Evaluation of the extent of phenotypic diversity in cell wall quality traits in *Miscanthus sinensis* experimental hybrids

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Abstract

Miscanthus is a promising crop for biobased economy as a sustainable lignocellulosic feedstock. Faced to the multiple industrial demands, not only high quality cell wall compositional traits are obligatory, but also a variety of biomass qualities are needed. In this work, the *Miscanthus sinensis* experimental hybrids in plant breeding group of Wageningen University & Research were characterized to assess the phenotypic variation within cell wall quality traits and among trials from different years. A large extent of variation was determined in cell wall properties and differed significantly. The range of hemicellulosic polysaccharides was from ~16% to ~35% with the coefficient of variance of 11.2% while lignin was from ~6% to ~14% with the CV% as 15.9%. The estimated heritability was calculated by linear parents-offspring regression and exhibited the hemicellulose as the highest heritability of 0.65 in the experiment. In general, this study reveals the potential of breeding *M. sinensis* hybrids for multiple industrial purposes.

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Introduction

Miscanthus

The growing depletion of conventional non-renewable fossil feedstocks (coal, oil, natural gas, etc.) and the concomitant environmental consequences of their over-exploitation have surfaced in the recent decades. (Allwright & Taylor, 2016; Nigam & Singh, 2011) Since energy, fuels and chemicals are mostly produced from petrochemical industries, developing alternatives to replace or reduce dependency on fossil fuels has become a pressing issue for society. Biomass, which is often regarded as a clean, renewable and low CO_2 emission feedstock, is an ideal choice contributing to the transition from the petrochemical to the biobased economy (Brosse et al, 2012; Lemus & Lal, 2005). As a biobased economy grows and strengthens, there is a need for reliable biomass feedstocks that can supplant the use of fossil fuels and here comes the Miscanthus. (Yamada, 2014)

Miscanthus spp. is a genus consisting primarily of perennial C4 rhizomatous grass species, some of which are considered to be the most promising biomass crops because of their broad adaptability to a wide range of agro-climatic conditions, high biomass yield, good ecological restoration and growing on marginal land ability, high water use efficiency, good abiotic stress tolerance and low management cost. The genus has been researched in Europe and USA for more than 20 years and is considered today one of the most promising species for the production of second generation biofuels and bulk chemicals(Lewandowski I, 1995; van der Weijde et al, 2017b; Wagenaar & Van den Heuvel, 1997).

Miscanthus is originally from East Asia and it is found across an extensive climatic, latitudinal, longitudinal and altitudinal range, and thus exhibits vast genetic diversity. Different genotypes contribute to the broad adaptability of Miscanthus to diverse environmental conditions, which today extends from Europe to Japan (Lewandowski et al, 2016; Yamada, 2014). For example, *Miscanthus sinensis* grows better in the northern Europe where winter can be severe, and *Miscanthus* x *giganteus* is more adapted to southern Europe with a more temperate climate (Christian et al, 2005).

In the context of a biobased economy, the most outstanding benefit of Miscanthus as a biomass crop is its high biomass yield. It shows an average yield of 22 t DM per hectare per year in some European and US sites, which is compared to 10 t DM per hectare per year of switchgrass (Van Der Weijde, 2013). According to Heaton et al. (2008), *M.* x *giganteus* shows a highest yield of 60.8 t DM per hectare per year in Illinois, U.S.. Moreover, given concerns over competition of land use between food crops and non-food crops worldwide, Miscanthus is an excellent choice for biomass production as it can grow on marginal lands with no competitive value for food production (Gabrielle et al, 2014). Miscanthus is generally considered an environment friendly crop since it has the ability to grow under different marginality factors, such as salinity, drought, and contaminated soils, and because it is an efficient crop because of its high level of biomass yield under low input levels. (Gabrielle et al, 2014; Xue et al, 2016) Also, when Miscanthus is planted once, there will be more than 15 years of continuous harvesting. During the winter, the nutrients are transferred to the rhizomes of Miscanthus and could be used for the next year. These traits can help to save a lot of labour and sources, and help to prevent soil erosion, improve soil condition, reduce environmental pollution and promote ecosystem restoration.

The Miscanthus genus has 17 species and several of them show a good prospect for biobased economy. The most well-known species are *Miscanthus* x *giganteus, Miscanthus sacchariflorus* and *Miscanthus sinensis*. (Brosse et al, 2012) *M.* x *giganteus* is currently the most widely used species for commercial biomass production in Europe because of its high yield of biomass, but it is a triploid and is sterile. *M.* x *giganteus* therefore always needs to be propagated by *in vitro* method, which means requiring cloning propagation and leads to a high cost of establishment of the program. (Xue et al, 2016; Yamada, 2014) Meanwhile, one genotype with a large-scale cultivation always means the instability from pets, disease and severe weather conditions.

