Characterization of the variation of a Miscanthus sinensis collection for biobased end-uses

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Abstract

In this research, a *M. sinensis* collection of the Laboratory of Plant Breeding from Wageningen University was characterized based on morphological and cell wall traits. To achieve a better insight miscanthus breeding, variation and trait correlations for morphological and biomass quality characteristics, relevant for the improvement of *M. sinensis* in the context of a bio-based economy, were investigated. Cell wall traits were predicted using NIR-based prediction models, which were actualized and improved using biochemical analysis. For both morphological traits as cell wall traits high variation was observed in the collection, together with plenty different trait combinations, resulting in a broad base of potential parental lines for all kind of breeding purposes. Dry matter yield showed a wide range of variation (~200 to ~3900 g) with an average of 1600 grams per 4 plants, of which 87% of dry mass consisted of cell wall material. From total dry mass, cellulose (~40 to ~54%), hemicellulose (~25 to ~34%) and lignin (~5 to ~11%) were the main constituents. For a mild pre-treatment, cellulose conversion ranged from ~30 to ~44%, which was about the same range as for hemicellulose conversion (~28 to ~43%). In general, accessions that represented a high sugar yield showed high dry mass yields together with a low cell wall conversion efficiency, except for one. This shows both the challenge as the high potential of *M. sinensis* breeding for bio-based purposes to combine these traits to develop miscanthus as a bioenergy crop.

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Introduction

Biobased crops

Renewable energy sources, such as biofuels, are becoming more important in the forthcoming future. The increased interest in biofuels is mainly ascribed to the finity of fossil fuels in combination with its harmful combustion to the environment. Also, majority of countries are highly dependent on oil producing countries, which can lead to political instability. While the amount of fossil fuels is finit, the worldwide energy demand is still increasing. This increase is predicted to rise even further in the near future, mainly ascribed to an growing industry in developing markets and a growing world population (Sorrel *et al*., 2010). The rise in energy demand is predicted to exceed the production in the coming years. The CO_2 emitted by burning fossil fuels is adding extra CO2 to the atmosphere, which was previously stored in underground layers, not being harmful to global warming. Since 40% of fossil fuel CO_2 emissions worldwide come from oil burning it is a major cause of global warming. By growing bioethanol crops to produce biofuels no extra CO2 is added to the atmosphere (Ciais *et al.*, 2014; Sandalow, 2008).

Nowadays, first-generation bioethanol crops are already used in high amount to produce renewable energy, mainly as a liquid energy carrier. However, these crops are not sustainable due to their high energy demand during cultivation and processing (Tilman *et al.*, 2006). Another drawback of these first-generation biofuels is that they are often cultivated on high quality farm land, which is suitable for food production. In this way, the ability of the world to produce enough food is compromised. Therefore, food prices are predicted to rise.

Due to these concerns, the attention to second-generation biofuels increased (Thompson and Meyer, 2011). Second-generation biomass is derived from crops producing high lignocellulosic biomass, having often limited function as a food crop. Lignocellulose is the most abundant carbon source in a plant, which is always present. It can be derived from food waste, algae, and non-food plant material such as grasses, wood and agricultural residues (Naik *et al.*, 2010). When producing this biomass on marginal soils, not using the high quality farm land and using low amount of fertilizers, biofuels can be produced having low-environmental impact (Weijde *et al.*, 2013). In conclusion, there is a demand for crops that can meet the biomass needs of upcoming biobased industries, having high lignocellulosic content, no competition with food crops and are produced in a sustainable way.

Miscanthus

A suitable crop to answer these needs is *Miscanthus* spp. Miscanthus is a highly productive C4 rhizomatous perennial, which originates from Southeast Asia and parts of tropical Africa

(Chung and Kim, 2012). The *Miscanthus* genus consists of about 15 species (Amarasekara, 2013) with *Miscanthus x giganteus* (*2n = 3x = 57*), *Miscanthus sinensis* (*2n = 2x = 38*) and *Miscanthus sacchariflorus* (*2n = 4x = 76*) as most important cultivated species. Miscanthus has several characteristics which makes it a suitable biomass feedstock. Its productivity (25t / ha / year) is high compared to other crops from which biofuels are produced. It has been calculated that 11.8 M ha of M. *x giganteus* is needed to produce 35 B gallons of ethanol. For this amount of ethanol 18.7 M ha of corn (grains and stems) or 33.7 M ha of switchgrass is needed (Heaton *et al.*, 2008).

The high production of miscanthus in Europe is mainly due to its C4 photosynthesis, which is a more efficient system to fixate carbon compared to the C3 photosystem. Miscanthus has the ability to perform its photosystem under lower temperatures than the C4 photosystem of maize and sorghum. For this reason, the growing season in Northern regions is longer for miscanthus compared to maize and sorghum. The rhizomes of miscanthus are able to vegetatively reproduce itself, but these rhizomes have another important function. In winter, during senescence, above ground minerals are relocated to the rhizomes. By doing this, the plant can immediately start growing in spring, when the new growing season starts (Christian *et al.*, 2008). The nutrient relocation to the rhizomes makes miscanthus a very nutrient efficient crop that does not need an annual amount of fertilizer, able to grow on poor soils (Davis *et al.*, 2010). A general advantage of the C4 photosystem is its high water use efficiency, resulting in a relative good drought tolerance compared with C3 crops (Weijde *et al.*, 2013). Together with its high genetic variation for salt tolerance, competition with food crops on high quality farm land can be avoided (Sun *et al.*, 2014). In general, no, or a limited amount of pesticides have to be used, since only a few mild natural pests are known (Jørgensen, 2011). However, there are still some challenges for establishing miscanthus as a biobased crop.

Challenges

Besides high yields, another important factor which makes a crop suitable for biofuel production are the characteristics of the biomass. This biomass quality is highly determined by the composition of the cell wall and its corresponding saccharification efficiency. Cell walls are made of lignocellulose, which consists of three polymers: cellulose, hemicellulose and lignin. Only cellulose and hemicellulose can be processed in bioethanol. The content in which cellulose, hemicellulose and lignin are available in miscanthus are ranging from 40-60, 20-40 and 10-30%, respectively (Hodgson *et al.*, 2011). The efficiency of saccharification depends on the ease of releasing monomers from the cell wall's polysaccharides by an enzymatic hydrolysis reaction. The monomers can subsequently be fermented into ethanol. The main limiting factor in this saccharification process is lignin. By restricting and absorbing hydrolytic enzymes such as cellulases, lignin decreases the binding of cellulases to cellulose (Zhao *et al.*, 2012). By genetically decreasing the lignin content the fractionation of lignocellulose into sugars can be improved (Van der Weijde *et al.*, 2016). In this way chemical pre-treatment can be reduced, which leads to a significant decrease in the production costs of bioethanol (Torres *et al.*, 2013).

Miscanthus in general is usually referred to M. *x giganteus*, which is a sterile species producing the highest amount of biomass compared to *M. sacchariflorus and M. sinensis*. However, this species is not farmer friendly, since it needs to be clonally propagated, resulting in high establishment costs. The establishment costs of hybrid rhizomes or plants are between € 2,381 – 4,762 (Christian *et al.*, 2005). As a consequence, once the plants established, switching to other crops is more expensive since a loss has to be taken. This gives the farmers currently less flexibility when growing miscanthus. For that reason, farmers are only willing to grow miscanthus when the biomass market is more stable or when contracts for the long-term are available (Wilson *et al.*, 2014). Compared to the species *M. x giganteus and M. sacchariflorus, M. sinensis* is able to produce fertile seeds because of its diploid background, which is one of the reasons to use *M. sinensis* in this research. However, since no homozygote hybrid seeds are on the market yet, it will result in a plant population having high morphological variation. When hybrid seed can be produced hybrid vigour may be released, which is currently not expressed in other miscanthus yet (Atkinson, 2009).

Miscanthus sinensis

M. sinensis has more advantages compared with *M. x giganteus* and *M. sacchariflorus*. At first, *M. sinensis* is most suitable for breeding for temperate climates such as present in Europe (Farrell *et al.*, 2006). Second, it is a diploid, while other important species are polyploid, making breeding tools used for *Miscanthus sinensis* easier and more accessible compared to polyploid breeding. As stated before, the major advantage of its diploid genome is the ability to produce fertile seeds. In combination with a large number of flowers per individual high multiplication rates can be achieved, which can result in a significant cost reduction in planting material.

A better cell wall quality, together with an increased biomass, can increase efficiency of bioethanol production. This increased efficiency will result in lower costs, which makes Miscanthus a promising and environment friendly alternative to fossil fuels for the near future. To achieve this higher efficiency, better insight in chemical and morphological aspects of the available genotypes is needed.

Miscanthus is only domesticated for some decades on a low scale (Sang, 2011). Also, the progress that can be made in a perennial crop is low. This means that there should still be a lot of unexplored genetic variation available, especially because of its outcrossing character and its existence in a lot of different environments (Zhao *et al.*, 2014).

A wide range of genetic variation in *M. sinensis* is available in the Miscanthus collection of the Laboratory of Plant Breeding at Wageningen University. For this reason, the extent of variation is investigated in *M. sinensis*, both on morphological as on chemical aspects. These insights can be used as a first step in future breeding programs.

Objectives

The main aim of this research project is to characterize the extent of phenotypic variation for both morphological characters as biomass compositional traits in the perennial C4 grass species *Miscanthus sinensis.* Insights from this project will be used in future breeding work aiming to advance superior *M. sinensis* varieties tailored for the production of biomass feedstock for a growing bio-based economy. To achieve this overreaching goal, the following tasks will be completed:

- Characterization of a *Miscanthus sinensis* diversity panel (property of the Laboratory of Plant Breeding WU) for morphological characteristics.

- Characterization of a Miscanthus *sinensis* diversity panel (property of the Laboratory of Plant Breeding WU) for biomass quality traits relevant to bio-based end-uses.

- Actualization, improvement and validation of NIR-based prediction models for the estimation of biomass quality characters in *M. sinensis*.

- Analysis of the extent of variation and trait correlations for morphological and biomass quality characteristics relevant for the improvement of *M. sinensis* in the context of a bio-based economy.

Materials and Methods

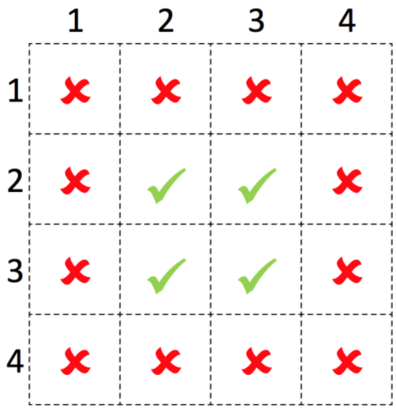
Germplasm

The field trial from the Collection of the Plant Breeding Department of WUR consist of 128 plots of miscanthus, consisting of 105 *M. sinensis*, 13 *M. sacchariflorus*, 5 *Miscanthus x giganteus,* 4 hybrids and one plot with an unknown background. These accessions are retrieved from diverse international gene banks around the world. Each accession has one, or in minor cases two, replications. A plot consists of 16 individuals from 5 years old, which are well established (Figure 1). In total 94 plots from the *Miscanthus sinensis* type are used in this research, consisting 91 different accessions.

This research consisted of two parts: a morphological analysis and biochemical characterization of the *M. sinensis* collection. A fresh harvest was used for the morphological part for which the 4 middle plants per plot are harvested (Figure 1). From each plot, 4 bundles of stems are harvested, each consisting of 3 randomly selected flowering tillers per plant. On the 10th and 12th of October, when > 90% of the accessions were flowering, the first and second harvest took place, resulting in two replications. Cutting of the stems took place just above the ground surface.

Harvested material from 2015 was utilized for biochemical analyses. Briefly, plants from the collection were harvested in a later stage of development and subsequently dried. The harvest took place when the plants were sufficiently senescent to use them for bioethanol purposes. To perform the chemical analysis, stem material of the different *Miscanthus sinensis* genotypes is milled using a 1 mm sieve. To achieve the most uniform samples the same milling machine is used.

Figure 1. Schematic representation of a field plot for the field of the Collection of the Plant Breeding Department of WUR.



	1	2	3	4
1	✗	✗	✗	✗
2	✗	✓	✓	✗
3	✗	✓	✓	✗
4	✗	✗	✗	✗

Individuals represented with ✓ are used for measurements, individuals with ✗ are not.

Morphological and agronomic traits

Field measurements

Flowering

The flowering date was measured using two distinct parameters: i) appearance of first flower and ii) when more than 50% of the plants from a plot had at least one flowering tiller. These traits were hereafter termed ‘initial flowering’ and ‘50% flowering’, respectively.

