia-CSIC (Salcedo, Pontevedra) in northeastern (42°03'N, 3°03'E) and northwestern Spain (42º25'N, 8º38'W) respectively, on a basic sandy loam soil in both locations. Experimental trials were carried out in 2015 using a randomized block design with two replicates. Each experimental plot consisted in two rows spaced 1.0 m apart in Girona and 0.80 m in Pontevedra. Each row had 13 one-kernel hill spaced 0.15 m apart in Girona and 0.18 m in Pontevedra, resulting in a plant density of approximately 67,000 and 70,000 plants/ha in Girona and Pontevedra respectively. Trials were irrigated, fertilized and controlled for weeds according to local agricultural practices.

2.2. Phenotypic characterization

Plants at the reproductive stage per plot were phenotyped by measuring stem diameter, plant height, number of internodes and RPS using five plants per plot. For dry weight percentage of the internodes, stalk sections below from healthy plants the main ear were weighed at harvest and immediately dried at 60ºC until weight remained unchanged. The stalk strength, measured as RPS, was evaluated in the centre of the flat side of the second internode below the primary ear-bearing node. Before evaluating with a penetrometer (Pontevedra: AMETEK, AccuForce CADET Force Gage; Girona: Facchini srl, FT 444), the leaf sheath was removed. In both devices, a 2 mm diameter pointed probe of 20 mm length was used. Stem diameters were measured in the same internode as above by using a Vernier Caliper (cm). After phenotypic data collection stem-pools were powdered using

a grinder (Retsch GM200; sieve: 1 mm). In addition, plots were examined for lodging at the harvest stage, measured as the percentage of plants including broken stalks below the main ear or leaning more than 45º from the upright, therefore, considering stalk and root lodging simultaneously. At the senescence stage, plants were also visually examined for fungal infection symptoms. Stalks and leaves were visually inspected for spread of damage and categorized using a 5 level scale from 100% (completely damage) to 0% (healthy).

2.3. Cell wall characterization

CW isolation from pooled-stems from each location was performed as previously described by Rebaque *et al*. [27]. Briefly, powdered stems were extracted with 70% ethanol (120 h) obtaining the alcohol insoluble residue (AIR) and then de-starched by α-amilase treatment (24 h). CWs were obtained from de-starched material by sequential treatment with phenol-acetic acid-water (2:1:1 by vol.) for 6 h, 70% ethanol and 100% acetone followed by air dried.

2.3.1. Fourier transform infrared (FTIR) spectroscopy

CWs were assayed using a JASCO 4700 instrument with an ATR module at a resolution of 4 cm^{-1}. For each sample the average FTIR spectra (n = 10) was obtained. Then all average spectra were normalized and baseline-corrected with Spectra manager v. 2.13.0 software. FTIR-spectra were selected for the 800-1800 cm^{-1} region corresponding to the wavenumbers associated with CW components [28].

2.3.2. Polysaccharide analyses

Cellulose was quantified in CWs with the Updegraff method [29], using the hydrolytic conditions described by Saeman *et al*. [30]. The glucose released after hydrolytic conditions was assayed by the anthrone method [31] using glucose as standard.

Tightly to loosely linked hemicelluloses ratio (KII/KI) was estimated after extracting CWs with 0.1 M KOH (10 mg/ml) for 24 h and 4 M KOH (10 mg/ml) for 24 h to obtain KI and KII fractions respectively.

Neutral sugar analyses were assayed as described by Albersheim *et al*. [32]. CW samples were hydrolyzed with 2 N trifluoroacetic acid at 121ºC for 1 h and the resulting sugars

were derivatized to alditol acetates and analyzed using a Supelco SP-2330 column and a Perkin-Elmer gas chromatography-flamed ionization detector (GC-FID).

Total sugar content was quantified from trifluoroacetic acid hydrolysate of CW by the phenol-sulfuric acid method [33,34] and expressed as glucose equivalents.

2.3.3. Lignin and cross-linking analyses

Lignin was quantified in CWs by Klason method accordingly to Dence [35] with minor modifications [27]. Lignin composition was assayed by thioacidolysis as described by Lapierre *et al*. [36].

In order to determine cross-linking properties CW-esterified FA, *p*CA and dehydrodiferulates isomers were analyzed from AIR after 2 N NaOH saponification by high performance liquid chromatography (HPLC) based on a method previously described by Santiago *et al*. [37]. The isomers of diferulic acid (diferulates, DFA) identified and quantified by this analytical method (8,5′-non-cyclic-DFA, 8,5′-cyclic-DFA or benzofuran, 8–*O*–4′-DFA and 5,5′-DFA) were added to obtain the total concentration of ester-linked-DFA (DFAT) [27].

2.4. Statistical analyses

Individual and combined by location analyses of variance (ANOVA) for the different traits were performed. As mentioned above, RPS was taken with different devices in the two locations, so in order to reduce any putative effects of using different devices and/or probe geometry on the measurements [38], the data were standardized (mean = 0 and standard deviation = 1) in each location to perform the combined analysis. Inbred lines were considered fixed factors, while location, replication nested within location and inbred line x location interaction were recognized as random factors. Inbred mean comparisons were performed by the least significant difference (LSD) method. We also conducted contrast analyses among H-RPS and L-RPS lines (RPS classification) and resistant (R-lines) and susceptible (S-lines) inbreds to lodging (lodging classification).

