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Straw digestibility in rice: Novel insights from pyrolysis GC-MS and biomass phenotyping

Mariana P. Silva [a,1], Caragh Whitehead [a,1], Reynante L. Ordonio [b], Trinidad C. Fernando [b], Mark Philip B. Castillo [b], Jeremias L. Ordonio [b], Tony Larson [c], Daniel J. Upton [a], Susan E. Hartley [d], Leonardo D. Gomez [a,*]

[a] *CNAP, Biology Department, University of York, Wentworth Way, Heslington, York, YO10 5DD, UK*
[b] *Philippine Rice Research Institute, Maligaya, Science City of Munoz, Nueva Ecija, Philippines*
[c] *Bioscience Technology Facility, Biology Department, University of York, Wentworth Way, Heslington, York, YO10 5DD, UK*
[d] *School of Biosciences, University of Sheffield, Sheffield, UK*

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ABSTRACT

Valorizing rice straw could mitigate the detrimental health and environmental consequences of disposal through field burning. An essential step for achieving this is reducing straw recalcitrance to digestion by either enzymes or animals to facilitate uses as fertilizer, animal feed, or conversion into fuels and chemicals. In the present work, we developed and characterized a Philippine rice diversity panel to explore the chemical basis of biomass recalcitrance. We used high throughput phenotyping of straw samples from the panel to identify chemical compounds that confer recalcitrance. We determined the saccharification potential, silica, ferulic, and p-coumaric acid content in each rice accession, as well as the chemical fingerprint of biomass composition using pyrolysis followed by GC/MS. Multivariate analysis of the phenotypic data allowed us to characterize the relationship between biomass components and straw saccharification establishing that Si, ferulic acid and coumaric acid are inversely correlated with saccharification. PCA analysis showed that pyrolysis products derived from lignin constitute the largest proportion of compounds inversely correlated with saccharification.

1. Introduction

Large quantities of rice straw are generated globally as rice is one of the main staple foods for human consumption and straw production is unavoidable. It is estimated that as much as 700 million tonnes of rice straw and husk are produced each year globally, and a large amount of this is burned to remove the crop waste from the fields [1–3] with negative impacts on human health and the environment. Straw represents approximately half of the total biomass of the rice plant [4] and is comprised of lignocellulosic material. Conversely, rice straw could represent a large renewable source of biomass with potential for valorization [5]. However, the high silica content and its poor digestibility represent a hurdle for rice straw to be used as feed, fertilizer, or feedstock for biorefineries [6]. Developing uses for rice straw remains a challenge, but given the importance of rice in human nutrition, improving rice straw quality is key to reducing the environmental impact of this essential staple crop.

One of the main obstacles to valorizing lignocellulosic biomass is its recalcitrance to hydrolysis. Plant cell walls have evolved to resist enzymatic digestion by establishing a network of polymers that are interconnected by covalent and hydrogen bonds, restricting the access of enzymes to their substrates [7]. Crystalline cellulose microfibrils are embedded in an interconnected network of complex matrix polysaccharides and lignin, a phenolic polymer that effectively seals the polysaccharides into a resistant macromolecular structure [8]. The grass cell wall presents substantial differences compared with dicots, particularly in matrix polysaccharides and lignin. In grasses, the major matrix polysaccharides are complex arabinoxylans, highly decorated with hydroxycinnamic acid esters appended to arabinosyl side chains [9]. The ferulic acid (FA) appendages of arabinoxylans can oligomerise with those of neighboring chains or with lignin [10] which, in grasses, contains hydroxycinnamic acid units in its structure [11]. These crosslinks

help to increase the resistance of the biomass to enzymatic attack. Reducing lignocellulose recalcitrance could facilitate alternative uses of rice straw, generating extra income for farmers. Changes in cell wall components in rice can affect straw recalcitrance, as shown by several studies to improve energy crops and processing alternatives [12,13]. The efforts to reduce recalcitrance in grasses have been directed at reducing lignin content [14] and reducing ester bonds between hydroxycinnamic acids and hemicellulose and lignin [15]. The availability of gene editing approaches that allow precise targeting of saccharification bottlenecks [16].

Rice is the highest Si accumulator in *Graminae*, and among the multiple beneficial effects that Si has in plants, it is regarded as a defense against herbivores due to the physical strengthening of the cell wall [17]. As a cell wall component, it interacts with cell wall polymers and co-regulates the composition and processing characteristics of rice straw [18]. Reducing the content of Si in rice cultivars by increasing N supply, increases saccharification of the resulting biomass [19]. Indeed, removal of the Si content using alkaline pretreated increases enzymatic hydrolysis of rice husks several folds [20].

Phenotyping large populations to assess differences in cell wall composition and digestibility requires high-throughput approaches that can cope with the large populations necessary to capture genetic diversity. Whilst such high-throughput approaches are now available for measurement of silica [21], the intrinsic chemical complexity of plant cell walls presents an analytical challenge. Spectroscopic approaches such as Fourier transformed infrared spectroscopy and near infrared spectroscopy (NIRS) have been applied to study plant populations and establish cell wall characteristics associated with saccharification [22, 23]. In rice, NIRS has been applied to identify lignocellulose recalcitrant factors in transformed lines and recombinant inbred lines [24,25]. These approaches, however, require the development of wet chemistry based models in order to gain precision.

Advances in mass spectrometry–based metabolomics and data processing techniques allow for large-scale analysis of untargeted metabolic profiles [26]. Analytical pyrolysis coupled with gas chromatography/-mass spectrometry (Pyr–GC/MS) has been successfully used for the characterization of complex natural polymers, such as lignin and polysaccharides [27–29]. The resulting pyrograms represent the fingerprint pattern of the samples, allowing a synthetic visualization of high-dimensional data and the relationships between different variables [27–29].

In the present study, we characterised the straw from field-grown rice varieties from the Philippines using analytical (Pyr-GC/MS) combined with phenotyping for saccharification potential, silica determination and hydroxycinnamic acid content. We explored the suitability of Pyr-GC/MS and PCA as a fingerprinting technique to identify the basis of straw recalcitrance.

2. Materials and Methods

2.1. Rice population and plant materials

The PhilRice association panel comprised 215 rice genotypes from the Philippines, which originate from two Oryza sativa subspecies: *indica* and *nerica*. These genotypes were selected from a number of different sources (76 released and 14 traditional varieties plus 125 elite lines). These genotypes are expected to be highly inbred lines with a homozygous genomic background. (See the Supplemental file Fig. S1 and Table S2 for the list of the population used).