Plant angle

The plant angle will be measured as an indication for lodging susceptibility by using a 0 to 90 scale, based on the degrees the average plant is lodging. A score of 0 reflects no lodging, while a score of 90 reflects maximum lodging. The measurements are performed based on visual observation. Lodging is measured in the second week of September on the middle of the day, when the plants didn’t carry droplets on its leaf surface, which could result in (extra) lodging.

Number of tillers per plant

The number of tillers per individual were counted manually in the field. Counting was performed on the middle 4 plants of the plot, one week before harvest (Figure 1).

Stem length, dry weight, internode information

Subsequently to the harvest the length of the plants were measured using a tapeline. Measurements were taken with an interval of 1 centimetre, not measuring the flower head. All stem lengths were written down separately. After length measurements the internodes were counted for the first repetition of stems, while the stem diameter was measured on the middle internode using a digital calliper. When no middle internode was present, the internode beneath the middle node was taken. By having these information, the average internode length was calculated. All stripped plant parts were weight both fresh and dry to exclude sample substitutions. By separately drying (70 °C, 12 hours) above ground plants parts, the leaf/stem ratio (LSR), the leaf/weight ratio (LWR) and the stem/weight ratio (SWR) was calculated.

Cell wall measurements

NIRS

Near-infrared spectrometry (NIRS) was used to predict the cell wall composition and the saccharification efficiency in a high-throughput way by using the spectrometer FOSS NIRS DS2500 (FOSS Company, Hillerd, Denmark), achieving output from the ISIscan software package. To improve prediction models, biochemical analysis was performed on a calibration- and validation sample set to achieve cell wall compositional- and saccharification efficiency data. To achieve the cell wall compositional data the Goering and Van Soest method is used (Goering and Van Soest, 1970). To achieve saccharification efficiency data the method from Van der Weijde *et al.* (2016a) is used. Equations are developed using the WinISI III software package version 1.60 (Infrasoft International LLC, PA, USA).

To select calibration accessions a PCA is performed on the NIRS spectra of 84 accessions of the *Miscanthus sinensis* diversity panel. This PCA gave 3 outliers, while 25 accessions were chosen manually because of their high spectra variation. These 28 accessions were added to the calibration set of the previous model. Subsequently, 15 genotypes were randomly selected from the remaining samples for external validation of the improved models. This method is used to develop both models. The number of principal component terms were considered when a new model was developed.

To select the best performing equation model, two sources of information were used. As first, the cross-validation statistics of the new model were compared with the previous model. As second part of selection, external validation was used to confirm proper predictions for a randomly selected set of genotypes. The aim was to create a balanced model having both convincing equation statistics as predictions close to actual biochemical data.

Goering and Van Soest method

Biochemical data was needed to calibrate the NIRS. Gathering these data was performed using the Goering and Van Soest method (Goering and Van Soest, 1970). This method consisted of three steps: the determination of neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL), consecutively. All steps were performed using the ANKOM filter bag method and were subsequently analysed using the Ankom 2000 Fiber Analyzer (ANKOM Technology, NY, USA). Ankom F57 filter bags were used to store the grinded biomass during washing. To each filter bag 450 – 500 mg of dry mass is added and subsequently sealed using a heat sealer (220v, 50/60 Hz). After 24 hours of incubation on 103 °C filter bags including dry mass were weighted again to correct for moisture content in the biomass sample. All determination steps were performed *in duplo*.

In the end, NDF and ADF content were calculated using the following equation:

$$\%NDF\ (or\ \%ADF) = \frac{W_3 - (W_1 * C_1)}{W_2} * 100\%$$

in which *W1 = Bag tare weight*

W2 = Sample weight

W3 = Dried weight of bag with fiber after the extraction process

C1 = Blank bag correction (running average of final oven-dried weight divided by original blank bag weight).

By calculation the NDF, ADF and ADL content of the total dry mass the cell wall components' dry weights were calculated using the following equations:

$Dry\ matter - NDF = Cytoplasm$

$NDF = Cell\ wall\ (lignocellulose)$

$NDF - ADF = Hemicellulose$

$ADF - ADL = Cellulose$

$ADL = Lignin$

Saccharification efficiency

Characterization of saccharification efficiency, using a Dionex to measure the amount of glucose and xylose (Thermo Fisher Scientific, ICS-5000), was performed *in triplo*. This biochemical analysis was performed on the same genotypes as used for cell wall composition measurements, using the method of Van der Weijde *et al.* (2016a). Cellulose- and hemicellulose conversion rates were calculated from the glucose- and xylose release using the following equations:

$$Hemicellulose\ conversion\ (\%) = \frac{Xylose\ release\ (mg)}{HC * 1.136} * 100\%$$

$$Cellulose\ conversion\ (\%) = \frac{Glucose\ release\ (mg)}{CC * 1.111} * 100\%$$

where HC is the hemicellulose content (mg) in the sample, CC is the cellulose content (mg) in the sample, 1.136 is the mass conversion factor that converts xylan to an equivalent of xylose and 1.111 is the mass conversion factor that converts cellulose to an equivalent of glucose (Dien, 2010).

Trait descriptions

The most important trait descriptions are listed in Table 1, providing information about measurement dates and units. The remaining traits are listed in Annex I.

Table 1. Trait descriptions for the most important traits.

Trait	Description
Cellulose (% DM)	Predicted cellulose % from total dry matter harvest 2-2016, predicted by model version 7
Hemicellulose (% DM)	Predicted hemicellulose % from total dry matter harvest 2-2016, predicted by model version 7
Lignin (% DM)	Predicted lignin % from total dry matter harvest 2-2016, predicted by model version 7
Flowering date, 50%	Flowering 50% in Julian days 2016
Cellulose conversion (%)	Amount of cellulose that is converted to glucose harvested in week 9, 2016, predicted by version 7
Hemicullulose conversion (%)	Amount of hemicellulose that is converted to xylose harvested in week 9 2016, predicted by version 7
Glucose release (% DM)	Amount of glucose released from total dry weight, harvested in week 9 2016
Xylose release (% DM)	Amount of hemicellulose released from total dry weight, harvested in week 9 2016
Glucose yield	Total amount of glucose yield in grams per 4 plants, harvested in week 9 2016
Xylose yield	Total amount of xylose yield in grams per 4 plants, harvested in week 9, 2016
Glucose + xylose yield	Total amount of glucose and xylose yield in grams per 4 plants, harvested in week 9, 2016
Stem length	Mean stem length (flowering stems from centre of plot) in week 41, 2016
Dry matter yield-15	Total dry weight (g) per plot (4 centre plants of plot) in week 9, 2016

Results

Morphological traits

Variation statistics morphological traits

To get more insight in the variation present in the collection, variation statistics are displayed in Table 2. The minimum, mean, maximum, range, standard deviation (SD), coefficient of variation (CV%) and skewness is estimated for 14 morphologic traits in 93 accessions. Morphological traits measured exhibit a broad variation with an average coefficient of variation of 36.7%. Plant organ dry weights showed high variation (stem dry weight, CV = 57.5%; leaf dry weight, CV = 54.7%; flower dry weight CV = 56.0%). Lower coefficients of variation were shown for initiation of flowering date and 50% flowering date (10.4 and 10.6%, respectively).

Table 2. Variation statistics for morphological traits of *M. sinensis* accessions in the growing season 2016.

	n	Min	Mean	Max	Range	SD	CV%	Skewness
Flowering date, initiation	88	183.0	228.0	281.0	98.0	23.6	10.4	0.40
Flowering date, 50%	86	186.0	234.8	307.0	121.0	25.0	10.6	0.35
Stem yield	93	25.8	174.3	455.7	429.9	100.2	57.5	0.78
Leaf yield	93	6.4	89.49	226.8	220.5	49.0	54.7	0.95
Flower yield	93	0.0	18.56	42.0	42.0	10.4	56.0	0.15
Total dry mass-15	84	213	1614	3945	3732	798	49.4	0.66
Stem/weight ratio	93	0.47	0.60	0.75	0.28	0.07	11.2	-0.11
Leaf/stem ratio	93	0.24	0.56	1.21	0.97	0.19	34.0	0.98
Leaf/weight ratio	93	0.17	0.32	0.53	0.37	0.07	22.0	0.59
Stem length	93	69.5	187.9	298.0	228.4	49.4	26.3	-0.05
Stem angle	93	0	12.9	80	80	15.6	120.7	2.06
Stem number	93	11.5	59.9	181.2	169.8	28.4	47.4	2.07
Internode number	93	4.3	9.4	16.0	11.7	2.4	26.0	0.25
Internode length	93	11.9	20.59	34.7	22.8	3.4	16.7	0.58
Internode diameter	93	2.887	5.285	7.657	4.77	1.1	20.3	-0.01

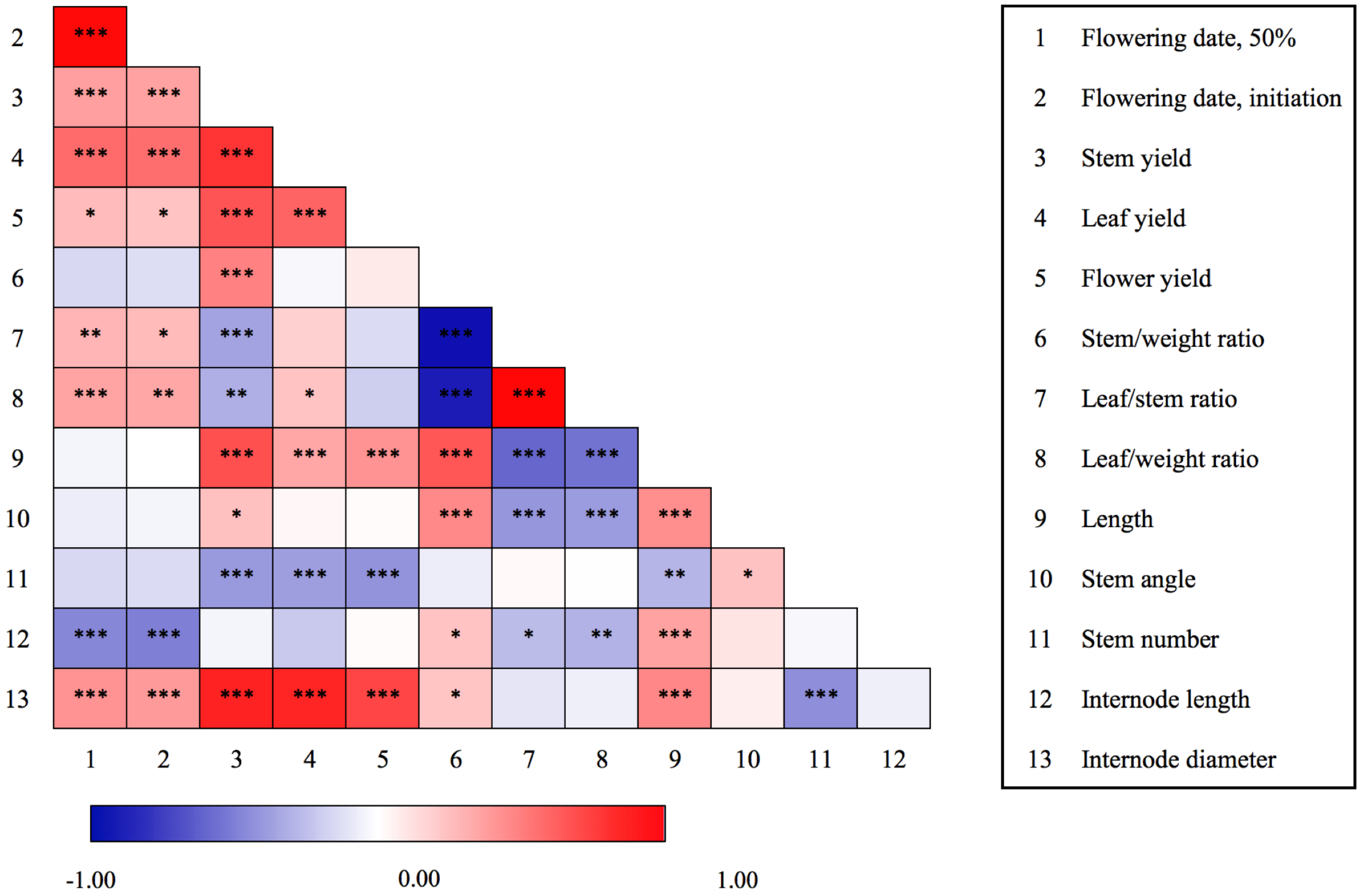
SD, standard deviation; CV%, coefficient of variation. Total dry mass-15 is measured in the 2015 growing season.

To visualise the variation present in the collections histograms are displayed (Annex II, Figure 15). The histograms show a normal distribution for the traits measured, of which a minority is positively skewed. Stem angle and stem number tend to show log-normal distributions.