Besides, multiple regression was carried out using stepwise with $p < 0.15$ for both input and output variable. RPS and lodging were considered dependent variables, while CW components were independent variables. Correlation between agronomic variables were tested using the Pearson correlation procedure.

Averaged FTIR-spectral data from both locations were analyzed in order to determine inbreds grouping. A Principal Component Analysis (PCA) with the number of components which explains the 95% of the variance followed by a Hierarchical Cluster analysis of Principal Component (HCPC) with a Ward method were carried out using factoextra [39] and FactoMineR [40] packages. Moreover, a PCA and HCPC similar than the mentioned above were carried out with CW traits that showed significant difference among inbreds in the previous ANOVA.

SAS software [41] (v.9.4) was used to perform individual and combined analyses of variance and the multiple regression, while the rest of the analyses were conducted by RStudio (v.3.6.3) [42].

3. Results

After analyzing our data set, it was found that inbreds were not influenced by inbred line x location interaction (data not shown), therefore, we analyzed the inbreds jointly without considering their origin.

3.1. Agronomic phenotyping

Twelve maize inbred lines previously evaluated for RPS and lodging resistance were phenotyped at the flowering stage for agronomic traits (data not shown). Significant differences ($p \leq 0.05$) among inbreds were found for RPS, resistance to lodging, plant height, stem diameter and fungal infection symptoms. No significant differences ($p > 0.05$) were found for the number of internodes and the dry weight of the internode percentage (Table 1). [Insert Table 1 here]

Additionally, in order to look for significant differences in agronomic variables after grouping maize inbreds according to RPS and lodging resistance respectively, contrast analyses were carried out (Table 1). Inbred lines with high RPS values were more resistant to lodging. Besides, we found that both H-RPS and R-lines showed significantly higher values for plant height, dry weight of the internode percentage and lower percentage of fungal infection symptoms when compared with the L-RPS and S-ones (Table 1). Stem diameter and number of internodes were not found significantly different, either for RPS or lodging groups (Table 1).

After a simple correlation analysis for agronomic traits (Table 2), it was found that RPS was positively correlated to the number of internodes and dry weight of the internode, and negatively correlated to the lodging percentage and the percentage of fungal infection symptoms. Conversely, the correlation analysis showed that lodging was positively correlated to fungal infection symptoms and negatively correlated to the number of internodes and dry weight of the internode (Table 2). [Insert Table 2 here]

3.2. FTIR monitoring

As a first insight into the relationship among bulk CW composition and stalk strength and lodging resistance, CWs obtained from maize inbred lines were characterized by FTIR

spectroscopy [28]. In order to monitor *in muro* structural and compositional changes in CW, FTIR-spectra were extracted for the 800-1800 cm^{-1} region corresponding to the wavenumbers associated with CW components (Fig. 1A). Although average spectra profiles from H and L-PRS groups were similar, regions with a large variability located between 1200 and 1300 cm^{-1}, and remarkably, in the 1500-1750 cm^{-1} band can be outlined. These FTIR regions correspond to the absorption zones of compounds such as phenolic compounds (1220-1235 cm^{-1} [43] and 1620-1630 cm^{-1} [44]), lignin (1505-1515 cm^{-1}, 1540 cm^{-1} and 1560 cm^{-1} [44,45]), S and G lignin monomers (1207 cm^{-1} [46]), proteins (1540-1560 cm^{-1} and 1650 cm^{-1} [44]) and pectins including polygalacturonic acid (1600-1630 cm^{-1} and 1730-1740 cm^{-1} [47,48]). FTIR-spectra from R- and S-lines in relation to lodging showed the same pattern as H- and L-RPS lines, respectively (data not shown).

FTIR-spectral data from CWs, were used to carry out a PCA followed by a Hierarchical Cluster analysis (Fig. 1B). The dendrogram obtained displayed two main branches (I and II) subdivided into four sub-branches (A, B, C and D). As shown in Fig. 1B, the cluster analysis grouped all H-RPS inbreds under branch I (H84, Mo20W, EP2008-20, B73, B14A and H106W) and most of L-RPS ones under branch II (B93, EA2024, B84 and EP126). Two L-RPS lines (PB55 and EP104) were clustered together with H-RPS lines into branch I (sub-branch B). Considering lodging classification, sub-branch A (branch I) arranged two R-lines, B sub-branch (branch I) grouped S-lines as well as R ones, and C and D sub-branches (branch II) grouped mainly S inbreds (Fig. 1B). Wavenumbers significantly contributing to CW-FTIR spectra clustering into branch I and II were located in 996-1016 cm^{-1}, 1572-1610 cm^{-1}, 1676-1790 cm^{-1} spectral bands and a wide region corresponding to 1104-1520 cm^{-1} wavenumbers (data not shown). These later spectral bands fitted with large variability regions outlined in Fig. 1A.