The association panel was grown in the field (N 15° 40′09″N 120° 53′45″E, 2017DS & 2018DS) at the Philippines Rice Research Institute, Science City of Muñoz, Philippines. The field trial was conducted in randomized plots of 10 m^2 at a plant density of 40 plants/m^2. Each straw sample was collected from the main tiller of five plants after harvesting for grain, and 5 replicates were grown for each genotype. The straw collected was dried for two days in the open air. Straw samples were

kept in separate paper bags and sent to the Centre for Novel Agricultural Products (CNAP), University of York, UK for characterisation. The rice stems (minus nodes) were cut into small pieces, then ground to a fine powder using a cyclone mill (Retsch UK, 1 mm sieve) and stored. These samples were used for assays including saccharification, Pyr-GCMS and total lignin content.

2.2. Ferulic (FA) and p-coumaric acid (CA) determination

Alkaline extraction of CA and FA was performed according to Fry 2000 [30]. Approximately 10 mg of dry plant material was ball milled and 1 ml of 1 M NaOH was added and the samples were incubated shaking at 140 rpm at 30 °C overnight. The reaction was neutralized by addition of 100 μl 99% TFA and phase extracted twice using butanol. The butanol was evaporated, and the pellet resuspended in 200 μl MeOH. Samples were analyzed by LC/MS. Separation was carried out on an Acquity I class LC system (Waters UK, Elstree) using a BEH C18-1.7u 2.1 × 100mm (Waters), the mobile phases were A) 0.1% acetic acid in H$_2$O, and B) 0.1% acetic acid in acetonitrile. The gradient started at 20% B, and increased to 100% B over 3.25 min, where it remained for 0.15 min. The gradient returned to 20% B over 0.05 min, and re-equilibrated over 0.55 min. The total duration of the program was 4 min. The flow rate was 0.5 ml min^{-1}, and the injection volume 3 μl. The detector used was a TSQ EnduraTM triple quadrupole mass spectrometer (Thermo Fisher Scientific, Altrincham, UK) with a HESI source, operated in negative MRM mode. Commercially purchased standards of CA and FA (Sigma) were used to construct standard curves in the range from 12 to 400 μM.

2.3. Saccharification determination

Saccharification potential was determined using an automated robotic platform as previously described [31]. In brief, loading of plant powder into 96-well plates was done using a custom-made robotic platform (Labman Automation, Stokesley, North Yorkshire, UK), and saccharification assays were performed after water pretreatment at 94 °C for 30 min. Enzymatic hydrolysis was carried out using an enzyme cocktail with a 4:1 ratio of Celluclast and Novozyme 188 (Novozymes Enzymes).

2.4. Analytical pyrolysis-gas chromatography-mass spectrometry (Pyr-GC/MS)

Individual powdered rice straw samples were subject to Pyr-GC/MS analysis. Pyr-GC/MS results were obtained using a CDS 5250-T Trapping Pyrolysis Autosampler, Agilent Technologies 7890B GC System as gas chromatography unit and Agilent Technologies 5977A MSD as mass spectrum unit. The samples were pyrolyzed at 450 °C for 15 s, under trapping mode. The volatile materials released were carried into the GC/MS unit, via a heated transfer line (340 °C) in a helium flow for GC/MS analysis. The following GC/MS parameters were applied: GC inlet temperature at 350 °C, the initial temperature at 40 °C for 2 min, ramp rate at 10 °C/min until 300 °C, holding at 300 °C for 12 min, split ratio 50:1. Helium carrier gas flow was 1 ml/min and the separation was performed using an Agilent HP-5ms Ultra Inert (30 m × 0.25 mm x 0.25 μm) column. Volatile compounds were identified by comparing the mass spectra with NIST 14 library database (Match ≥85%).

Three spectra for each sample were collected, and the triplicate-averaged spectrum was used for principal component analysis (PCA). PCA was carried out using The Unscrambler X 10.5.1 software (CAMO). Peak identification was initially done manually using MassHunter Qualitative Analysis B.07.00 and NIST 14 library.

Before PCA analysis, the acquired data were processed according to the methodology previously described [32–36]. This process produced a matrix of baseline corrected peak areas for samples x unique masstags (*m/z* and retention time), for further analysis.
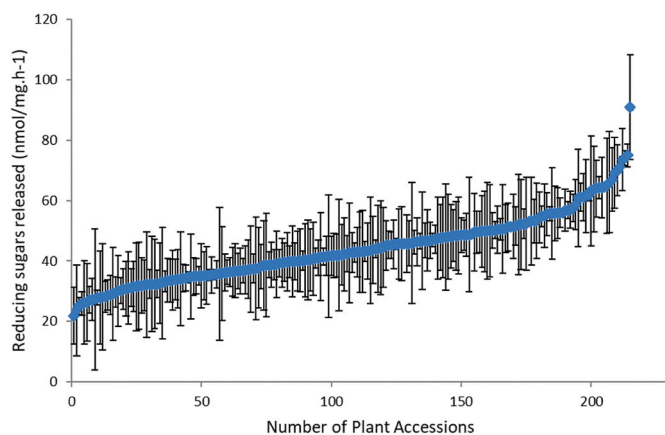
Fig. 1. Range of saccharification values obtained for the rice association panels.

2.5. Determination of silicon and lignin

Silicon (Si) in pellets of ground straw was determined using a portable X-ray fluorescence spectrometer and ground biomass pellets following the method validated by Reidinger et al. [21]. Lignin content was quantified using acetyl bromide [37]. Three replicates (4 mg) from each straw sample were used for lignin determination following the procedure previously described [38]. Lignin content ($\mu g.mg^{-1}$ cell wall) was determined using the following formula: (Absorbance ÷ (coefficient × path length)) × ((total volume × 100%) ÷ biomass weight)). The coefficient for grasses (17.75) was used for rice.

3. Results

3.1. Saccharification

Lignocellulose recalcitrance to digestion was measured by incubating ground straw from 215 individual genotypes with a commercial cellulase cocktail following a water pre-treatment at 94 °C using an automated platform as reported by Gomez et al. [31]. The straw was harvested in the spring season of 2017 and the material used for saccharification was carefully selected to include stem tissue of

equivalent age. The saccharification results show values between 21.48 and 90.84 nmol of reducing sugar equivalents/mg of biomass per hour of hydrolysis (nmol/mg.h) (Fig. 1). These values present a 4.2-fold variation in saccharification potential, indicating that saccharification represents a trait with a wide range of phenotypic variation in our association panel. In a recent study, Nguyen et al. used the same platform to determine the saccharification potential in a rice association panel from Vietnam [38]. The Vietnamese association panel presented values ranging between 20 and 134 nmol/mg.h in two consecutive years (2013 and 2014) under field conditions, therefore showing a phenotypic variation similar to the PhilRice association panel.