Trait correlations

To get more insight in morphological correlations, which can influence the breeding process, a correlation plot is displayed (Figure 2). In general, organ dry weight traits were positively correlated with late flowering, high stem length and a big diameter of the middle internode, while organ dry weights were negatively correlated with stem number.

Figure 2. Correlation plot for morphological traits of *M. sinensis* accessions from the growing season 2016.



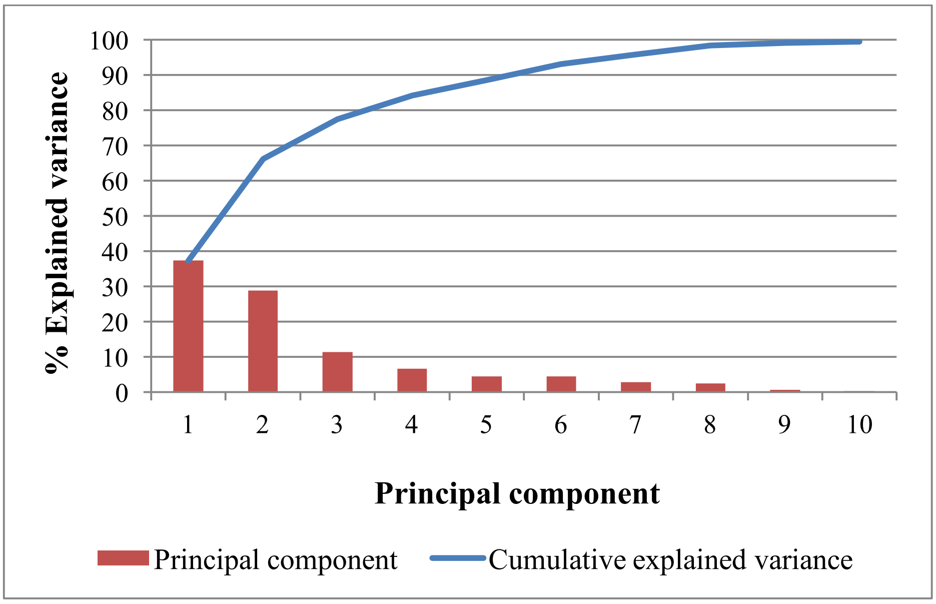
	1	2	3	4	5	6	7	8	9	10	11	12
2	***											
3	***	***										
4	***	***	***									
5	*	*	***	***								
6			***									
7	**	*	***			***						
8	***	**	**	*		***	***					
9			***	***	***	***	***	***				
10			*			***	***	***	***			
11			***	***	***				**	*		
12	***	***				*	*	**	***			
13	***	***	***	***	***	*			***		***	

*Cells that are designated with a * exhibit a significance level of P<0.05, ** for P<0.01 and *** for P<0.001.*

Principal component analysis

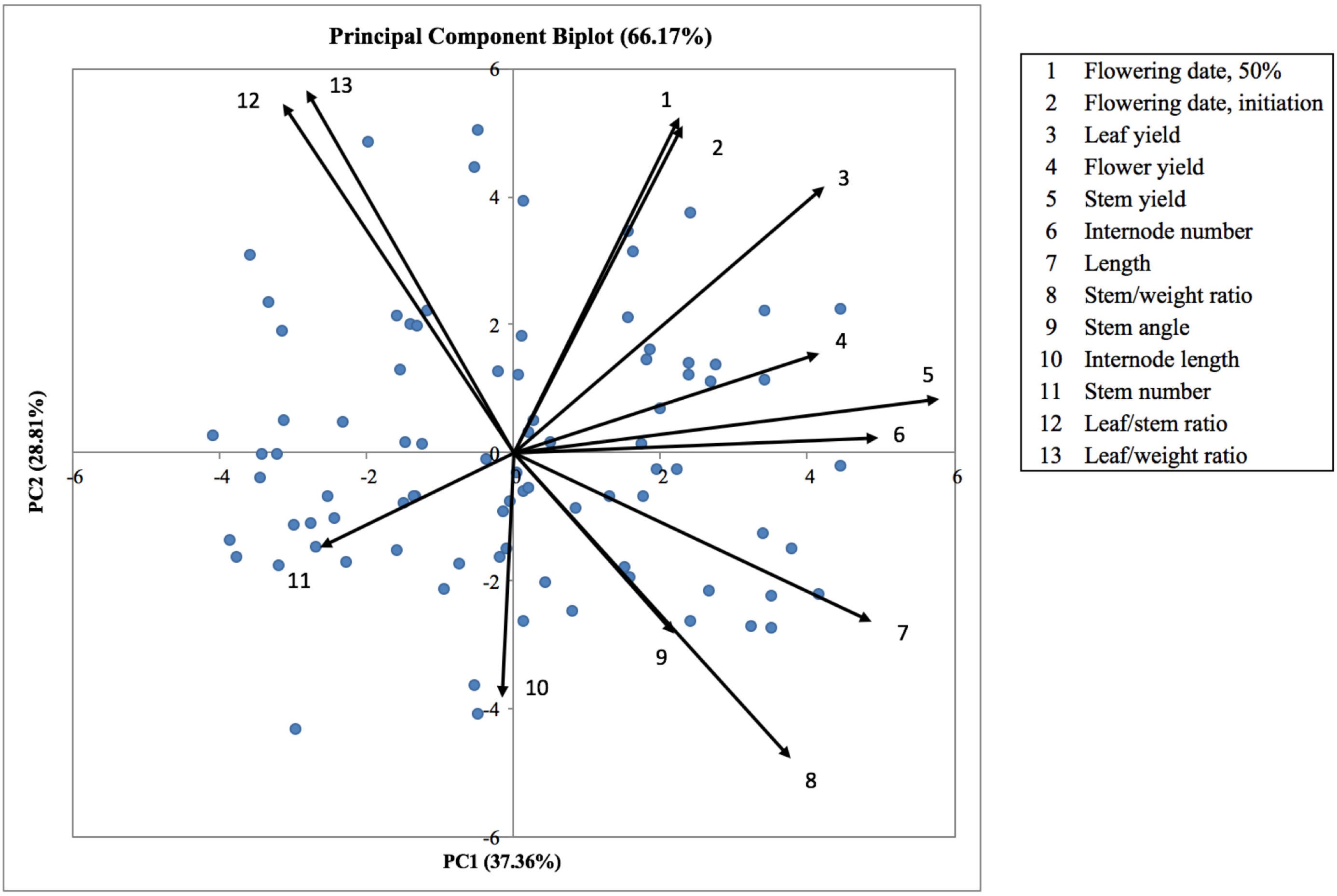
Principal component analysis shows that morphological traits can be largely resolved through two internode variables (Figure 4). The first component is dominated by internode number, while the second component is dominated by internode length. Traits with the highest variation for the first principal component were stem yield, internode diameter, internode number and stem length, while leaf/weight ratio, leaf/stem ratio, 50% flowering and initiation of flowering showed high variation for the second component axis. From the variation present, the principal components 1 and 2 explained 37.36 and 28.81 % of the variation (Figure 3).

Figure 3. Scree plot of principal components explaining the observed morphological variation.



The principal component analysis based on morphological traits reveals that internode information (PC1: internode number, total stem weight; PC2: internode length) are suitable markers to separate *M. sinensis* varieties present in the diversity panel of the Laboratory of Plant Breeding WU, on characteristics related to the accumulation of biomass, with an explained variance of 66.2%.

Figure 4. Principal component biplot for morphological traits measured in the 2016 growing season.



Flowering

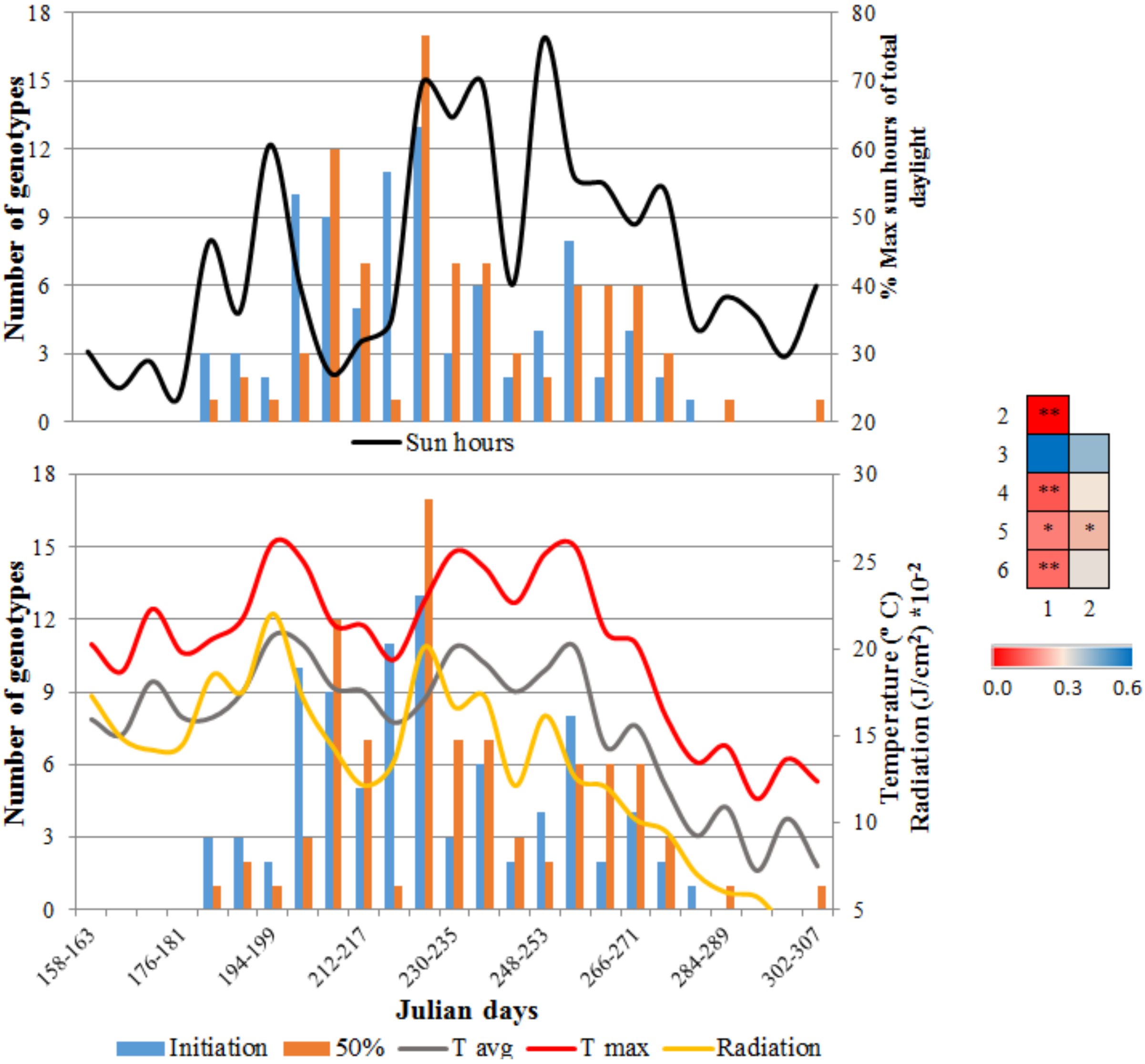
Due to the importance of flowering in cross-pollinated breeding and the significant correlations of flowering date with the majority of morphological traits (Figure 2) flowering is briefly highlighted in this section.

Flowering started at the end of June and ended in October. During this flowering period 6 out of 94 accessions did not reach the initial flowering phase. 2 out of the 88 remaining accessions were not able to reach the 50% flowering phase. On average, it took 7.7 days from initial flowering to 50% flowering, with a minimum of 2 and a maximum of 38 days. 50% flowering showed to be consistent over these years with a coefficient of determination of 0.811 (Figure 2). However, in 2015 flowering started on average 15.8 days later. The order of accessions reaching 50% flowering between the growing season 2015 and 2016 showed a correlation of 0.922.

To get more insight in flowering behaviour related to the environment the initiation of flowering and 50% flowering over the flowering period is correlated with the maximum sun hours of total daylight, the average- and maximum temperature and the radiation (Figure 5).

Both initiation of flowering and 50% flowering showed significant (*$P < 0.05$*) correlations with majority of the environmental parameters included, except for the maximum amount of sun per total daylight. The initiation of flowering was more sensitive to the environmental parameters than the 50% flowering date.

Figure 5. Environmental parameters related to flowering characteristics of *M. sinensis* accessions in the growing season 2016 and its correlations.