3.3. Cell wall analyses

After the FTIR monitoring, an in-depth CW characterization was carried out, together with its appropriate statistical analysis (Table 3). Attending to the wet chemistry characterization of the CWs, significant differences among inbreds were observed for *p*CA, FA, 8,5′-non-cyclic-DFA (DFA85l), 8–O–4′-DFA (DFA8o4), H, G, S, S/G ratio

and loosely cross-linked hemicelluloses (KI). In addition, although 5,5´-DFA (DFA55), cellulose, tightly cross-linked hemicelluloses (KII) and arabinose content showed non-significant differences for inbred lines, they showed a significant inbred x location interaction (data not shown). Therefore, all traits which have been significant for inbreds or interaction among sources of variation have been included in Table 3 for subsequent analysis. The rest of the CW traits which showed non-significant differences were included in supplementary Table MS1. [Insert Table 3 here]

Some inbred lines can be highlighted from the analyses: The inbred PB55, classified as L-RPS and S-line, showed the lowest amounts of *p*CA, FA, DFA55 and arabinose content, but the highest amounts of cellulose. In contrast, H84 inbred, classified as H-RPS and R-line, revealed the highest amount of *p*CA, DFA55 and tightly cross-linked hemicelluloses (KII). With regard to lignin composition, B93 inbred, classified as L-RPS and S-line, showed the highest percentage of S subunits and hence the highest S/G ratio, as well as the largest loosely cross-linked hemicelluloses (KI) value. Finally, Mo20W inbred, classified as H-RPS and R-line, presented the highest percentage of G subunits, and thus, the lowest S/G ratio, and the lowest amount of tightly cross-linked hemicelluloses (KII) (Table 3).

Contrasts analyses between both RPS and lodging groups for CW traits are also shown in Table 3. CWs obtained from H-RPS inbred lines showed higher esterified phenolics such as *p*CA, FA and DFA55, H and G lignin subunits, cellulose and tightly cross-linked hemicelluloses (KII) than L-RPS, although significant differences were only found for H subunit percentage. On the other hand, L-RPS CWs showed significant higher concentrations for DFA85l and DFA8o4. In addition, although not significant, a trend in higher contents was observed for S lignin subunit percentage, (and the S/G lignin ratio), and for the loosely cross-linked hemicelluloses (KI) and arabinose content (Table 3). When CW variables were compared between maize inbred lines regarding lodging resistance, *p*CA, FA and KII contents were significantly higher in R-lines compared to the S-lines. The amounts of arabinose and DFA85l were higher in S-lines compared to R-lines. Other CW parameters such as DFA55, DFA8o4, H, G, S, S/G ratio, cellulose and loosely cross-linked hemicelluloses (KI) were not significant for contrast analyses considering lodging behavior (Table 3).

298 To better understand the interaction among CW traits and their distribution regarding the
299 inbreds, a PCA analysis was carried out using the variables of Table 3. PC1 and PC2
300 accounted for ca. 55% of total variance (Fig. 2). PC1 was explained by *p*CA, FA, DFA55,
301 H and DFA85l traits, with a correlation of 0.89, 0.81, 0.66, 0.61 and -0.60, respectively.
302 PC2 was explained by S, KII and G variables, with 0.86, 0.78 and -0.88 correlation values,
303 respectively.

304 Considering both RPS and lodging resistance groups, maize inbred lines seem to
305 distribute along PC1 (Fig. 2). With the exception of inbred B73, all maize inbreds which
306 combined H-RPS and lodging resistance (H/R) were distributed at the positive side of
307 PC1; whereas all the inbreds which combined L-RPS and S-lines (L/S) were found at the
308 negative side of this PC.

309 Finally, stepwise multiple linear regression analyses were performed in order to get
310 knowledge about which CW traits contribute to RPS and which ones to lodging
311 resistance. Our results indicate that the 34% of RPS strength variation could be explained
312 by the percentage of H subunits (RPS = -1.74874 + 1.09509 * H ($r^2 = 0.34$)). On the other
313 hand, the best predictor of lodging was ferulic acid (FA: 53% explained variation),
314 followed by total diferulates (DFAT: 14% explained variation) and cellulose (8%
315 explained variation) content. According to linear regression analysis, and increase in FA
316 and cellulose would improve resistance to lodging, whereas DFAT would positively
317 contribute to lodging (Lodging = 266 - 0.06759 * FA ($r^2 = 0.53$) + 0.13521 * DFAT ($r^2 =$
318 0.14) - 0.17514 * cellulose ($r^2 = 0.08$)).

4. Discussion

Mean comparison, contrast, and correlations showed that maize inbreds with a high RPS were usually resistant to lodging, which is in agreement with other studies [49]. RPS is an agronomic parameter easily measurable due to environmental conditions, such as wind, are not required to evaluate it. Therefore, it could be a suitable trait for improving lodging indirectly as was proposed by Martin *et al.* [26].

In our study, plants with a high RPS and lodging resistance were taller, accumulated more dry weight in the internodes and showed lower fungal infection symptoms than L-RPS and S-lines. Contrary, characters such as stem diameter and number of internodes did not seem to contribute to distinguish either for RPS or lodging among inbred lines analyzed (Table 1 and 2). RPS and the dry weight percentage of the internodes were factors inversely related to maize lodging as previously reported [7,26,50]. Along with this, a negative correlation between lodging susceptibility and plant height was found in this study. This result disagrees with studies reporting a positive correlation between lodging and plant height [49,51,52], those showing the lack of association between lodging resistance and short plants [53] or the marginal relationship between stalk lodging and plant height in maize [54]. Some studies have shown that stem diameter is negatively correlated to lodging [55]. Contrary, our results showed a lack of significant correlation between these two parameters (Table 2). It must be pointed out that differences attending to stem diameter within our genotypes set were marginal, and hence it would hinder the identification of a significant correlation, although a negative orientation has been found (Table 1 and 2).