3.2. Pyr-GC/MS analysis of rice straw

The PhilRice association panel was characterized by Pyr-GC/MS fingerprinting of each of the rice varieties in the panel to identify pyrolysis peaks that associate with the recalcitrance of rice straw to enzyme digestion.

The composition of the straw in the PhilRice population was typically 30% cellulose, 20% matrix polysaccharides, and the lignin content varied between 13.72 and 20.87%, while Si had an average content of 7% (Supplementary Table S1). FA and CA content ranged between 0.78 and 3.99 mg/g and 2.85–8.95 mg/g respectively (Supplementary Table S2). The role of feedstock composition in determining pyrolysis yields has been shown in numerous studies [39]. The differences in composition of the straw material in our rice panel (cellulose, hemicellulose, lignin, FA and CA) will determine the pyrolytic behavior of the materials.

Seventy compounds, comprising 92% of the total area in the pyrogram, could be compared through GC-MS analyses among the 167 peaks detected initially (Supplementary Table S3 and Table S4). A total of 37 compounds, representing 55.88% of the total area in the pyrogram, were identified through GC-MS analyses, using the NIST Lab database with a Match ≥85% from the 70 peaks. Among them, twenty compounds (26.27% of the total area) were categorized as aromatic hydrocarbons, furans, hydrocarbon derivatives, oxygenated, and nitrogen-containing substances (NIST Lab database with a Match ≤85%), based on their fragmentation pattern. Thirteen compounds (constituting 9.91% of the total area) remained unidentified within the 70 peaks compared. Fig. 2 illustrates a typical chromatogram for rice straw samples analyzed using the pyrolysis conditions described in the Methods section.
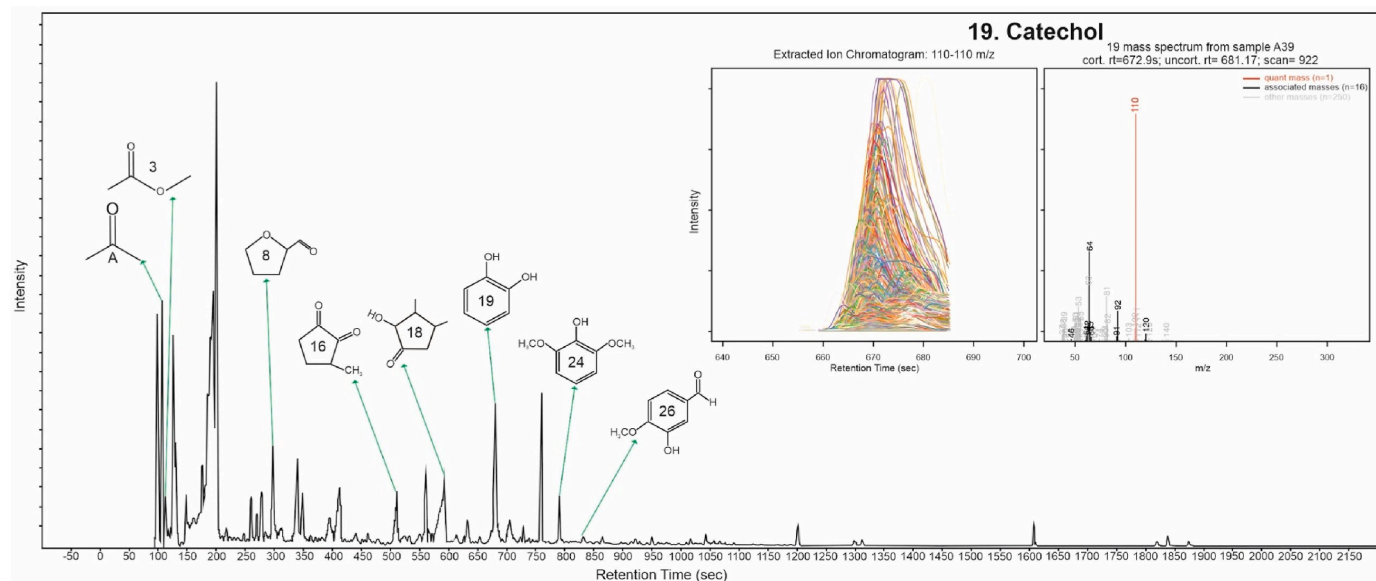

Fig. 2. Chromatogram of pyrolyzed rice straw. A: acetone, 3: acetic acid, methyl ester, 8: furfural, 16. 3-methylcyclopentane-1,2-dione, 18. 2-cyclopenten-1-one, 2-hydroxy-3,4-dimethyl, 19. catechol, 24. 2,6-dimethoxy, phenol, 26. 3-hydroxy-4-methoxy-benzaldehyde.

Table 1

Peak list from Pyr-GC/MS from the PhilRice population samples. Retention time (RT), ID numbers, compound names (NIST 14 library database -Match ≥85%-), peaks are derived from Carbohydrate (C), Lignin (L) and Other (O) and *m/z* of detected fragments.

RT (secons)	ID	Name	From	*m/z*
102	M44T102	Acetaldehyde	C	44 43 42 41 45 40 49 51 37
103	M47T103	Unknown	U	47 48 46
110	M56T110-M68T110	acetone	C	58 43 42 39 41 38 56 44 37
112	M59T112	acetic acid, methyl ester	C	74 43 42 59 44 41 45 75
113	M58T113	2-propen-1-ol	C	57 58 55
150	M150T750+M150T760	2-methoxy-4-vinylphenol	L	150 77 135 107 79 51 78 53 63 39
159	M45T159	3-hydroxy-2-butanone	C	45 43 88 42 44 46 41
159	M60T159	acetic acid	C	45 60 44
176	M60T176	Unknown	U	60 45 43 42 41 44 61 40 46
177	M45T177	oxygenated compounds	O	43 45 42 41 39 69 73 81 80 59
189	M75T189	Unknown	U	74 38 57 37 53 55 40 56 58
189	M74T189+ M74T173	organic nitrogen-containing compounds	O	74 42 45 43 44 41 39 38 37 75
197	M90T197	acetic acid, hydroxy-, methyl ester	C	59 61 42 90 60 62 44 75 40 68
234	M91T234	Unknown	U	86 91 58 92 44 63 62 87 93 52
241	M89T241	Unknown	U	88 56 41 39 58 55 53 38 37 59
246	M88T246	Unknown	U	88 56 41 39 58 55

Table 1 (*continued*)