*Initiation (1), initiation of flowering; 50% (2), 50% of plants flowering; Sun hours (3), % hours sun of total daylight; T avg (4), average temperature (° C); T max (5), maximum temperature (° C), Radiation (6), radiation (J/cm^2). Environmental data are retrieved from KNMI (De Bilt) on 21-12-16. Cells that are designated with a * exhibit a significance level of P<0.05, ** for P<0.01 and *** for P<0.001.*

Cell wall traits

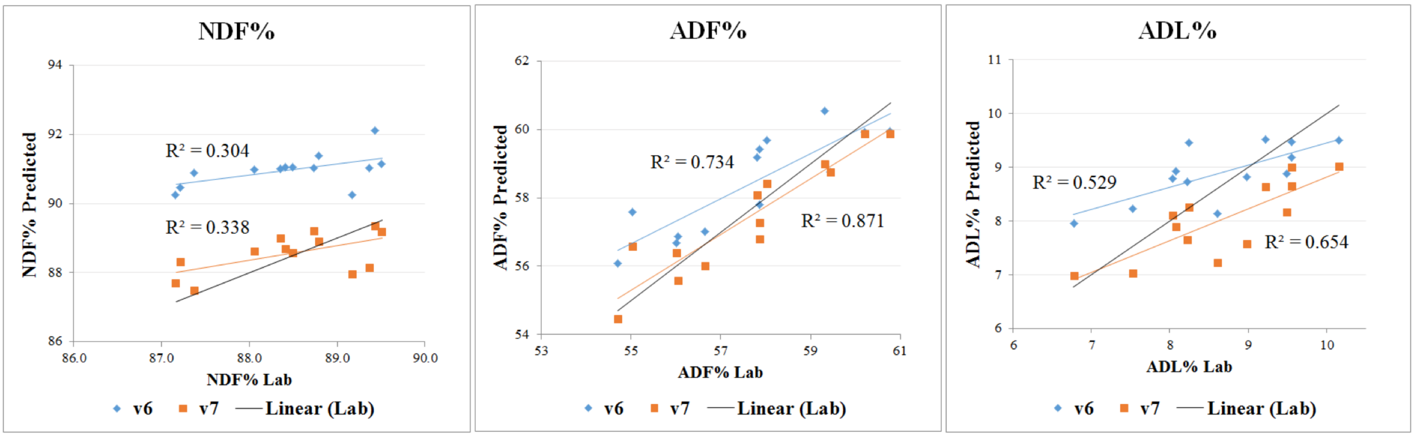
Improvement of NIR prediction models

By integrating biochemical measurements to the calibration set of the previous prediction models (v6), accuracy and reliability of the cell wall composition and saccharification efficiency model is improved. Both improved prediction models had 9 principal component terms with an explained variance of 98.12% and 97.98% for the cellular composition and the saccharification efficiency model, respectively.

Cell wall composition

For all detergents measured the coefficient of determination of the new model improved compared to v6, however, still a bias is present. The predicted validation samples for v6 and v7 are displayed in Figure 6, together with the data retrieved from biochemical analysis.

Figure 6. Predicted validation samples for model v6 and v7 compared with the data from biochemical analysis.

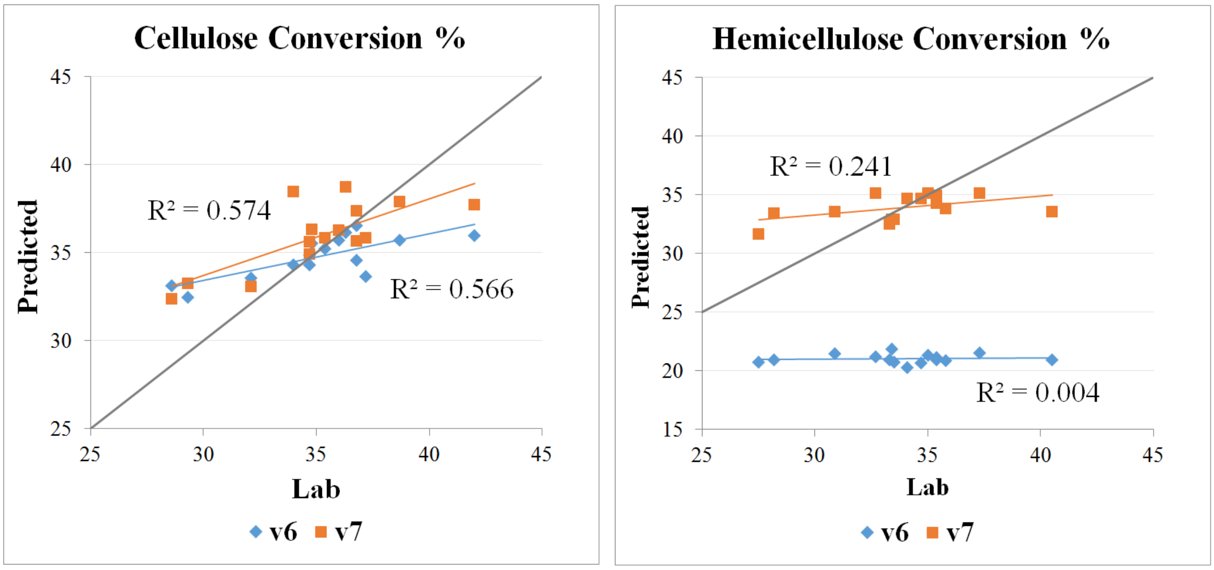


NDF, ADF and ADL are detergents of total dry matter. The grey line is representing data from biochemical analysis.

Saccharification efficiency

For both cellulose- and hemicellulose conversion the new model improved. However, there is a strong bias present, especially for hemicellulose conversion. For hemicellulose conversion, an improvement was made in predicting the right range of values compared to the previous model. The predicted validation samples for v6 and v7 are displayed in Figure 7, together with the data from biochemical analysis.

Figure 7. Predicted validation samples for model v6 and v7 compared with the data from biochemical analysis.



The grey line is representing data from biochemical analysis.

The output of the prediction models was used for analysis. Since the predictions of the conversion efficiency model for hemicellulose conversion were weak, biochemical data in combination with predicted data is used for statistics.

Variation statistics for cell wall traits

Cell wall traits exhibit a broad variation with an average coefficient of variation of 14.9% (Table 3). On average, about 87% of dry mass consisted of cell wall material. From this cell wall material a large range of variation was observed for cellulose (~40 to ~54%), hemicellulose (~25 to ~34%) and lignin (~5 to ~11%). Cellulose and lignin content showed about the same coefficient of variation (5.8 and 5.7%, respectively), while lignin content showed a CV of 12.8%. Cellulose conversion ranged from ~30 to ~44%, which is about the same as for hemicellulose conversion (~28 to ~43%), with a CV of 8.1 and 8.5% respectively.

Table 3. Summary statistics for cell wall traits of *M. sinensis* accessions in the growing season 2015.

	n	**Min**	**Mean**	**Max**	**Range**	**SD**	**CV%**	**Skewness**
Cellulose (% DM)	84	39.89	48.24	54.09	14.20	2.79	5.8	0.26
Hemicellulose (% DM)	84	24.87	30.95	34.58	9.72	1.78	5.7	0.26
Lignin (% DM)	84	5.23	8.18	11.19	5.97	1.05	12.8	0.26
Cellulose conversion (%)	94	29.88	36.03	44.11	14.23	2.92	8.1	0.09
Hemicellulose conversion(%)	94	27.54	34.32	43.34	15.80	2.93	8.5	0.50
Glucose release (% DM)	94	15.71	19.26	22.07	6.36	1.27	6.6	0.03
Xylose release (% DM)	94	9.52	15.32	22.60	13.09	3.83	25.0	0.19
Glucose + xylose yield / 4 plants	94	78	542	1180	1102	253	46.8	0.48

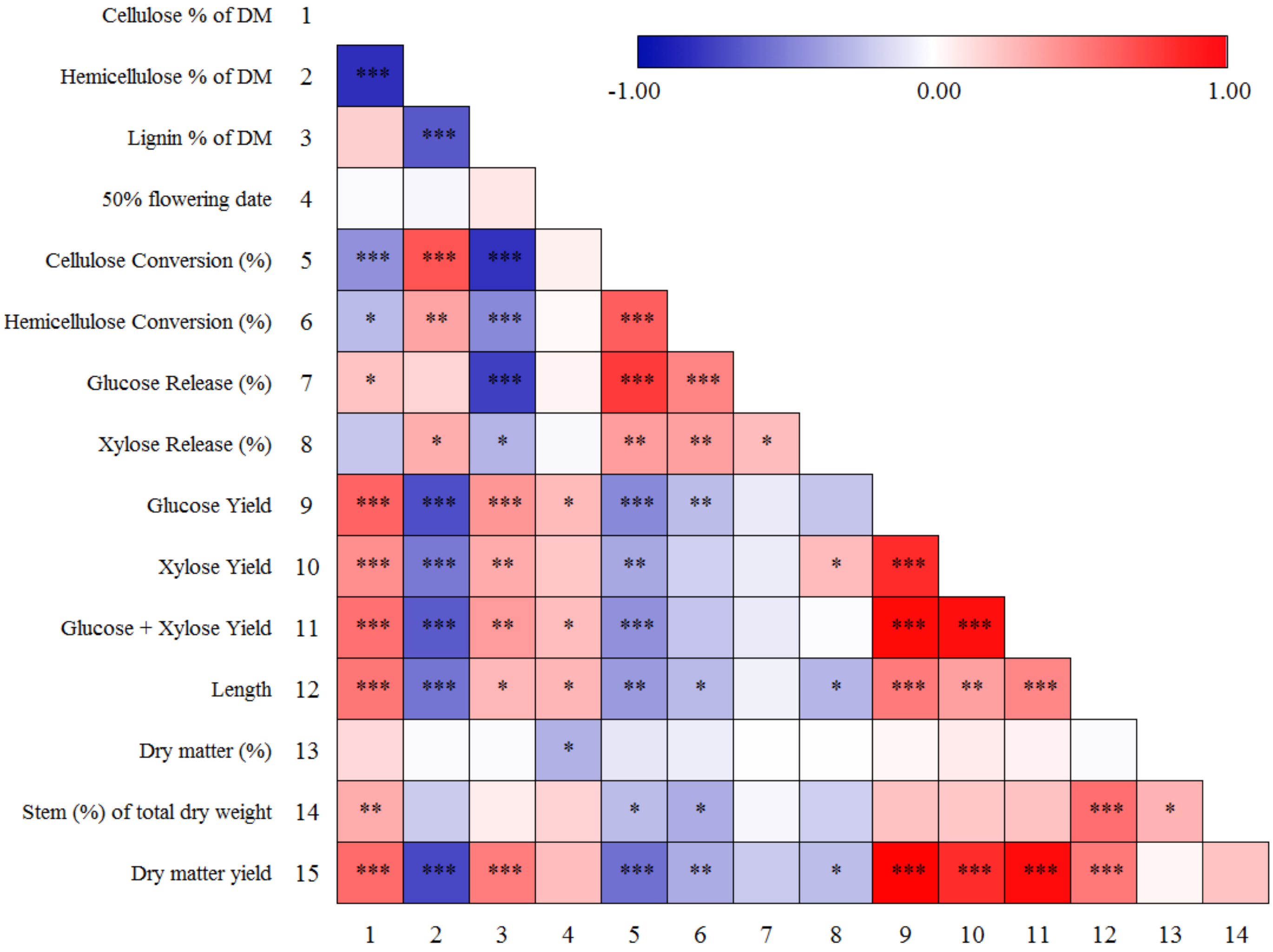
DM, dry matter; SD, standard deviation; CV%, coefficient of variation.

The visualise the variability for cell wall traits in the collections histograms are displayed in Annex II (Figure 16). The histograms show a normal distribution for the traits measured, except for xylose release, which tended to show a bi-modal distribution.

Trait correlations

To get more insight in cell wall correlations which can be of interest in breeding a correlation plot is displayed Figure 8. Strong negative correlations were found between cellulose- and hemicellulose content (*r=-0.818*) and cellulose conversion and lignin content (*r=-0.805*). Dry matter yield showed a positive correlation with cellulose content (*r=0.586*) and lignin content (*r=0.514*), while it had a negative correlation with hemicellulose content (*r=-0.719)*. This resulted in a negative correlation between dry matter yield and cellulose conversion (*r=-0.557)*. The amount of dry matter of the total weight did not show any significant correlation with cell wall traits, while the flowering date has no significant correlation with the cell wall composition and conversion efficiency.

Figure 8. Correlation plot for cell wall traits and morphological traits of *M. sinensis* accessions from the growing season 2015.