The cluster analysis of FTIR-spectra allowed us to suggest a putative relationship between the composition and/or structure of the CW, RPS and lodging resistance (Fig. 1B). Thus, these results invited us to carry out a more in-depth characterization of CW composition and structure for better understanding their role in the rind penetration and lodging resistance (Fig. 1B).

Deepening into the study of the CW parameters, significant differences among inbreds were found for CW esterified phenolics, both monomers and dimers, loosely cross-linked hemicelluloses (KI) and lignin composition by ANOVA analysis (Table 3). The lack of significance in core components of maize secondary CW (Table MS1), such as Klason

lignin content, cellulose, hemicelluloses and matrix sugars (such as arabinose, xylose or glucose, among others) points to minor differences among maize inbreds in terms of quantitative composition of CW. In light of these results, we suggest that variation between close genotypes are more likely to occur in the arrangement and interaction among CW components (qualitative differences) than in the gross amount of each particular constituent.

After a contrast and stepwise multiple linear regression analyses for RPS, our results indicated that the amount of a minor CW components such as H subunits are a key factor explaining high RPS. This could be achieved through increasing the number of bonds that can be generated among CW polysaccharides as it has been previously pointed out [56]. However, only 34% of the RPS variability is explained by the contribution of H units, indicating that other characteristics, in addition to these lignin subunits, contribute to the variability of RPS.

The contribution of diferulates to hemicellulose cross-linkage can be associated to higher tissues toughness [57], and thereby, pest resistance properties [58,59]. Surprisingly, our results indicated that 1) a significantly higher level in DFA85l and DFA8o4 was found in L-RPS when compared to H-RPS ones (Table 3), and 2) a non-significant increase in total diferulates (DFAT) was associated with RPS groups (Table MS1). Although unforeseen, the relationship between increased DFA contents and L-RPS inbreds could be understood as a coping strategy to overcome a weakened CW. It is worth mentioning that CWs show a remarkable structural/compositional plasticity and that compensatory mechanisms involving DFA and other CW components have already been shown as in the case of weakened cellulose-deficient cells [60,61].

As indicated above, no significant differences in DFAT content were found in our inbred collection (Table MS1). However, the regression analysis suggests the existence of a positive correlation between the DFAT content and lodging susceptibility in maize stalks analyzed. As explained above, it is likely that a high DFAT content increases the hemicellulose cross-linking degree. To what extent this may contribute, both directly or indirectly to the unexpected relationship between DFA and lodging susceptibility found here will need of a further investigation.

It has been proposed that FA acts as a nucleation point for lignification, contributing to cross-coupling hemicelluloses and lignin, and increasing the strength of the CW [60,62].

Apart from this, FA also contributes to cross-linking CW polymers, through ester and ether bonds with hemicelluloses (arabinose) and lignin, respectively [60]. In this sense, some authors have reported that the CW becomes thinner and firmer as the amount of cross-linked feruloylated arabinoxylans increases [63]. Regarding our results on lodging classification, a relationship between increased levels of CW-esterified FA and lodging resistance can also be established (Table 3 and Fig. 2). The significant higher FA content found in R-lines compared to S-lines (Table 3), together with results obtained from stepwise multiple linear regression analyses for susceptibility to lodging, led us to propose that increased levels CW-esterified FA associates with lodging resistance in maize.

Differently to FA, *p*CA does not seem to participate in CW cross-linking and therefore its role in CW reinforcement and lodging resistance is far to be elucidated [60]. However, our contrast analysis revealed the existence of a positive relationship between esterified *p*CA in the CW and the lodging resistance of maize stalks.

On the other hand, the lignin composition, particularly S/G ratio, may condition the interaction with the hemicellulosic matrix. Paying attention to B93 and Mo20W inbreds, high or low S/G ratios (Table 3) seems to weaken the linkage of CW hemicelluloses being associated with low KII/KI ratios (Table MS1). However, B93 inbred (high S unit %) was considered L-RPS and S-line, whereas Mo20W (high G unit %) was classified as H-RPS and R-line. Thus, attending to lignin monomeric composition it seems that a larger percentage of G subunits, predominates over matrix polysaccharide role in determining RPS. This result seems to contradict recent findings that would relate S lignin with a more extensive xylan-lignin interaction and hence a higher CW strengthening [23]. In this sense, and as previously noted, the lignin composition could affect some other features in the CW matrix that would depend on the particular genotype, so it is difficult to point out a global and precise role for those lignin subunits.

Finally, the relationship between cellulose and hemicellulose contents and lodging resistance is still unclear due to the fact that positive, negative or no correlations have been proposed [64] and refs. therein. Data obtained with our inbred collection indicated that a weak relationship or no relationship at all exists between cellulose and hemicellulose contents and lodging resistance of maize stalks (Table 3). Although cellulose was included in the regression equation, the orientation of the effect for lodging could be

414 conditioned by the residual variability explained in the model (8%). Moreover,
415 hemicellulose content was previously rejected since it was found as non-significant CW
416 variable through combined analysis (Table MS1). Thus, our results would point to a lack
417 of association between lodging resistance and cellulose and hemicellulose net amounts
418 agreeing with some of the last above mentioned results [64] and refs. therein.