RT (secons)	ID	Name	From	*m/z*
265	M102T265	Unknown	U	53 38 37 59 102 42 44 41 39 59 38 55 37 103
275	M96T275	3-furaldehyde	C	95 96 39 41 38 67 37 86 80 40
300	M96T300	furfural	C	96 82 95 39 38 53 54 41 37 98
309	M96T309	oxygenated compounds	C	43 39 96 95 82 54 38 41
328	M96T328	oxygenated compounds	C	42 53 98 55 39 69 41 53 42 70
334	M98T334	2-furanmethanol	C	96 57 94 66 65 39 40 55 63 38
341	M116T341	1- acetyloxy, 2-propanone	C	95 50 86 42 116 73 44 96 57
349	M95T349	Furans	C	68 74 40 43 95 110 38 55 51 50 37 52
360	M98T360	Unknown	U	96 98 68 83 112 57 58 45
363	M95T363	1-(2-furanyl)- ethanone	C	95 110 39 43 38 37 50 111
373	M98T373	Furans	C	41 98 53 67 51 50 95 52 81
403	M98T403	1,2 cyclopentanedione	C	109 98 55 42 69 43 41 39 70 99 40
431	M106T431	aromatic hydrocarbons	L	110 39 105 77 106 42 51 40 54 82

Table 1 (*continued*)

RT (secons)	ID	Name	From	m/z
436	M130T436	Unknown	U	110 109 57 43 53 67 39 81 51
450	M101T450	2,4-dihydroxy-2,5-dimethyl-3(2H)-furanone	C	43 55 39 101 41 73 144 45 98 69
459	M94T459	Phenol	L	94 66 65 39 55 63 38 95 50
465	M140T465	2-furanmethanol, acetate	L	81 98 53 140 52 56 43 80 97 122
475	M114T475	organic nitrogen-containing compounds	O	114 58 57 42 85 115 98 59 86
505	M112T505	3-methylcyclopentane-1,2-dione	C	112 55 69 57 41 83 56 84 43 39
535	M109T535	oxygenated compound	C	126 43 95 39 69 111 41 55 83 51
553	M128T553	Furans	C	43 128 57 45 58 44 72 129
553	M158T553	Furans	C	43 57 128 124 69 54 45 53 123 67
573	M43T573	2-Cyclopenten-1-one, 3-ethyl-2-hydroxy-	C	43 44 39 57 55 41 42 126 53 83
579	M44T579	Pentanal	C	44 57 43 39 41 42 55 45 53 40
591	M126T591	2-cyclopenten-1-one, 2-hydroxy-3,4-dimethyl	C	126 69 111 43 55 41 83 56 39 97

Table 1 (*continued*)

RT (secons)	ID	Name	From	m/z
617	M134T617	1-ethenyl-4-methoxy-benzene.	L	134 91 119 65 63 135 89 92 62 103
621	M102T621	Unknown	U	102 56 57 45 42 103 58 61 46 38
654	M110T654	aromatic hydrocarbons	L	138 123 95 67 77 53 51 65 43 66
666	M142T666	oxygenated compound	O	142 68 85 71 113 67 140 53 40 69
672	M110T672	Catechol	L	110 64 81 53 111 82 54 79 68
675	M85T675	oxygenated compound	C	85 43 57 86 42 56 44 58
679	M120T679	2,3-dihydro-benzofuran,	L/C	120 91 65 39 63 119 62 51 50 94
702	M97T702	Unknown	U	97 125 51 43 128 44 79 85 91 56
730	M137T730	aromatic hydrocarbons	L	137 166 81 109 51 53 77 135 138 91
734	M132T734	2,3-dihydro, 1H-inden-1-one	O	104 132 78 103 51 77 131 67 50 76 180
737	M100T737	Furans	C	43 100 55 99 39 85 124

Table 1 (*continued*)

RT (secons)	ID	Name	From	m/z
				41 42 73
771	M182T771	Unknown	U	108 39 43 126 136 111 52 53 78 41
791	M154T791	2,6-dimethoxy, phenol	L	154 139 96 111 93 65 39 68 51 53
797	M151T797	Unknown	U	57 41 109 69 81 59 68 151 87 135
811	M151T811	Unknown	U	39 151 150 94 66 67 78 74 146 149
814	M127T814	oxygenated compound	O	57 55 127 99 39 100 41 53 101 42
820	M130T820	Unknown	U	123 130 152 124 131 109 68 81 67 153
833	M151T833	3-hydroxy-4-methoxy-Benzaldehyde	L	151 166 123 150 178 107 77 108 79 52
865	M168T865	hydrocarbons derivative	L	168 125 153 53 41 107 110 169 82 118
866	M164T866	2-Methoxy-4-(1-propenyl)-phenol	L	164 149 91 77 103 131 121 55 133 104

Table 1 (*continued*)

RT (secons)	ID	Name	From	m/z
922	M167T922	aromatic hydrocarbons	L	167 182 79 77 107 168 53 123 65 121
927	M180T927	1-(4-hydroxy-3-methoxyphenyl)-2-propanone	L	137 180 122 43 94 138 66 51 77 65
951	M180T951	3′,5′-dimethoxyacetophenone	L	180 165 137 77 122 91 65 181 94 51
972	M170T792	Unknown	U	138 57 94 66 147 148 81 27 89 90
1014	M190T1014	hydrocarbons derivative	L	147 176 148 45 41 181 73 81 92 177
1044	M194T1044	(E)-2,6-dimethoxy-4-(prop-1-en-1-yl)phenol	L	194 91 119 179 77 131 79 103 151 195
1140	M242T1140	pentadecanoic acid	O	73 60 43 41 55 57 129 71 69 199
1203	M256T1203	n-hexadecanoic acid	O	73 60 43 55 57 41 129 69 71 256
1301	M67T1301	octadecanoic acid isomer	O	67 81 95 82 68 96 79 54 109 110
1304	M264T1304	octadecanoic acid isomer	O	55 69 41 83 97 43 57 84 70 56
1313	M43T1313	octadecanoic acid	O	73 43 60 57 55 41

Table 1 (*continued*)

RT (secons)	ID	Name	From	*m/z*
				129
				69 71

The 70 compounds selected were classified into three distinct classes based on the fraction from which they originated: Carbohydrate (C), Lignin (L), Others (O), and unknown (U) (Table 1). C and L compounds were the most abundant in all samples, contributing 38.03% and 25.35% of the total peaks respectively. O compounds were ca. 15.49% and U 22.53% (Table 1 and Supplemental Table S1).

The lack of information on the pyrolytic behavior of all the components of the network of polymers in the 215 rice straw accessions precludes the identification of 44.12% of the compounds. However, the metabolite fingerprinting approach that we selected does not require a complete metabolite identification to assess the quality of rice straw related to saccharification [32,40]. A principal component analysis (PCA) was applied to the 167 distinct peaks to visualize and characterize the differences or similarities between samples.