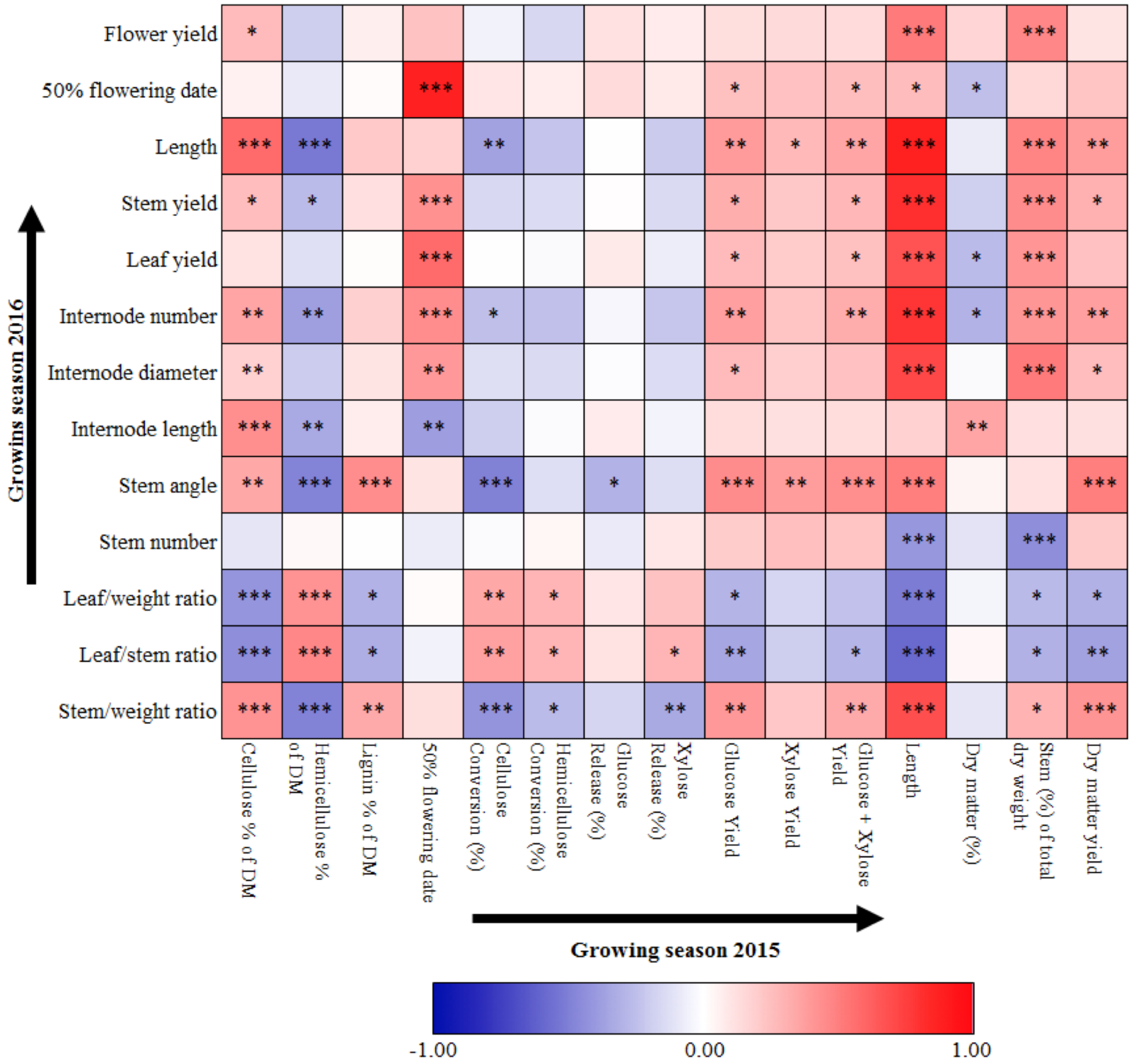


*Cells that are designated with a * exhibit a significance level of P<0.05, ** for P<0.01 and *** for P<0.001.*

Morphological- and cell wall trait correlations

Correlations between the 2015 and 2016 growing season, for both morphological as cell wall traits, are summarised in Figure 9. Cell wall traits were measured in the 2015 growing season, most morphological traits were derived from the 2016 growing season. Length in the growing season 2015 showed a positive correlation with length in the growing season 2016 (*r=0.875*), while flowering date (>50% flowering) showed a correlation of 0.871 between the different growing seasons.

Figure 9. Correlation plot for morphological- and cell wall traits of *M. sinensis* accessions from the growing season 2015 and 2016.



*Cells that are designated with a * exhibit a significance level of P<0.05, ** for P<0.01 and *** for P<0.001.*

Positive correlations between cell wall traits and morphological traits were measured between cellulose content and internode traits (length, diameter, number) and cellulose content and length, while these correlations were negative for hemicellulose content. In general, the higher the fraction of leafs the higher the hemicellulose content.

Principal component biplot

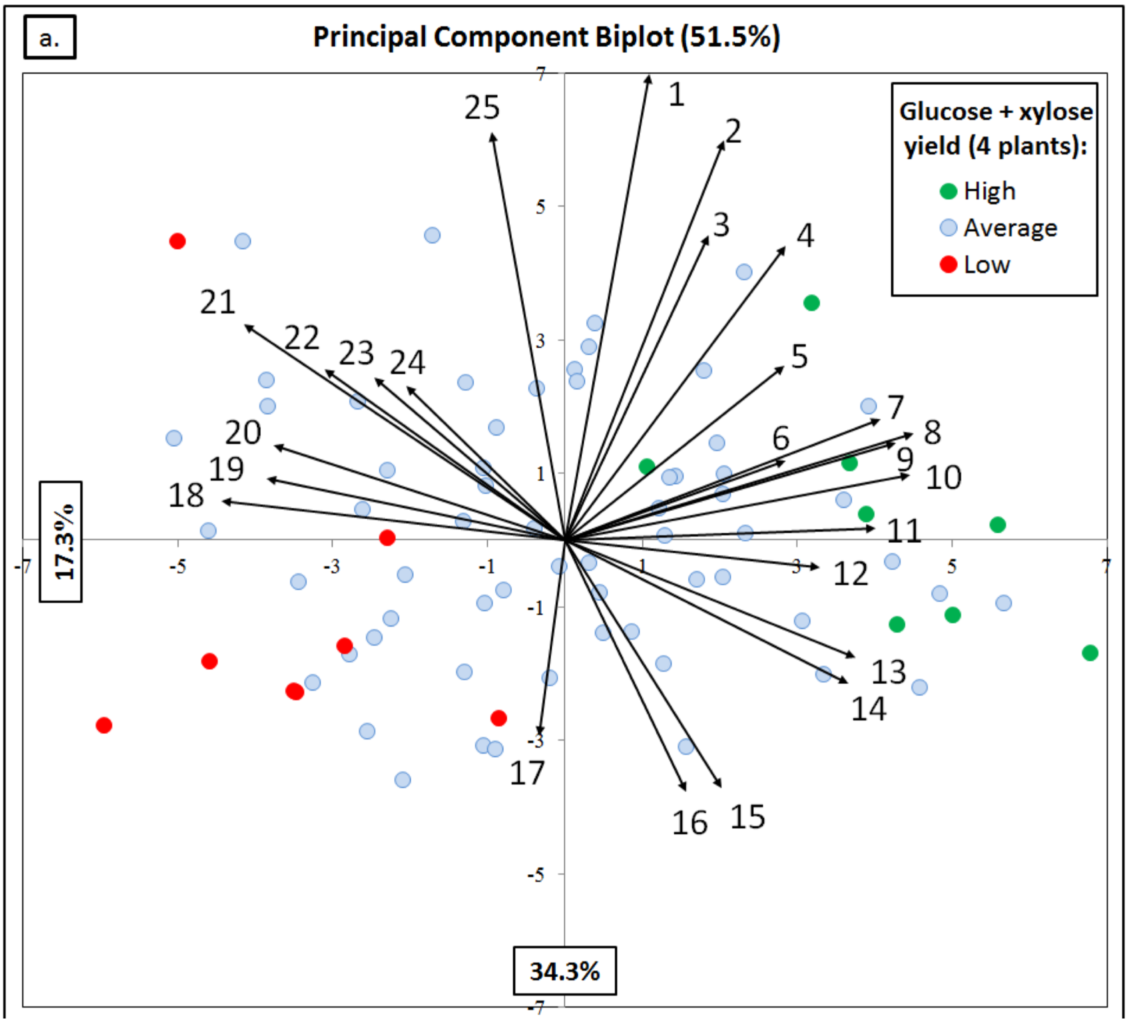
In Figure 10 principal component biplots are displayed with both morphological as cell wall traits from both growing seasons, based on groups of accessions that showed high (top 10%), average and low (lowest 10%) sugar yields per 4 plants (Figure 10-a) and cell wall conversion quality (Figure 10-b). Sugar yields were calculated by the multiplication of sugar release and dry matter yield, while the cell wall conversion quality was a summation of cellulose conversion and hemicellulose conversion.

Both principal component biplots explain about the same range of variation (~51%). Correlations observed between traits were also observed in previous correlation plots. Principal components of both plots are dominated by the same traits. For PC1 most traits are related to yield and hemicellulose content, while PC2 is dominated by flowering date and stem number.

The average coefficient of variation of morphological traits (36.7%) is smaller than the average coefficient of variation of quality traits (14.9%), except for glucose + xylose yield (46.9%). This can be explained by the fact glucose + xylose yield is the sum of the multiplication of dry weight yield (CV = 49.4%) with both glucose- and xylose release (CV = 6.6 and 25.0% respectively), which makes it a combination of a morphological trait and a cell wall trait. Dry weight yield seemed to be dominant over sugar release according to the similar range of coefficients of variation of 46.9 and 49.4% for glucose + xylose yield and dry weight yield, respectively. This assumption is strengthened by Figure 10-a, in which high glucose + xylose yielding accessions show low values for glucose and xylose release.

Grouping based on total sugar yield results in a cluster of high sugar yielding accessions which all have a high score for principal component 1, which results in relatively high dry matter yields and cellulose- and lignin contents combined with low hemicellulose content and conversion efficiency (Figure 10-a). Grouping based on cell wall conversion efficiency resulted in a cluster of accessions which are all in the first quadrant, except for one. This results in relatively high hemicellulose content, high leaf to stem ratios and late flowering in combination with low dry matter yields, cellulose- and lignin contents and a low number of stems per plant (Figure 10-b).

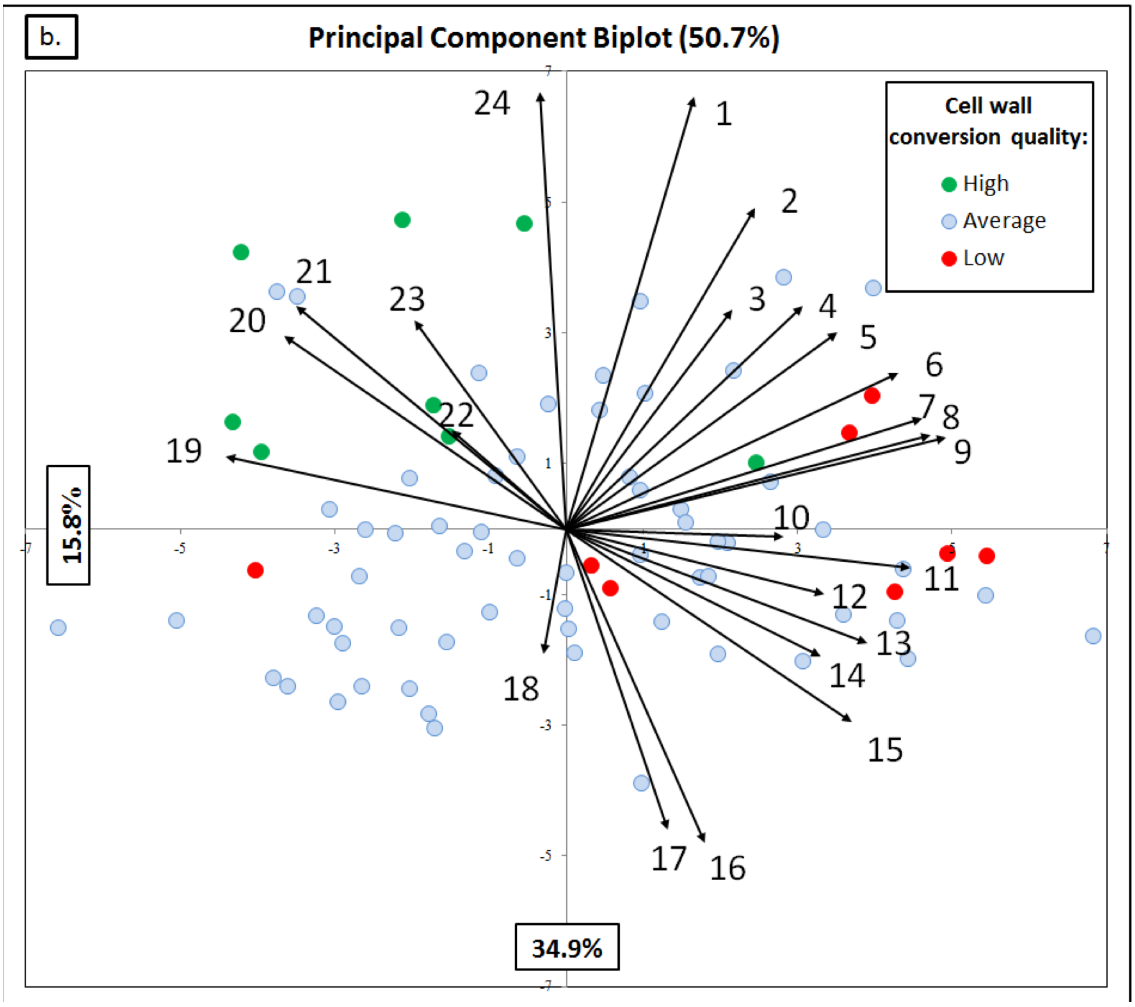
Figure 10-a. Principal component biplot for all morphological and cell wall traits grouped on glucose + xylose yield.