419 Previous works have demonstrated the positive correlation between arabinose-
420 substitution of xylans and the amount of amorphous cellulose regions [65,66]. Therefore,
421 a reduction in the arabinose content and/or in the arabinose/xylose ratio contribute to
422 decreasing the amount of cellulose crystallinity [65,66] and hence, to an increase of the
423 lodging resistance. In line with this, the higher arabinose content found in S-lines, could
424 be associated with an increase in cellulose amorphous regions rather than with variations
425 in the cellulose content (Table 3 and Fig. 2). In addition to that observation, lodging
426 resistance was also related to hemicellulose extractability, as hemicelluloses tightly cross-
427 linked to CW (KII) were found in higher concentrations in R-lines (Table 3) agreeing
428 with its PCA vector (Fig. 2). Therefore, our results indicate that compositional features
429 of cellulose and hemicellulose could have in fact a role in the maize lodging resistance.

430 In this study, twelve representative maize genotypes with different genetic background
431 have been analyzed. The composition of the CWs is a complex matter which may depend
432 on the particular genotype, and the experimental design may impact on the final results
433 likewise. In an attempt to overcome these limitations, these twelve inbred lines were
434 grown in two locations using a randomized block design with two replications at each
435 one, and a wide set of CW parameters has been evaluated. In our opinion, these results
436 provide valuable information about the impact of the CW composition on RPS and
437 lodging resistance in maize, enabling us to establish the basis for future studies.

5. Conclusions

The characterization of this maize inbred collection performed in this work has allowed us to shed light on the complex relationships existing between CW components and the properties of maize stalk, such as strength and lodging resistance.

Results provided here point to less differences among maize inbreds in terms of bulk composition of major CW components (cellulose, hemicelluloses and lignin), allowing us to highlight the importance of minor CW components and their effects on CW microstructure. Thus, our results revealed that H subunits, although present in minor amounts in the lignin polymer, can play an important role in strengthening the maize stalk, while some types of diferulates (DFA85l and DFA8o4) are associated with L-RPS inbreds. On the other hand, lodging behavior can be explained by ferulic acid and dimers in an opposite way. Ferulic acid would improve resistance to lodging, whereas total diferulates would relate to lodging susceptibility. This knowledge provides new biotechnological tools for breeding programs aimed at improving maize resistance to lodging.

The results provided here pave the road for future works in which a more in depth characterization of the CWs from selected inbreds for RPS and lodging susceptibility will be needed. Considering that the amount of main CW components seem not to explain clearly either RPS or lodging susceptibility, further studies need to be carried out, focusing specifically on the CW microstructure and interactions among its constituents.

Author contributions

AE, PG, MC, SF, JA, JM, DC, RS and RM carried out the experimental design of the work and obtained the funding for the research. AM contributed to compile and analyse the data and wrote the manuscript. AE, PG, RM and RS contributed to analyse the data and supervised the manuscript writing. RS, RM, VM and AM conducted statistical analyses. AE, PG, MC, SF, JA, JM, DC, IL, RS, RM and AM performed the experimental analyses. MC, SF, JA, JM, DC, VM and IL revised the writing manuscript. All authors read and approved the manuscript.

Declaration of Competing Interest

We have no conflict of interest to declare.

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Availability of data and materials

The data sets used and/or analysed during the current study together with their corresponding scripts are available from the corresponding author on reasonable request.

Appendix A. Supplementary data

TableMS1. Mean values for the non-significant cell wall traits evaluated through combined and contrast analyses (AppendixA.docx).

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Figures and tables

Figure 1. (A) FTIR-spectra profiles of cell walls from maize inbreds cultivated at two locations-classified as low (L-RPS; gold line) and high (H-RPS; green line) rind penetration strength. FTIR-spectra showed the typical features of a secondary CW, namely: (i) the 800-1200 cm^{-1} band (fingerprint region) where absorption of polysaccharides such as cellulose and hemicelluloses is found [67], (ii) the 1200-1400 cm^{-1} band where cellulose (1320-1385 cm^{-1}) and lignin (1260-1440 cm^{-1}) absorb; and (iii) the 1600-1800 cm^{-1} band assigned to phenolic ester and ether bonds [28]. Data represent $\overline{X}$ ± SD (n = 4). **(B)** Dendrogram of FTIR-spectra data profiles of cell walls from maize inbred lines cultivated at two locations. Cluster analysis was carried out using the means corresponding to each line. Green circles represent maize inbred lines with high rind penetration strength (H) which were classified as resistant to lodging (R). Gold triangles represent maize inbred lines with low rind penetration strength (L) which were classified as susceptible to lodging (S). Blue squares represent maize inbred lines with high rind penetration strength (H) which were classified as susceptible to lodging (S). Pink crosses represent maize inbred lines with low rind penetration strength (L) which were classified as resistant to lodging (R). A-D are clusters discussed in the text.