3.2.1. Principal component analysis of saccharification and pyrolysis

Separate PCA were conducted for the pyrolysis peaks of the 215 rice accessions (Fig. 3A), or in combination with saccharification potential data (Fig. 3C). In these PCA scores plots, each point represents the data obtained for one accession. When the pyrolysis data is analyzed alone, the scores plot shows that 68% of the total variation is explained by the first two principal components (PCs), with PC1 describing 44% of the

validated variance and PC2 describing 24% (Fig. 3A). When we added the saccharification data into the analysis, the scores plot shows that an overall 87% of the total variation is explained by the two first components, where PC1 explains 76% of the validated variance and PC2 explains 11% (Fig. 3C). There are no distinct clusters of accessions in the score plot without added saccharification data (Fig. 3A) indicating that the overall pyrolysis data does not define any sub-groupings for straw composition. However, when saccharification was included in the analysis, the score plot shows that the samples are not randomly distributed (Fig. 3C). In Fig. 3D, the angle between saccharification and PC1 is close to zero, therefore PC1 completely describes saccharification. Moving from left to right (PC1) along the score plot in Fig. 3C–a pattern can be observed; indicating that samples with the same color and shape have similar average values for saccharification. Using the loading plots in Fig. 3B and D, we selected between 41-36 peaks with loadings ranging from −1/-0.5 or 1/-0.5 (Table 1). Out of the 41 peaks selected, we identified a subset of 36 compounds based in their mass spectra using the NIST Lab database (Match ≥85%) (Table 2). The fingerprints of loading plots Fig. 3B and D were determined by 41 and 36 compounds, respectively (Table 2), with important differences in the percentage in which each class of compounds was represented. The loading plot in Fig. 3B is characterised by a similar contribution of C (34.15%) and L (34.15%) compounds, followed by O (14.63%) and U (17.07%) compounds. In contrast, the loading plot in Fig. 3D is characterised by a major contribution of L (34.14%) followed by C (24.39%), and a similar contribution of O (12.20 %) and U (12.20%).

The main compounds identified in the C group were, 3-methyl-1,2-cyclopentanedione (16), 2-hydroxy-3,4-dimethyl-2-cyclopenten-1-one (18), furans (23), 1- acetyloxy 2-propanone (10), 1,2
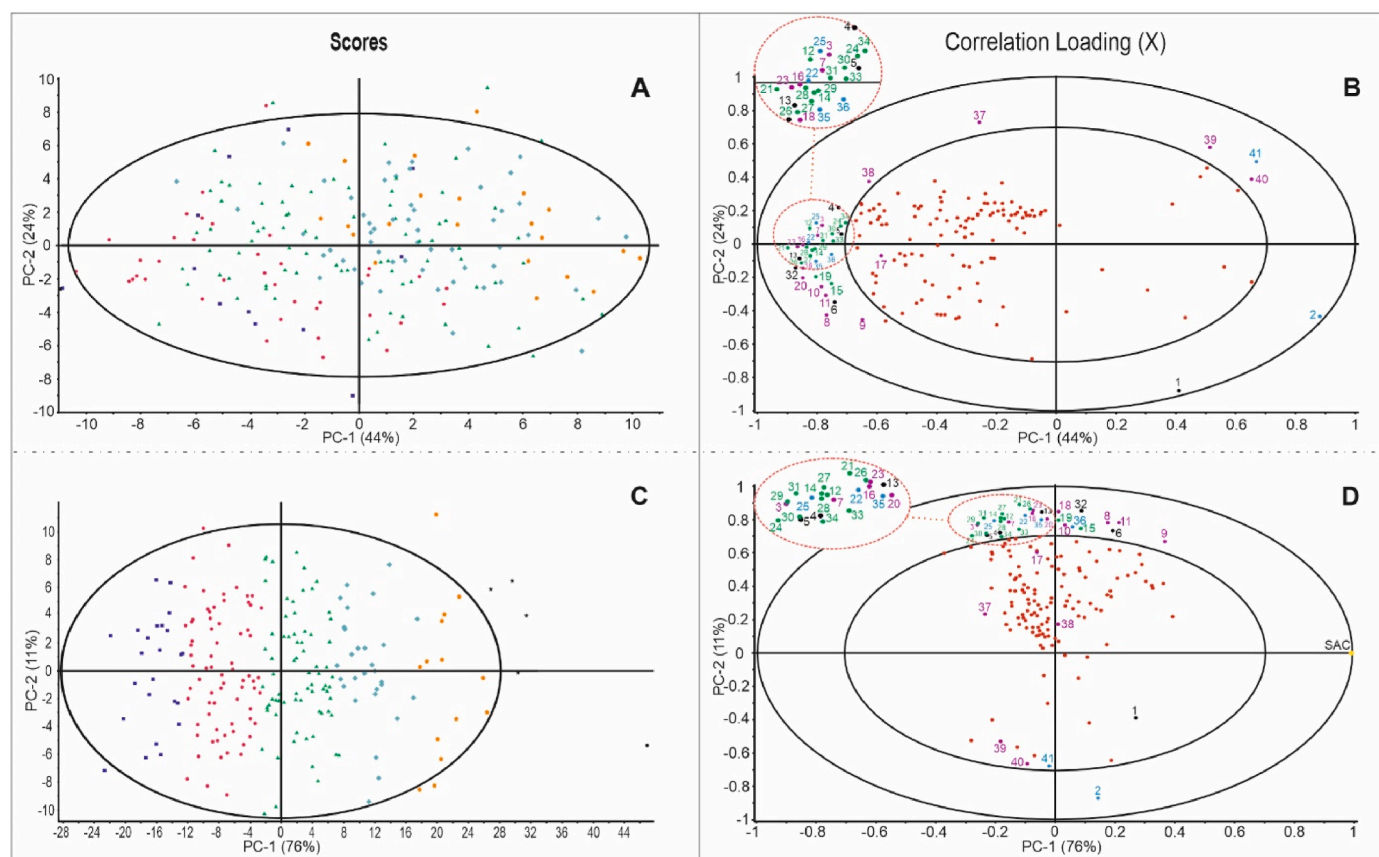

Fig. 3. Principal component analysis of pyrolysis derived compounds and saccharification in the PhilRice population. A–C: Score plots, the clusters of accessions are represented by dots of the same color and shape. B–D: Loading plots, peaks are labelled in different colours according to their origins: derived from Carbohydrate (purple), Lignin (green), Other (blue), and Unknown (black).Unselected peaks (red) and saccharification (yellow). The areas inside the red ellipses are amplified to facilitate the identification of the samples. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2

Peak list of subsets of 41 compounds from PCA of pyrolysis derived compounds and saccharification. Number PCA (NP), Retention time (RT), ID numbers, compound names (NIST 14 library database -Match ≥85%-), peaks are derived from Carbohydrate (C), Lignin (L) and Other (O).