1	Leaf yield-16
2	Internode diameter-16
3	Flower yield-16
4	Number of internodes-16
5	Xylose yield (g) per 4 plants
6	Stem DM% of total dry matter
7	Glucose Yield (g) per 4 plants
8	Stem yield-16
9	Dry weight yield (g) per 4 plants
10	Stem length-16
11	Stem:Weight Ratio-16
12	Stem angle-16
13	Cellulose % of DM
14	Lignin % of DM
15	Internode length-16
16	Dry matter (%) of total weight
17	Number of stems per plant-16
18	Hemicellulose % of DM
19	Leaf:Stem Ratio-16
20	Leaf:Weight Ratio-16
21	Cellulose Conversion (%)
22	Hemicellulose Conversion (%)
23	Xylose Release % of DM
24	Glucose Release % of DM
25	Flowering date (>50% plot)

High, top 10% performing accessions; low, weakest 10% performing accessions; average, remaining accessions. A traits name followed by '-16' represents a trait measured in the 2016 growing season, other measurments are derived from material of the 2015 growing season.

Figure 10-b. Principal component biplot for all morphological and cell wall traits grouped on cell wall conversion quality.



1	Leaf yield-16
2	Internode diameter-16
3	Flower yield-16
4	Number of internodes-16
5	Xylose yield (g) per 4 plants
6	Glucose + xylose yield
7	Glucose Yield (g) per 4 plants
8	Dry weight yield (g) per 4 plants
9	Stem yield-16
10	Stem DM% of total dry matter
11	Stem length
12	Stem angle-16
13	Stem:Weight Ratio-16
14	Lignin % of DM
15	Cellulose % of DM
16	Internode length-16
17	Dry matter (%) of total weight
18	Number of stems per plant-16
19	Hemicellulose % of DM
20	Leaf:Stem Ratio-16
21	Leaf:Weight Ratio-16
22	Glucose Release % of DM
23	Xylose Release % of DM
24	Flowering date (>50% plot)

High, top 10% performing accessions; low, weakest 10% performing accessions; average, remaining accessions. A traits name followed by '-16' represents a trait measured in the 2016 growing season, other measurments are derived from material of the 2015 growing season.

Discussion

By performing different kind of measurements, for both morphological as cell wall traits, the data is used to characterize the variation present in the *M. sinensis* collection of the Laboratory of Plant Breeding Wageningen University. The characterized variation can be used in future breeding projects. Correlation analysis is performed to investigate correlations between traits influencing the breeding process, while principal component biplots give insight in the variation between accessions for different trait combinations which is based on grouping of accessions posessing preferred charactics for biobased end-uses.

Variation for morphological- and cell wall traits present in the collection

Variation for traits

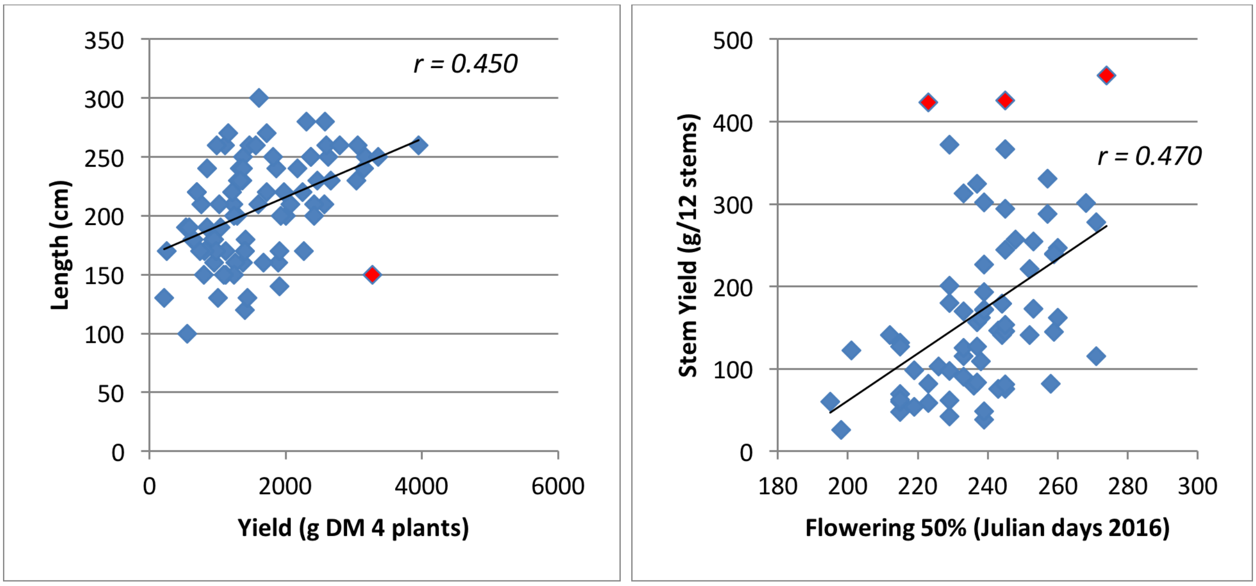
Overall, substantial variation was identified for morphological traits, cell wall composition and cellulose- and hemicellulose degradation efficiency in the *M. sinensis* collection of the Laboratory of Plant Breeding of Wageningen University. In general, morphological variation is higher than cell wall trait variation with an average coefficient of variation of 36.7% and 14.9%, respectively (Table 2 and 3). Large variation was found for lignin content, which is a key factor in determining lignocellulose recalcitrance (Van Der Weijde *et al.*, 2016a). For cell wall traits the range of observed values is broad, however, the lowest and highest intervals presented in the histograms of Annex II represent in majority one accession. This decreases the possibilities to use different accessions possessing extreme cell wall characteristics in breeding. Cellulose- and hemicellulose conversion show about the same range of statistics (Table 3), which indicates that the accessibility for holocellulose degrading enzymes rely on the same process for cellulose and hemicellulose in a mild pretreatment.

Since the accessions are retrieved from different collections around the world, the observed variation can be explained by the rich variety of ways in which plants answer growth, survival and reproduction in different parts of the world (Díaz *et al.*, 2016). The outer reaches of the PCA plots (Figure 10) represent the more extreme combinations of traits, however, a wide gradient of intermediate trait combinations is also present. The observed variation in combination with the high amount of observed trait combinations indicate the potential for using natural variation to breed optimised varieties for biobased- and other breeding purposes, of which cell wall characteristics are highly heritable (Van der Weijde*, unpublished). The results of this research complement these findings and shows that the collection is a valid genetic resource for the improvement in biobased *M. sinensis* breeding programs.

Variation for trait combinations

Regressions between yield and other traits, such as length and stem diameter, show low but significant correlations. This is expected since yield is a complex trait based on multiple simple traits (Clifton-Brown, 2008). Accessions that do not follow this trend indicate that these traits allow access to a diversity in traits independent of another. These outliers could represent a desirable combination of traits. Figure 11 and 12 provide examples that breeding for certain trait combinations is possible, such as a high yield in combination with a small length, early- and late flowering in combination with a high stem yield and high cellulose conversion in combination with low- and high lignin content.

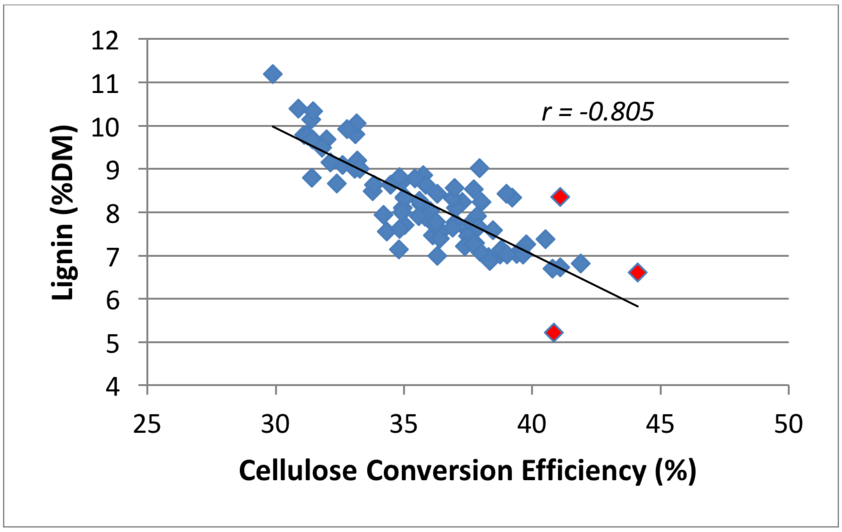
Figure 11. Correlation between yield and length and between flowering 50% and stem yield.



Outliers are marked in red.

As observed from Figure 12 the negative effect of lignin on cellulose conversion efficiency is variable as several accessions with a relatively low amount of lignin were not more efficient in cellulose conversion efficiency. This variation can be explained by the cell wall architecture and its cross-links to hemicellulose (Pauly & Keegstra, 2008; Torres *et al.*, 2014).

Figure 12. Correlation between cellulose conversion and amount of lignin.



Outliers are marked in red.

Correlations influencing *M. sinensis* breeding for biobased end-uses

Miscanthus biomass quality

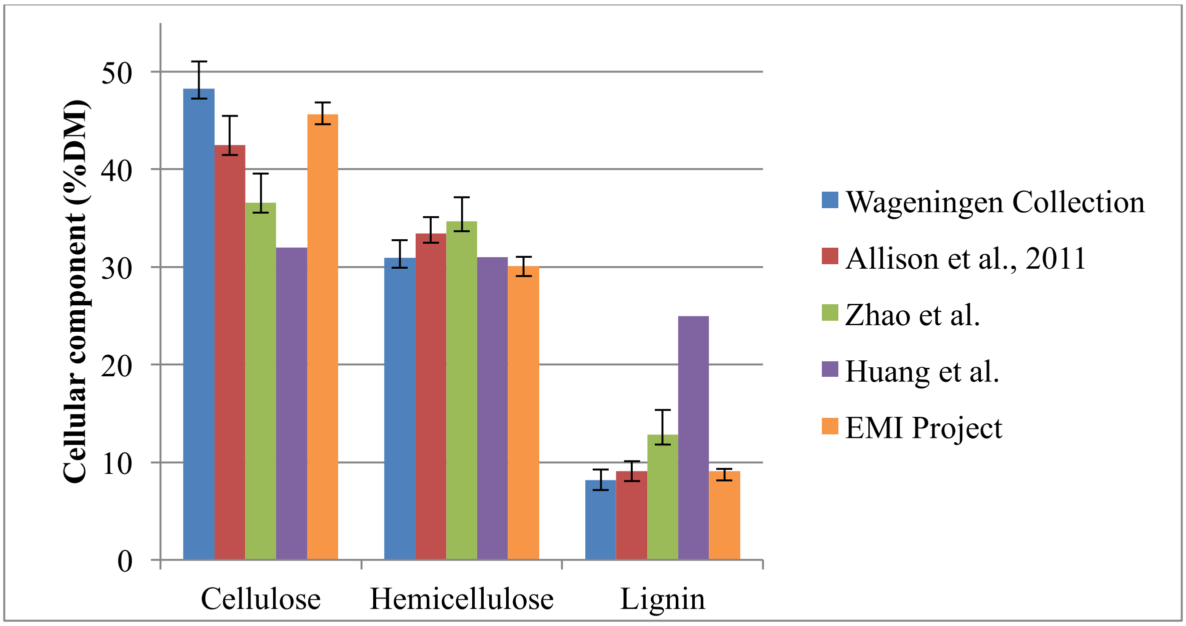
To untangle the cell wall, in which lignin is a hardly degradable component, thermochemical pretreatment is performed. However, this increases production costs drastically (Aresta *et al.*, 2012). The correlation study shows that high lignin content was responsible for low cellulose- and hemicellulose conversion efficiency rates, while a higher amount of hemicellulose results in higher conversion efficiency rates. This shows that differences in the composition of the cell wall have effect on the saccharification efficiency, which is in line with findings established in literature (De Souza *et al.*, 2015; Akin, 2008). For that reason, conversion efficiency can be improved by optimizing biomass composition and decrease pretreatment costs. Due to the high heritability of biomass composition, selection to advance miscanthus feedstocks to improve efficiency- and economics of conversion processes is a feasible approach (Van der Weijde*, unpublished).