Figure 2. Principal component analysis (PCA) of maize inbreds cultivated at two locations. A plot of the first and second components (PCs) is represented based on the following variables: *p*CA (*p*-coumaric acid), FA (ferulic acid), DFA55 (5,5′-DFA), DFA85l (8,5′-non-cyclic-DFA), DFA8o4 (8–*O*–4′-DFA), H (*p*-hydroxyphenyl), G (guaiacyl), S (syringyl), S/G ratio, cellulose (µg/mg CW), KII (4 M KOH), KI (0.1 M KOH), ARA (arabinose). Green circles represent maize inbred lines with high rind penetration strength (H) which were classified as resistant to lodging (R). Gold triangles represent maize inbred lines with low rind penetration strength (L) which were classified as susceptible to lodging (S). Blue squares represent maize inbred lines with high rind penetration strength (H) which were classified as susceptible to lodging (S). Pink crosses represent maize inbred lines with low rind penetration strength (L) which were classified as resistant to lodging (R).

Table 1. Mean values for the agronomic traits on twelve inbreds evaluated at two locations and H (H-RPS lines) versus L (L-RPS lines) and R (lodging resistant lines) *versus* S (lodging susceptible lines) contrast.

Inbred line	Rind penetration strength classification	Lodging classification	Rind penetration strength	Lodging (%)	Plant height (cm)	Stem diameter (cm)	No. of internodes	Fungal infection symptoms (%)	Dry weight of the internode (%)
B14A	H	R	0.98	0.00	194	2.37	13.13	12.50	23.98
B73	H	R	0.49	8.33	207	2.40	13.25	0.00	16.57
H106W	H	R	1.04	0.00	193	1.94	14.08	0.00	20.12
H84	H	R	1.04	0.00	167	2.08	13.33	0.00	20.66
Mo20W	H	R	1.12	0.00	172	2.17	14.58	0.00	16.39
EP2008-20	H	S	0.13	23.33	147	2.36	11.75	12.50	14.92
B84	L	R	-0.74	0.00	177	2.23	11.63	50.00	15.25
B93	L	S	-0.52	43.75	123	1.95	12.44	75.00	14.97
EA2024	L	S	-1.14	88.75	159	1.83	9.65	87.50	14.97
EP104	L	S	-0.49	27.00	177	2.17	13.17	12.50	14.34
EP126	L	S	-0.38	33.75	122	1.99	9.06	87.50	11.87
PB55	L	S	-0.93	62.50	151	2.18	9.88	100.00	10.54
LSD ($p \leq 0.05$)			0.88	27.95	32	0.28		35.03	
Rind penetration strength									
Mean H			0.81 a	5.00 b	181 a	2.23	13.57	4.17 b	18.77 a
Mean L			-0.68 b	40.81 a	151 b	2.05	10.90	68.75 a	13.66 b
Lodging									
Mean R			0.51 A	1.47 B	187 A	2.22	13.17	12.50 B	19.32 A
Mean S			-0.34 B	40.33 A	149 B	2.07	11.40	53.57 A	14.00 B

LSD Least significant difference ($p \leq 0.05$).
[a-b] Significant differences between the two rind penetration strength class (H and L lines).
[A-B] Significant differences between the two lodging class (R and S lines).
Rind penetration strength data were standardized (mean = 0 and standard deviation = 1) in each location to perform the combined analysis.

721 **Table 2.** Simple correlation coefficients (Pearson) among agronomic traits on twelve
722 inbreds evaluated at two locations.
723

	Lodging	Plant height	Stem diameter	No. of internodes	Fungal infection symptoms	Dry weight of the internode
Rind penetration strength	-0.79 **	0.51	0.31	0.79 **	-0.84 ***	0.77 **
Lodging		-0.54	-0.51	-0.75 **	0.81 **	-0.62 *
Plant height			0.47	0.61 *	-0.69 *	0.61 *
Stem diameter				0.31	-0.49	0.20
No. of internodes					-0.88 ***	0.66 *
Fungal infection symptoms						-0.68 *