NP	RT (secons)	ID	Name	From
1	176	M60T176	Unknown	U
2	177	M45T177	oxygenated compounds	O
3	197	M90T197	acetic acid, methyl ester	C
4	234	M91T234	Unknown	U
5	246	M88T246	Unknown	U
6	265	M102T265	Unknown	U
7	275	M96T275	3-furaldehyde	C
8	300	M96T300	furfural	C
9	334	M98T334	2-furanmethanol	C
10	341	M116T341	1- acetyloxy, 2-propanone	C
11	403	M98T403	1,2 cyclopentanedione	C
12	431	M106T431	aromatic hydrocarbons	L
13	436	M130T436	Unknown	U
14	459	M94T459	Phenol	L
15	465	M140T465	2-furanmethanol, acetate	L
16	505	M112T505	3-methylcyclopentane-1,2-dione	C
17	535	M109T535	oxygenated compound	C
18	591	M126T591	2-cyclopenten-1-one, 2-hydroxy-3,4-dimethyl	C
19	672	M110T672	Catechol	L
20	675	M85T675	oxygenated compound	C
21	730	M137T730	aromatic hydrocarbons	L
22	734	M132T734	2,3-dihydro, 1H-inden-1-one	O
23	737	M100T737	Furans	C
24	791	M154T791	2,6-dimethoxy, phenol	L
25	814	M127T814	oxygenated compound	O
26	833	M151T833	3-hydroxy-4-methoxy-Benzaldehyde	L
27	865	M168T865	hydrocarbons derivative	L
28	866	M164T866	2-Methoxy-4-(1-propenyl)-phenol	L
29	922	M167T922	aromatic hydrocarbons	L
30	927	M180T927	1-(4-hydroxy-3-methoxyphenyl)-2-propanone	L
31	951	M180T951	3′,5′-dimethoxyacetophenone	L
32	792	M170T792	Unknown	U
33	1014	M190T1014	hydrocarbons derivative	L
34	1044	M194T1044	(E)-2,6-dimethoxy-4-(prop-1-en-1-yl) phenol	L
35	1140	M242T1140	pentadecanoic acid	O
36	1203	M256T1203	n-hexadecanoic acid	O
37	573	M43T573	2-Cyclopenten-1-one, 3-ethyl-2-hydroxy-	C
38	579	M44T579	Pentanal	C
39	102	M44T102	acetaldehyde	C
40	113	M58T113	2-propen-1-ol	C
41	189–173	M74T189+ M74T173	organic nitrogen-containing compounds	O

cyclopentanedione (11), 2-furanmethanol (9), acetic acid methyl ester (3), and furfural (8). Lignin derived compounds were represented by syringol and guaiacol derivatives such as, catechol (19), 3-hydroxy-4-methoxy-benzaldehyde (26), phenol (14), and 2,6-dimethoxyphenol (24).

Similar relationships between pyrolysis products were found in the PCA loading plots in Fig. 3B and D. Compounds such as 3-methylcyclopentane-1,2-dione (16), 2-cyclopenten-1-one, 2-hydroxy-3,4-dimethyl (18) and furfural (8), included in the C group, show a positive correlation between themselves and with the L derived compounds Catechol (19), 3-hydroxy-4-methoxy-benzaldehyde (26) and 2,6-dimethoxyphenol (24). All these compounds present a negative correlation with 1, 2, 39, 40 and 41.

A further selection of 36 peaks within the 41 peaks was performed based on a combination of loading plot in Fig. 3B and D. These 36 peaks showed a range of correlations with saccharification (Fig. 3D). Fig. 3D displays the PCA relationships between all peaks detected and saccharification. Unknown (6), furfural (8), 2-furanmethanol (9) and 1,2

cyclopentanedione (11) (mostly C-derived compounds) grouped together in the top of right-hand side in Fig. 3 D, showing that they are positively correlated with themselves and with saccharification. While acetic acid, methyl ester (3), 3-furaldehyde (7), phenol (14), 2,6-dimethoxyphenol (24), 2-methoxy-4-(1-propenyl)-phenol (28), 1-(4-hydroxy-3-methoxyphenyl)-2-propanone (30), 3′,5′-dimethoxyacetophenone (31) and (E)-2,6-dimethoxy-4-(prop-1-en-1-yl) phenol (34) (group 2, mostly L-derived compounds) are situated in the top of left-hand side are positively correlated with themselves, but inversely correlated with saccharification.

3.2.2. Combined analysis of saccharification, silica content, hydroxycinnamic acids and pyrolysis peaks

A series of biomass components are well known to determine biomass saccharification. Among them, silica and hydroxycinnamic acids are some of the best-known factors that determine digestibility. In order to integrate these biomass components into our Pyr based analysis, we determined the Si content by XRF, analyzed hydroxycinnamic acids in all the samples, and integrated them in our PCA. Si content in the PhilRice association panel ranges between 3.6 and 8.7 %, showing a variation of 2.4-fold (Supplementary Table S1). This indicates that silica content is genotype specific and could be selected as an independent trait. FA and CA content ranged between 0.78 and 3,99 mg/g and 2.85–8.95 mg/g respectively. Principal component analysis was performed to establish the relationships between biomass components and saccharification. Fig. 4 A and B, show the score and loading plots between Si content, FA, CA, and saccharification. Using a combination of both (correlation and loading plot) we found that Si, CA and FA, are positively correlated with one another and negatively correlated with saccharification.

In Fig. 3 B and D we showed that compounds in the C group (3, 8, 9, 11, 16, 18, 23) are positively correlated with themselves and with compounds in the L group (14, 19, 24, 26) (Fig. 3 B and D). A similar relationship between pyrolysis products was found in the PCA correlation loading in Fig. 4 D. Fig. 4C and D show the relationships between the subset of 36 pyrolysis derived compounds, Si, FA, CA and saccharification. The peaks that group together in the left quadrant (lower) have similar weight in the analysis and correlate. Compounds such as 3-methylcyclopentane-1,2-dione (16), 2-cyclopenten-1-one, 2-hydroxy-3,4-dimethyl (18), furfural (8), catechol (19), 3-hydroxy-4-methoxy-benzaldehyde (26) and 2,6-dimethoxyphenol (24) are positively correlated with Si, FA and CA and a significant negative correlation with saccharification (Fig. 4C). The same is true for peaks 1 and 2 in the left quadrant (upper) indicating that they are inversely related to saccharification but in a different manner to either of others picks, the hydroxycinnamic acids, or silica content (Table 3). Peaks are closer to the center of the plot indicating a weak correlation with saccharification.