As seen in Figure 8, cellulose content showed no significant correlation with lignin content and a negative correlation with hemicellulose content (*r=-0.612*), while hemicellulose and lignin were negatively correlated with a correlation coefficient of -0.653. These correlations are in line with Van Der Weijde *et al.* (2016c), but differ from the results of Allison *et al.* (2011). In the research of Allison *et al.* the correlation between cellulose and lignin content was higher (0.46) and significant. The strong negative correlation between hemicellulose- and lignin content suggests that most lignin is going to be substituted by hemicellulose when future breeding is performed aiming for lower lignin content. Lignin and hemicellulose both provide rigidity to the cell wall and its interactions are established in literature (Qin, 2012; Torres, 2014). For that reason, selection of accessions having high hemicellulose can take place due to its negative correlation with lignin (*r=-0.653*) and its positive correlation with conversion efficiency (*r=0.671*). Since cell wall architecture influences conversion efficiency, accessions with high cellulose- and hemicellulose content should be selected in combination with a higher than expected degradation efficiency. In this way selection of accessions possessing a high amount of potential sugars, in combination with a favourable cell wall architecture, takes place. The combination of these characteristics increases the total sugar release per amount of dry matter for a mild pretreatment. The same conclusion is drawn for a mild acid pretreatment performed in maize, while for severe pretreatments the amount of polysaccharides, that can be converted to monomers, is most important to achieve a high sugar release (Torres *et al.*, 2013).

Cell wall composition compared to other collections

Compared to other *M. sinensis* collections found in literature, the Wageningen Laboratory of Plant Breeding collection possesses preferable characteristics regarding cell wall composition in perspective of biofuel production. Figure 13 displays the cell wall composition of different collections based on one year measurements in the winter cut, measured on one location, except for the data of Allison *et al.* (3 years) and the EMI project (several locations across Europe). As preferred characteristics, the Wageningen collection possesses a high cellulose content in combination with low lignin content, while possessing an average amount of hemicellulose content. However, since cell wall phenotypic plasticity is a phenotypic trait influenced by environment and management effects, comparison between the same environment and management conditions is more reliable (Hodgson *et al.*, 2010; Van Der Weijde, 2013).

Figure 13. Cell wall composition of other *M. sinensis* collections.



Error bars represent the standard deviation. No standard deviation is known for the collection of Huang et al.

Flowering

A key morphological trait in breeding is flowering. Insight in flowering time is important in cross-pollinated breeding to make crosses, however, flowering date shows also significant correlations with most morphological traits measured (Figure 2). The challenge with flowering is its dependency on environmental stimuli, which in their turn has effect on the morphological traits (Figure 5). According to Jensen *et al.* (2011) flowering time is a key trait regarding to yield, since the transition from vegetative to reproductive growth diverts photosynthates away from the accumulating biomass. The positive correlation between flowering and yield was also observed in this research, however, not significant (Figure 2). For that reason, early flowering accessions produce lower yields than the late- or even not flowering genotypes, in that sense late flowering *M. sinensis* is preferred (Clifton-Brown *et al.*, 2001). However, late flowering has negative impact on combustion quality, since plants do not retain their nutrients to their rhizomes completely (Lewandowski *et al.*, 2003). This correlation between yield and combustion quality can be challenging in breeding, since high yield in combination with high combustion quality is preferred. For that reason, a flowering date leaving enough time for senescence is preferred, with the cost that dry matter yields are not optimal.

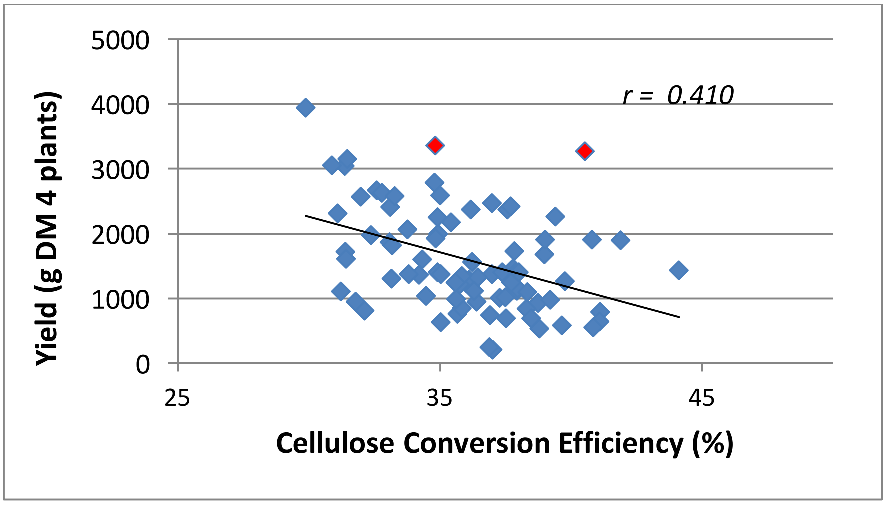
Breeding prospects

Yield and cell wall degradability

According to Figure 10-a in combination with Figure 10-b an answer to growth, reproduction and survival in different regions of the world did not results in the combination of easy cell wall digestibility and a high dry matter yield. In terms of fitness this makes sense, because plant lignocellulose evolved to withstand chemical and enzymatic degradation. The combination of high cell wall digestibility and high yield would therefore result in plants which are easy an easy target for organisms and microbes which are degrading cell walls by enzymatic saccharification or anaerobic digestion. The theory of Xu *et al.* (2012) supports this theory by the statement that hemicellulose content is positively associated with cell wall degradability.

Accessions in the *M. sinensis* collection are high sugar yielding due to high dry matter yields, not due to high conversion efficiencies (Figure 10), which is an undesired combination for biobased end-uses. However, this reflects the potential for *M. sinensis* as a biobased crop when breeders manage to combine these traits. In the collection some variation is present for this desired trait combination (Figure 14), which implies that breeding for this trait combination should be feasible and would presumably not result in too low fitness.

Figure 14. Correlation between cellulose conversion efficiency and dry matter yield.



Outliers are marked in red.

Breeding program

As a start in breeding, a cross between parental lines, which are highly contrasting in yield and conversion efficiency, results in a F1 population in which outliers could possess both high yields and easy degradability. Ideally, the preferred yield characteristics would be combined with the preferred conversion efficiency. However, since both dry matter yield and conversion efficiency are based on quantitative genetics a high amount of progeny should be sown. In this way the likelihood that outliers, present in the F1 population, possess the preferred characteristics. At the end of the second growing season cell wall characteristics are rather stable and biochemical analysis can be performed, while yield potential can be scored from the third year onwards (Van Der Weijde *et al.*, 2016b; Atienza *et al.*, 2003b).

Field trials for *M. sinensis* are difficult and expensive, while selection times are long. Therefore, the development of a marker assisted selection (MAS) program could be considered. By using the highly contrasting accessions as parental lines a mapping populations can be formed. When this data is combined with phenotypic data, such as data gathered in this research, QTLs can be identified in combination with markers. In this way, MAS can predict the mature phenotype already at the seedling stage. For a perennial where the mature phenotype cannot be fully measured before the third year MAS has high potential to accelerate miscanthus breeding (Vermerris, 2008)

Reliability of data

Reliability of NIR for the prediction of cell wall traits

To perform predictions on cell wall composition the improved model is able to predict both in the right range as with a usable coefficient of determination, especially for ADF and ADL predictions (Figure 6). There is still a bias compared to the actual biochemical data, however, the coefficients of determination is high enough to allow comparisons between samples. A high correlation with a high bias is for breeding more useful than a low correlation having a high bias. When a high correlation is present a correction can be made for possible over- or underestimation of the prediction model, based on biochemical data. However, biochemical measurements should still be performed to determine the bias and its coefficient of determination. By using the Goering and Van Soest method a fraction of the lignin is solubilized during the detergent- and acid reactions, in this way lignin could be underestimated in the end (Hatfield *et al.*, 1994). By subsequently integrating this data to the calibration set of a prediction model the accuracy of the prediction model is negatively influenced.

Conversion efficiency predictions are more complicated, resulting in a strong bias in combination with low coefficients of determination (Figure 7). For that reason, predictions are not as accurate as desired. The range in which predictions are made improved drastically compared to the previous model. The improved model ‘learned’ to recognise samples with a certain absorption and reflection phenotype, which can be explained by the fact that the phenotype is the interaction of its genotype with the environment. Therefore, the improvement of the ability to predict in the right range can be explained by the fact that samples were added to the calibration set that had a common genetic background, environment or both, as the samples that were predicted.

In conclusion, NIRS is a usable tool for specific traits, of which the reliability is dependent on the recognition of the sample set. To predict in the right range NIRS is most reliable when a small number of samples measured using biochemical analysis are added to the calibration set of the previous model. By using the NIRS a bias is insuperable and should be accepted, with the advantage that laborious and expensive biochemical analysis is not needed. However, this consideration is probably dependent on the aim of the research. Also, a combination of NIRS and subsequent biochemical analysis can be used, using the NIRS to make a rough selection. In this manner the number of samples to be measured by biochemical analysis can be drastically reduced.

Cell wall composition model equation statistics compared

In literature two equation models for cell wall component prediction are found: the model of Allison (Allison *et al.*, 2011) and the model used in the EMI project (Hodgson *et al.*, 2010). These models show in general the same characteristics as the model used in this research in which ADL is harder to predict than ADF and NDF. Based on statistics the model used in this research seems most reliable, which can be explained by the fact that the model is built using data from multiple experiments across many years and locations, mostly from the experiment of Van der Weijde *et al*. (2016b). For that reason, a lot of environmental variation is included in the model, which increases the model's spectrum to 'recognise' samples, resulting in more accurate predictions.

Combustion quality

To achieve a better and more complete overview of the collection the elemental composition of the accessions should be investigated. The elemental composition has effect on the combustion efficiency, since it can form ash and can be corrosive, making the combustion process more expensive (Atienza *et al.*, 2003a). Other research established that early flowering accessions senesce early in the growing season, which enables them to translocate elements to its rhizomes and shed leafs before harvest take place (Lewandowski *et al.*, 2003). For that reason, flowering date can be used as an indicator for combustion quality.

Conclusion

In conclusion, this study made clear that a high amount of variation and trait combinations is present in the collection of the Laboratory of Plant Breeding from Wageningen University, for both morphological as cell wall traits. This variation offers a broad base of potential parental lines for both bio-based as other breeding purposes. The reliability of a NIRS prediction model is dependent on the trait, genetic background of the sample set and the environment in which the miscanthus has grown. For that reason, using NIRS to predict specific traits should be carefully considered dependent on the aim of the research. In general, accessions that represent a high sugar yield show high dry mass yields together with a low cell wall conversion efficiency, except for one. This shows both the challenge as the high potential of *M. sinensis* breeding for bio-based purposes to combine these traits. A next step in this breeding process could be a cross between highly contrasting parental lines in dry mass yield and conversion efficiency.