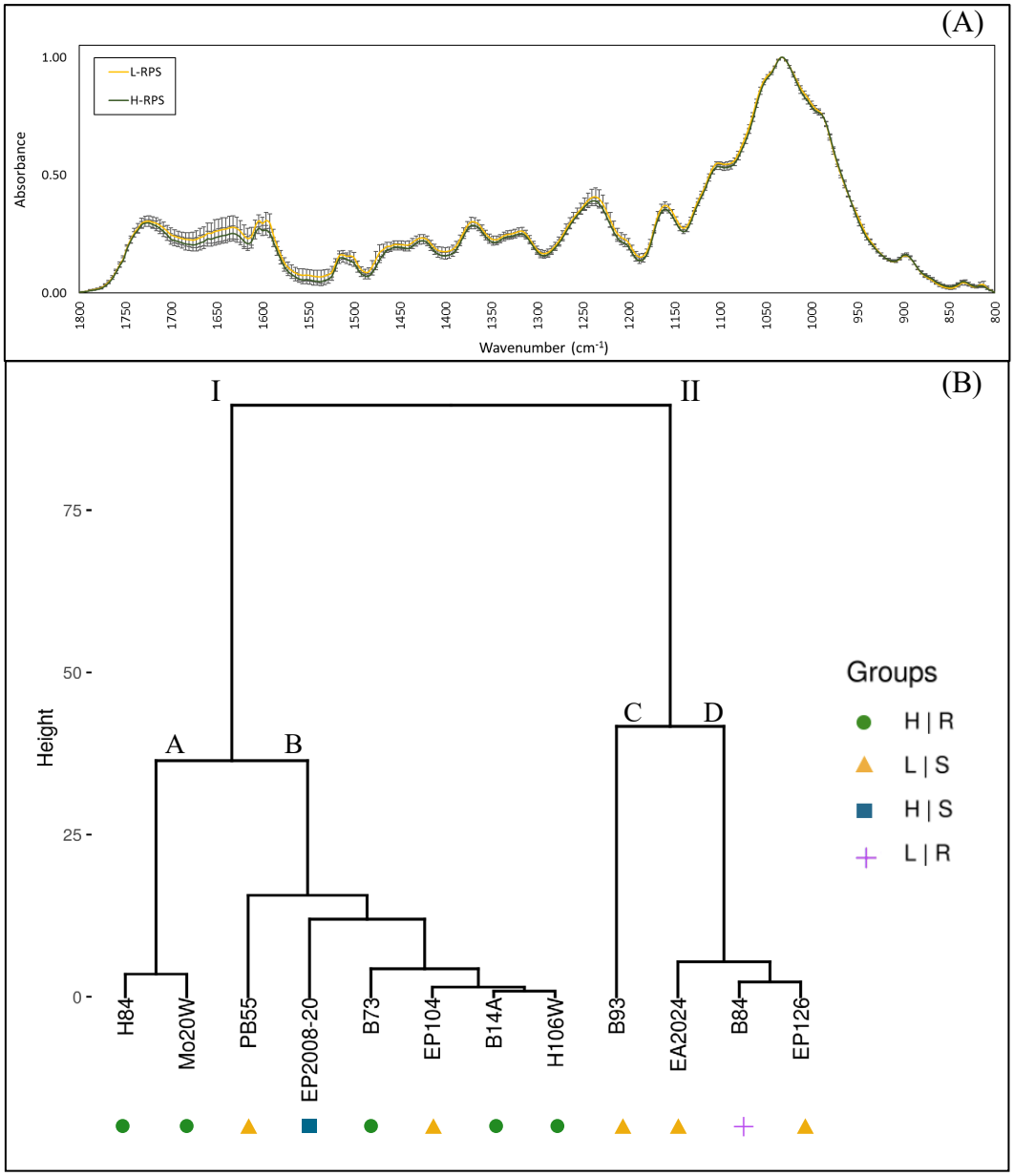
724
725 * Probability level of 0.05.
726 ** Probability level of 0.01.
727 *** Probability level of 0.001.

Table 3. Mean values for the significant cell wall variables analyzed through combined analyses on twelve inbreds evaluated at two locations, and H (H-RPS lines) *versus* L (L-RPS lines) and R (lodging resistant lines) *versus* S (lodging susceptible lines) contrast. Variables which had a significant interaction inbred line x location are also shown (cellulose, KI and ARA).

Inbred line	Rind penetration strength classification	Lodging classification	*p*CA (µg/g AIR)	FA (µg/g AIR)	DFA55 (µg/g AIR)	DFA85l (µg/g AIR)	DFA8o4 (µg/g AIR)	H (%)	G (%)	S (%)	S/G	Cellulose (µg/mg CW)	KII (µg/mg CW)	KI (µg/mg CW)	ARA (µg/mg CW)
B14A	H	R	14265	3175	101.2	34.37	91.31	1.23	43.25	55.52	1.29	486.0	232.9	97.7	28.29
B73	H	R	13988	3298	97.0	47.34	90.01	1.42	38.47	60.11	1.56	494.4	230.7	112.4	37.88
H106W	H	R	16802	3202	127.1	39.05	107.9	2.68	29.56	67.76	2.32	494.0	259.2	113.6	32.90
H84	H	R	17873	3810	159.3	32.47	100.6	2.05	30.77	67.18	2.19	438.1	290.7	104.7	27.27
Mo20W	H	R	16946	3172	121.3	47.79	116.1	2.23	44.52	53.25	1.21	525.3	207.3	108.1	31.00
EP2008-20	H	S	14928	3420	145.8	79.71	170.1	1.42	36.17	62.41	1.76	518.8	220.9	115.9	38.09
B84	L	R	17482	4101	145.9	61.59	136.5	1.53	35.71	62.76	1.77	343.6	214.2	93.0	26.99
B93	L	S	13215	2842	132.4	58.67	129.4	1.26	26.55	72.19	2.96	419.3	243.1	133.3	38.88
EA2024	L	S	15485	2727	91.5	55.53	116.9	1.50	33.66	64.84	2.01	390.5	228.2	125.6	29.15
EP104	L	S	15703	3210	113.3	77.13	128.9	1.74	39.91	58.35	1.51	512.4	208.1	107.6	50.49
EP126	L	S	14094	2888	99.1	61.30	119.8	1.34	33.08	65.58	2.12	475.0	211.4	103.4	36.78
PB55	L	S	12352	2583	74.3	61.95	131.3	1.29	26.96	71.75	2.67	591.2	225.7	111.7	25.16
LSD ($p \leq 0.05$)			2392	485		23.41	31.61	0.43	7.08	3.18	0.43			23.5	
Rind penetration strength															
Mean H			15720	3337	124.0	46.14 b	111.6 b	1.84 a	37.12	61.04	1.72	492.41	239.9	108.2	32.34
Mean L			14938	3102	112.6	62.76 a	126.7 a	1.45 b	32.98	65.57	2.14	441.47	221.9	112.9	35.37
Lodging															
Mean R			16058 A	3532 A	125.8	43.55 B	106.3	1.78	35.55	62.67	1.83	446.94	243.0 A	103.3	30.31 B
Mean S			14763 B	2984 B	112.3	63.20 A	129.2	1.55	34.78	63.67	2.00	479.51	221.2 B	116.2	36.61 A