4. Discussion

In the present work, we have used a novel approach to study cell wall components in a rice diversity panel by combining Pyr-GC/MS with high throughput saccharification, Si determination and hydroxycinnamic acid content. Pyr-GC/MS is a technique used in polymer analysis that was adapted to characterize large sets of lignocellulose samples [27–29]. In the present paper we used Pyr-GC/MS combined with PCA to analyse 645 samples (the rice association panel comprises 215 accessions) and explore the suitability of identify fingerprinting related with straw recalcitrance. Being this is the first time that this "phenomics" approach is used in rice straw.

The pyrolysis of rice straw involves the cleavage of carbohydrates and lignin in complex reactions through several pathways [41–43]. A total of 71 compounds were identified through GC-MS and classified into three classes according to the fraction from which they originated: Carbohydrate (C), Lignin (L) and Others (O) (Table 1). C and L compounds were the major classes present in all samples, contributing 38.03% and 25.35% respectively. O compounds were ca. 15.49% and U
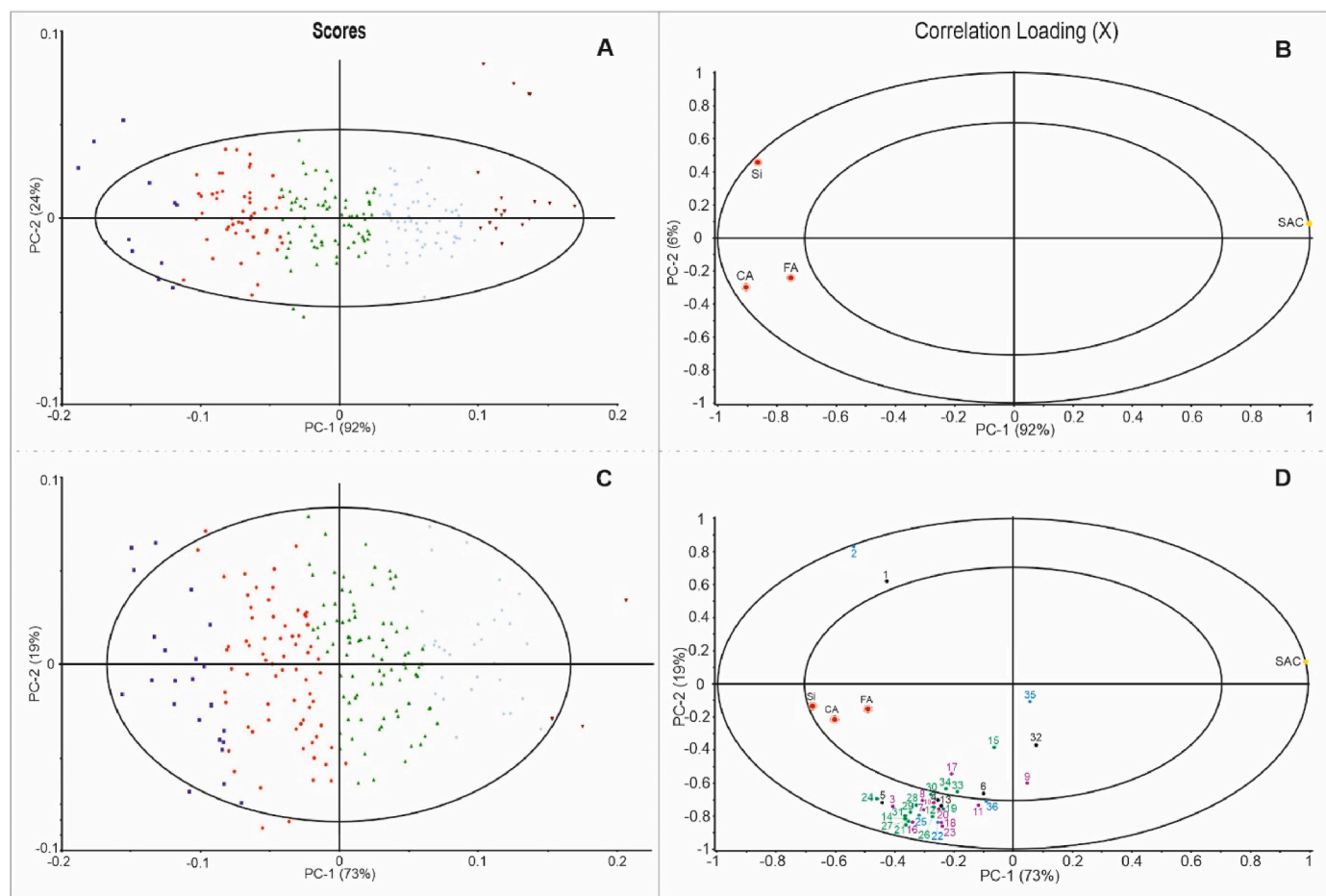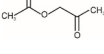
Fig. 4. Principal component regression analysis of Si, ferulic acid, p-coumaric acid, saccharification and a subset of 36 pyrolysis derived compounds. A–C: Score plots, the clusters of accessions are represented by dots of the same color and shape. B–D: Loading plots, peaks are labelled in different colours according to their origins: derived from Carbohydrate (purple), Lignin (green), Other (blue), and Unknown (black). Si, ferulic acid, p-coumaric acid (red) and saccharification (yellow). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
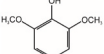
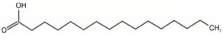22.53%. The decomposition of lignin generated a mixture of syringol and guaiacol derivatives such as, catechol (19), 3-hydroxy-4-methoxy--benzaldehyde (26) and 2,6-dimethoxyphenol (24). Derivatives of syringol and guaiacol have been found to form between 200 and 450 °C [43,44]. Syringol is typically detected at low temperatures (275–350 °C) [45,46] and the maximum rate of phenol formation occurs between 360 and 400 °C [47,48]. The importance of lignin composition in the determination of saccharification has been underlined by several publications in monocotyledonous species, in particular the non-alkaline extractable fraction of lignin [49,50]. The products of cellulose and hemicellulose pyrolysis identified in the C group included, 3-methyl-1,2-cyclopentanedione (11), 2-hydroxy-3,4-dimethyl-2-cyclopenten-1-one (18), acetic acid, methyl ester (3), acetone and furfural (8). Within this C-derived group, anhydrosugars did not represent a significant fraction. This observation indicates that the formation of anhydrosugars can be inhibited by the interaction of hemicellulose and cellulose during pyrolysis, reducing their yields [48]. In parallel to phenotyping the PhilRice association panel using Pyr-GC/MS, we determined the saccharification potential using a HT automated approach [8]. The saccharification values found in the PhilRice panel are within the range found for rice in these previous studies [38]. To explore associations between pyrolysis products and saccharification we used principal component analysis (PCA). The combination of pyrolysis, PCA and saccharification allowed to identify 36 compounds as specific markers for carbohydrates and lignin derivatives (Fig. 3). C-derived compounds such as furfural (8), 2-furanmethanol (9) and 1,2

cyclopentanedione (11), were correlated directly with saccharification. While L-derived compounds such as phenol (14), 2,6-dimethoxyphenol (24), 2-methoxy-4-(1-propenyl)-phenol (28), 1-(4-hydroxy-3-methoxyphenyl)-2-propanone (30), 3′,5′-dimethoxyacetophenone (31) and (E)-2,6-dimethoxy-4-(prop-1-en-1-yl) phenol (34) showed an inverse correlation with saccharification, acknowledging that saccharification could be restricted by L-derived compounds.