Annex

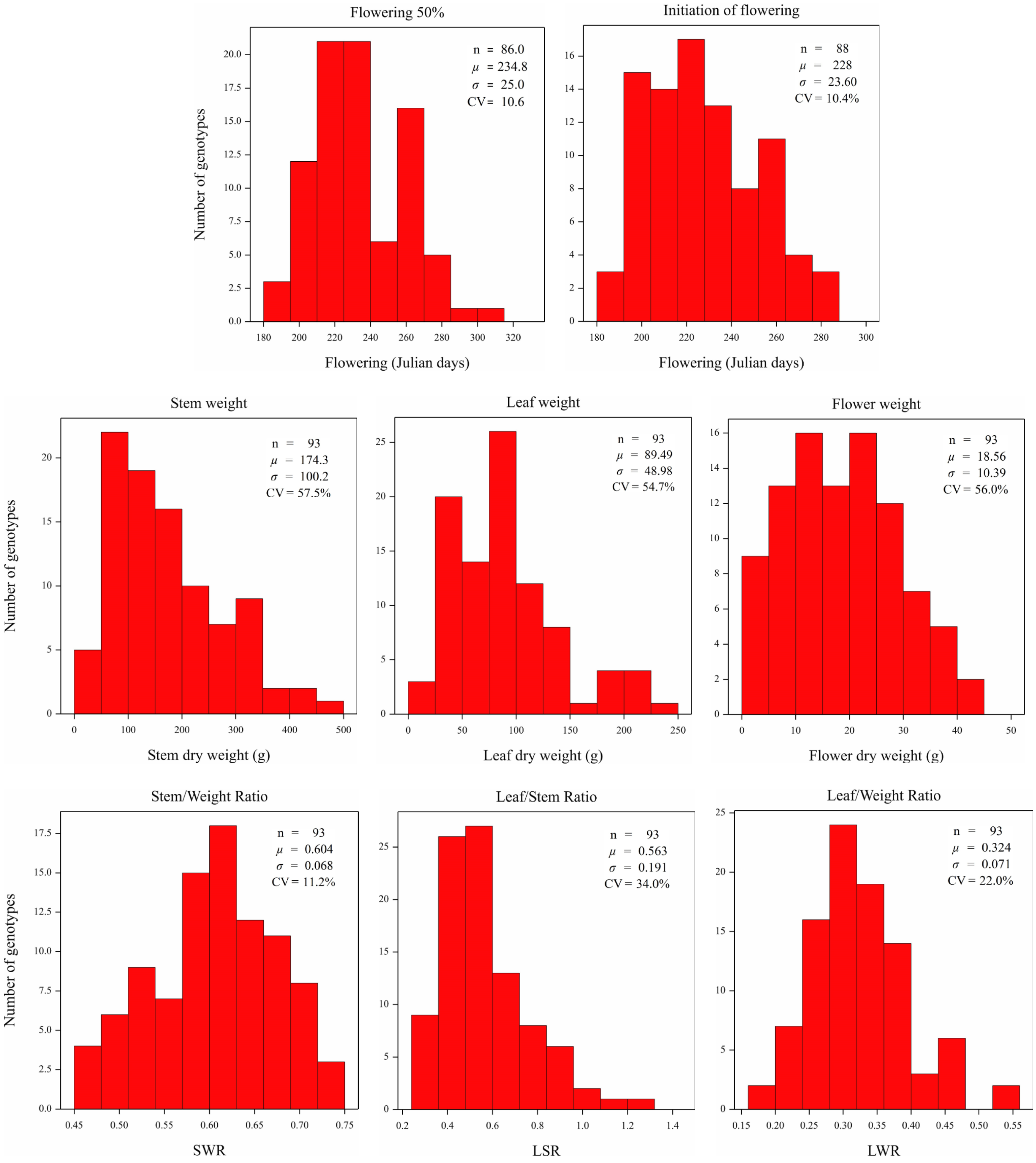
Annex I. Trait descriptions

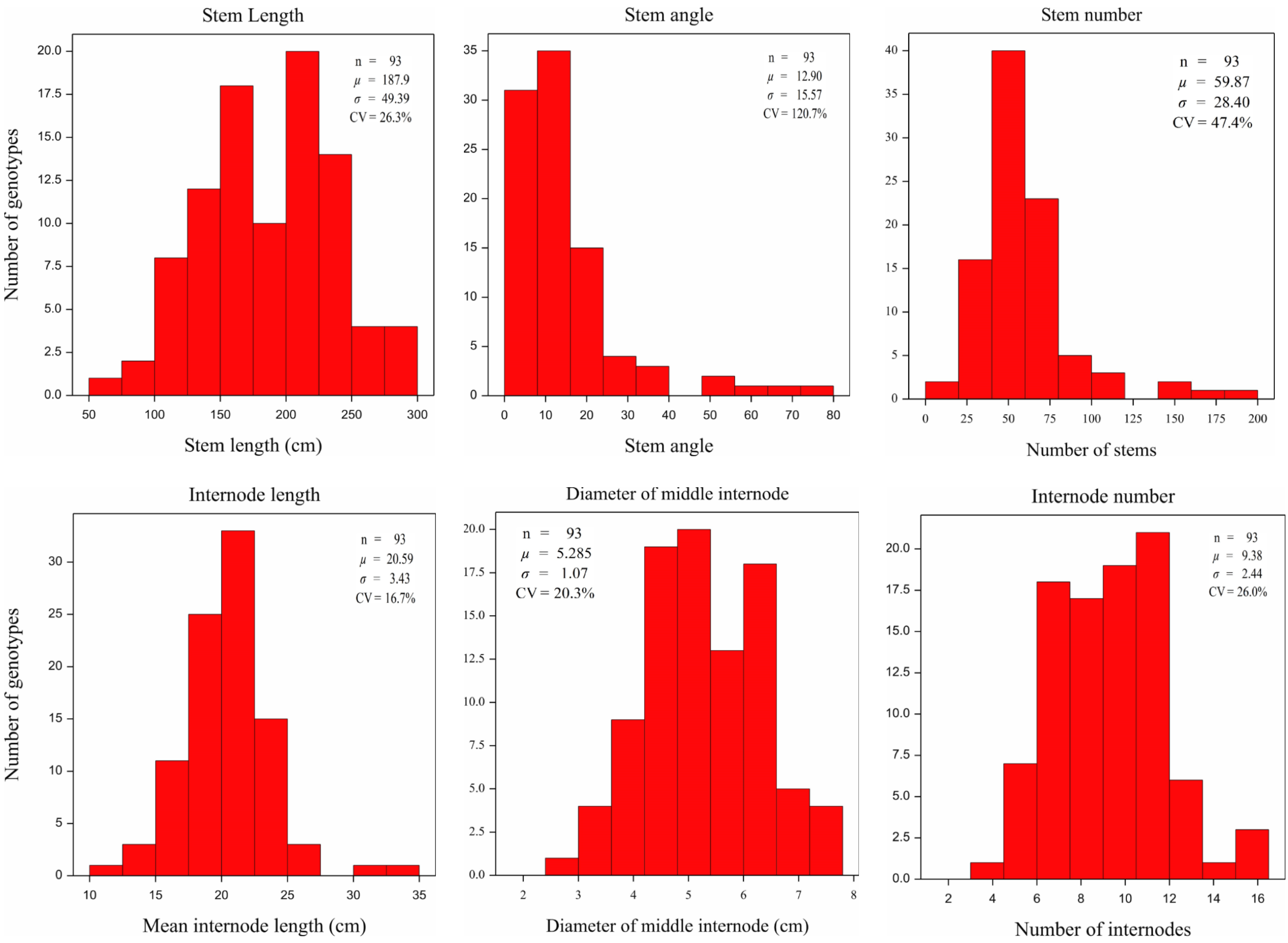
Table 3. Trait descriptions in addition to Table 1.

Trait	Description
Flowering date, initiation	Flowering initiation in Julian days 2016
Flowering date-15, 50%	Flowering 50% in Julian days 2015
Stem length-15	Plant height (cm), 3-5 tallest stems from average plant on 10-12-2015
Dry matter content (%)	Dry matter content % in week 9, 2016
Stem biomass content (%)	Stem content % of total dry matter in week 9, 2016
Total stem yield-15	Total stem yield (g) per plot (4 centre plants of plot) in week 9, 2016
Stem yield	Mean stem dry weight of 12 stems (flowering stems from centre of plot) in week 41, 2016
Flower yield	Mean dry weight of 12 flower heads (on stems from centre of plot) in week 41, 2016
Leaf yield	Mean leaf dry weight of 12 stems (flowering stems from centre of plot) in week 41, 2016
Internode number	Mean amount of internodes per stem (flowering stems from centre of plot) in week 41, 2016
Internode diameter	Mean diameter of middle internode (flowering stems from centre of plot) in week 41, 2016
Internode length	Mean internode length calculated by mStm_L/mInt_No
Stem angle	Mean stem angle border plants of plot in week 37, 2016
Stem number	Mean number of stems per plant (flowering stems from centre of plot) in week 37, 2016
Leaf/weight ratio	Mean leaf/weight ratio calculated by mDW_Lf/(mDW_Fl + mDF_Lf + mDW_St)
Leaf/stem ratio	Mean leaf/stem ratio calculated by mDW_Lf/mDW_St
Stem/weight ratio	Mean stem/weight ratio calculated by mDW_St/(mDW_Fl + mDF_Lf + mDW_St)

Annex II. Histograms

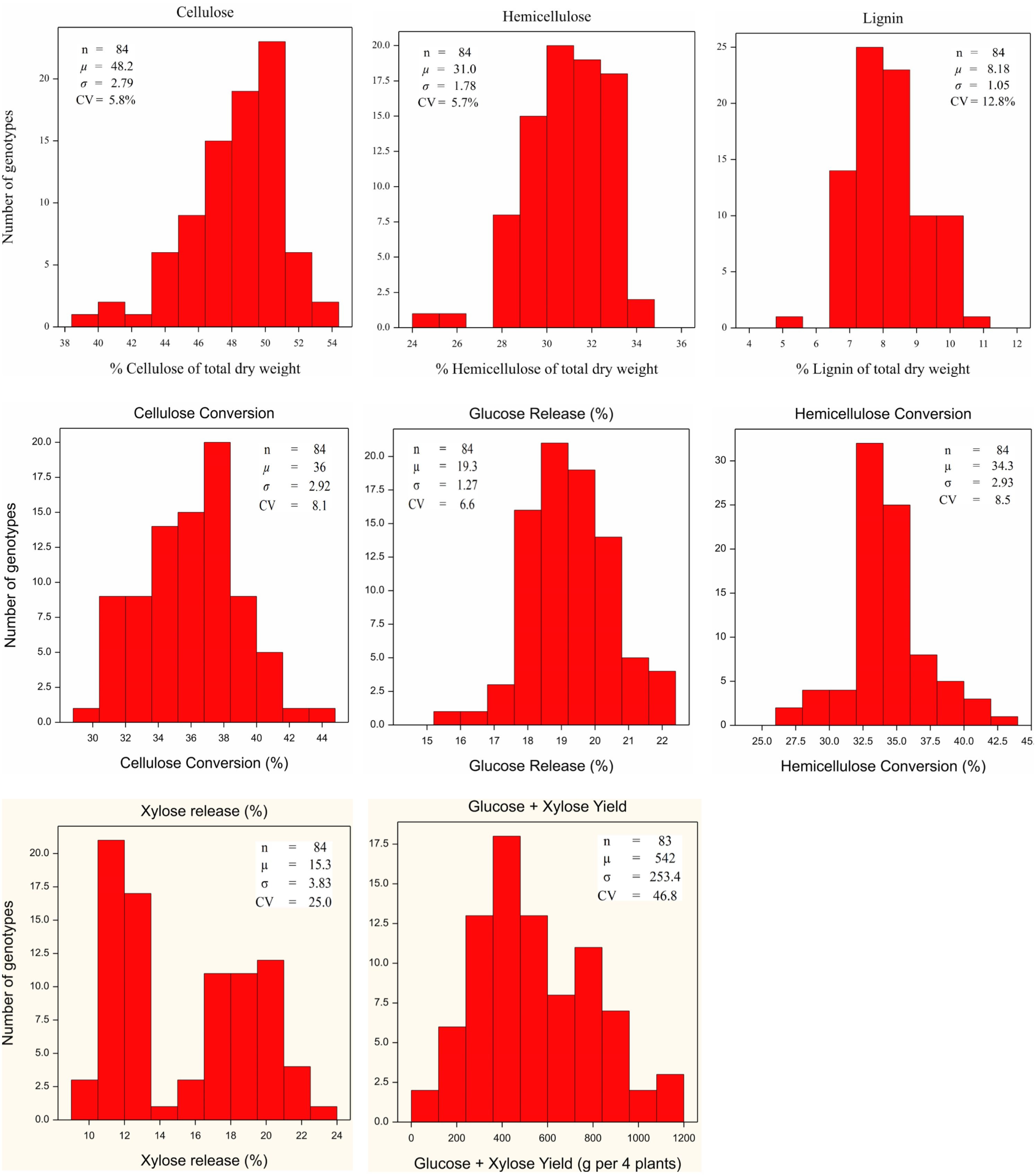
Figure 15. Histograms for morphological traits of *M. sinensis* accessions in the growing season 2016.





n, number of genotypes; μ, mean; σ, standard deviation; CV, coefficient of variation (%)

Figure 16. Histograms for cell wall traits of *M. sinensis* accessions in the growing season 2016.



n, number of genotypes; μ, mean; σ, standard deviation; CV, coefficient of variation (%)

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