LSD Least significant difference ($p \leq 0.05$).
[a-b] Significant differences between the two rind penetration strength class (H and L lines).
[A-B] Significant differences between the two lodging class (R and S lines).
Legend: *p*CA (*p*-coumaric acid), FA (ferulic acid), DFA55 (5,5′-DFA), DFA85l (8,5′-non-cyclic-DFA), DFA8o4 (8–*O*–4′-DFA), H (*p*-hydroxyphenyl), G (guaiacyl), S (syringyl), S/G ratio, KII (4 M KOH), KI (0.1 M KOH), ARA (arabinose).

736 **Figure 1.**
737



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740
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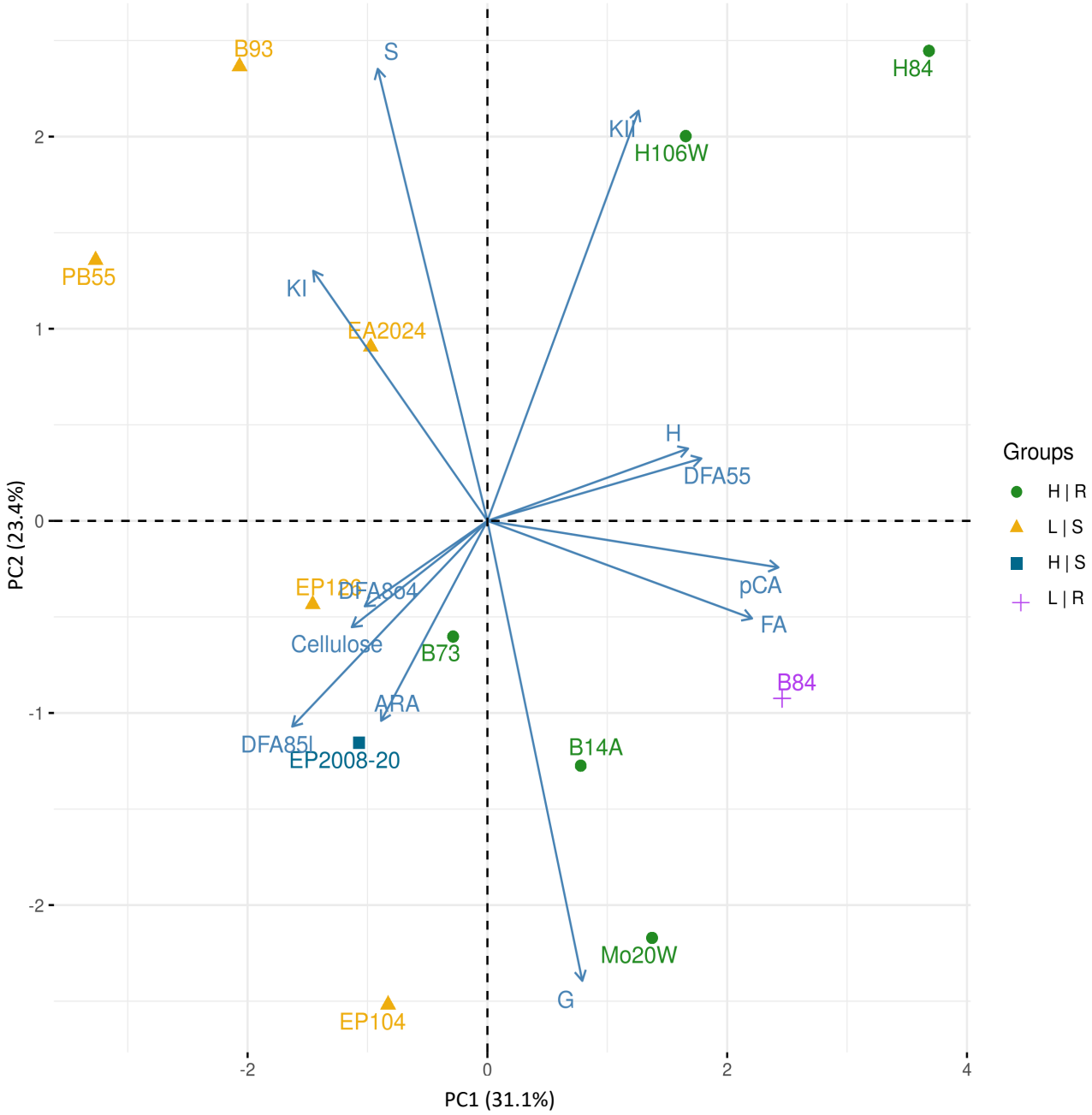
Figure 2.


B93
S
H84
KII
H106W
PB55
KI
EA2024
H
DFA55
pCA
FA
EP108
DFA804
Cellulose
B73
B84
ARA
DFA85I
EP2008-20
B14A
Mo20W
G
EP104
PC2 (23.4%)
PC1 (31.1%)
2
1
0
-1
-2
-2
0
2
4
Groups
H | R
L | S
H | S
L | R