Since FA, CA and Si are the most studied components of biomass in relation to recalcitrance and digestibility properties to biomass [51], we evaluated the correlations of the content of FA, CA and Si with saccharification and the 36 compounds by PCA. Our correlation analysis shows that saccharification is significantly restricted by FA, CA and Si across the association panel (Fig. 4A–B). Hydroxycinnamic acids can be covalently linked to different components of the cell wall, and the carboxyl group at the end of its propenyl group provides the ability to esterify hemicellulose and lignin [52]. FA strengthens the plant cell wall by crosslinking arabinoxylans and lignin [53]. CA, in turn, has been proposed to facilitate lignin polymerisation by acting as a radical catalyst [54]. On the other hand, Si is a physical barrier to saccharification that is mostly located in the cuticle on the rice straw and protects the inner tissues of the straw [55]. In our work, Si represents a large proportion of rice straw and in our association panel it ranges between 3.6 and 8.7%. The relationships between the subset of 36 pyrolysis derived compounds, Si, FA, CA and saccharification (Fig. 4C–D) allowed to identify 16 compounds as fingerprints for recalcitrance and digestibility properties in the rice straw investigated (Table 3). Compounds such as

Table 3

Peak list of subsets of 28 compounds from PCA of saccharification, silica content, hydroxycinnamic acids and pyrolysis peaks. Number PCA (NP), compound names (NIST 14 library database -Match ≥85%-), peaks are derived from Carbohydrate (C), Lignin (L) and Other (O).

NP	Name	From	Structure	Correlated Si, FA CA	SAC
1	Unknown	U		+	−
2	oxygenated compounds	O		+	−
3	acetic acid, methyl ester	C		+	−
4	Unknown	U		+	−
5	Unknown	U		+	−
7	3-furaldehyde	C		+	−
8	furfural	C		+	−
10	1- acetyloxy, 2-propanone	C		+	−
11	1,2 cyclopentanedione	C		+	−
12	aromatic hydrocarbons	L		+	−
13	Unknown	U		+	−
16	3-methylcyclopentane-1,2-dione	C		+	−
18	2-cyclopenten-1-one, 2-hydroxy-3,4-dimethyl	C		+	−
19	catechol	L		+	−
20	oxygenated compound	C		+	−
21	aromatic hydrocarbons	L		+	−
22	2,3-dihydro, 1H-inden-1-one	O		+	−
23	furans	C		+	−
24	2,6-dimethoxy, phenol	L		+	−
25	oxygenated compound	O		+	−
26	3-hydroxy-4-methoxy-benzaldehyde	L		+	−
27	hydrocarbons derivative	L		+	−
28	2-methoxy-4-(1-propenyl)-phenol	L		+	−
29	aromatic hydrocarbons	L		+	−
30	1-(4-hydroxy-3-methoxyphenyl)-2-propanone	L		+	−
31	3′,5′-dimethoxyacetophenone	L		+	−
36	n-hexadecanoic acid	O		+	−

3-methylcyclopentane-1,2-dione (16), 2-cyclopenten-1-one, 2-hydroxy-3,4-dimethyl (18), furfural (8), catechol (19), 3-hydroxy-4-methoxy-benzaldehyde (26) and 2,6-dimethoxyphenol (24) positively correlated with Si, FA and CA and negatively correlated with saccharification (Fig. 4C) can be used as fingerprints for recalcitrance.

5. Conclusion

In the present work, we explored the chemical basis of biomass recalcitrance using a Philippine rice association panel, involving high throughput pyrolysis followed by GC/MS and multivariate data analysis. This novel approach to lignocellulose analysis allowed for the identification of metabolite fingerprinting of lignocellulose has the advantage of providing a fingerprint of the complex chemical nature of rice straw.

This study highlights the insights generated by using PCA on Pyr-GC/MS data to correlate pyrolysis derived products with biomass composition. We have determined the correlation of biomass components such as Si and hydroxycinnamic acids with saccharification and pyrolysis products. The compositional variation in the population, reflected in the abundance of pyrolysis products derived from different fractions of the biomass, determines the saccharification potential.

Our results shed light not only on the biomass components that underpin the recalcitrance in rice straw, but also to the pyrolysis derivatives that are associated with this trend. This knowledge represents a toolkit for the selection of more digestible varieties, as well as a new trait that can assist in the speeding of breeding programs that target biomass valorization.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its Additional file 1.

Declarations

Ethics approval and consent to participate.
Not applicable.

Consent for publication

Not applicable.

CRediT authorship contribution statement

Mariana P. Silva: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Caragh Whitehead:** Formal analysis, Investigation, Methodology, Writing – review & editing. **Reynante L. Ordonio:** Conceptualization, Data curation, Investigation, Methodology, Resources, Writing – review & editing. **Trinidad C. Fernando:** Investigation, Resources. **Mark Philip B. Castillo:** Investigation, Resources. **Jeremias L. Ordonio:** Formal analysis, Investigation, Resources. **Tony Larson:** Data curation, Validation, Writing – review & editing. **Daniel J. Upton:** Formal analysis, Investigation, Writing – review & editing. **Susan E. Hartley:** Conceptualization, Formal analysis, Supervision, Writing – review & editing. **Leonardo D. Gomez:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft.

Declaration of competing interest

The authors declare that they have no competing interests.

Data availability

Data will be made available on request.

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Abbreviations

Pyr-GC/MS   analytical pyrolysis-Gas chromatography-mass spectrometry
PCA   principal component analysis
C   Carbohydrate
L   Lignin
O   Others
GHs   glycosyl hydrolases
2,6-DO   2,6-Dimethoxyphenol
COBL   cobra-like gene
GPI   encode glycosylphosphatidylinositol
A   acetone
SAC   saccharification potential
Si   silica
FA   ferulic acid
CA   p-coumaric acid

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biombioe.2024.107099.

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