M. sinensis is reported has adapted to the climate in northern Europe (Clifton-Brown et al, 2001) and large genetic variation exist due to the adaptability to various environmental conditions while is available for the future genetic improvement. At the same time, it is diploid and possible to flower and produce fertile seeds, which can reduce a large amount of establishment cost (Christian et al, 2005). The diploid genome is easier to utilize present breeding tools than diploid and the available hybrids also can have hybrid vigour with better performance Therefore, the most attractive virtues of *M. sinensis* are the large genetic complexity and the possibility of breeding through hybridization. Molecular breeding could be used to help to develop attractive traits such as tolerance for abiotic and biotic stress, higher yield, saccharification efficiency, better biomass quality, etc. (Yamada, 2014).

Breeding for improved cell wall composition

Faced with the increasing demands for traditional and novel applications of biomass, the demands for Miscanthus in different industries are complex and diverse. The suitability of Miscanthus as a biomass feedstock ultimately depends on the quality of lignocellulose, which is mostly composed of cell walls. There is a large quantity of carbon in the lignocellulose, majorly existing in cellulose, hemicellulose and lignin. Cellulose is the main constituent of the cell wall and made of glucose. Because of the multiple utilization of polysaccharide cellulose, it is the focus of almost all areas, such as bioenergy, paper making, but it is inserted in a hemicellulose and lignin cross-linked matrix structure. Hemicellulosic polysaccharides also could be used to produce xylose or bioethanol and have been reported with the ability of replacing lignin to increase cell wall degradability. The aromatic polymer lignin is considered to hinder the saccharification efficiency in the pretreatment process to produce bioethanol product. (De Souza et al, 2015; Zhao et al, 2014) Also in paper pulp making process, lignin is the main reason of a black liquor, which is always treated as a waste. (Haverty et al., 2012) Cell wall compositions hold an important position is because breeding could be operated to increase the amount of certain component which is relevant to the industry. Secondly, cell wall composition is a promising breeding target which could also reduce the cost of pre-treatment of lignocellulose and make the separation of lignocellulose in its basic components more energy and material-use efficient. Thirdly, the properties of cell walls could be changed to create material properties unique feedstock to multiple industrial utilizations. The changes that need to be made depend on the industry and what it desires. Therefore, characterization of the content of cellulose, hemicellulose and lignin is important for evaluating the Miscanthus biomass behaviour and is favourable to Miscanthus breeding to develop targeting specific market.

As Miscanthus is always grown for the lignocellulosic biomass, which is regarded as a key player of the biobased economy, it has become a hot topic of concern and research for diverse applications, such as bioenergy, industrial applications (paper pulp, bioplastic, 3D printing, packaging materials, fibre materials, bioethylene and other chemical derivative), building materials (lightweight concrete, insulation board, roof covering), horticulture (peats, substrates), livestock bedding and feed (cow, horse), etc. (OPTIMISC, 2016).

Nevertheless, there are so many traits could be improved for Miscanthus, such as biomass yield and some food crop relevant traits (biotic and abiotic tolerance, source use efficiency) and bioproduct-specific traits which is necessary for meeting the requirements of some particular utilization. As Miscanthus lignocellulosic biomass is an optimizing choice for second generation biofuel, there is another example for the attractive traits to be bred for. The second generation biofuel, which is based on the cellulose and hemicellulose, is a renewable energy and is concerned a way to solve the problems of energy and environmental crisis and competition between food and bioenergy.(Nigam & Singh, 2011) As previously mentioned, lignin avoid cellulose to be separated out from the cell wall, thus lignin-reducing Miscanthus, which could decrease the cost of pretreatment during producing the bioethanol, is a desired target of breeding.

WUR *Miscanthus sinensis* breeding program

To breed for Miscanthus cell wall quality and conversion efficiency for biobased feedstock, it is important to have a well understanding of the plant. Miscanthus is indicated as a self-incompatible plant. The germplasm collections of Miscanthus are available and in its original area in Asia and also there are accessions collected in Europe.

However, there are seldom breeding and selection work for Miscanthus previously compared to other food crops. Recent studies show a result that the cell wall composition and conversion efficiency of *M. sinensis* could be genetically improved through breeding. Genotyping-by-Sequencing was developed by several people before and 86 QTLs for cell wall composition and conversion efficiency were first developed in Miscanthus, which is helpful for the future MAS programs. (van der Weijde et al, 2017a)

Meanwhile, the probable drawbacks of breeding in Miscanthus are necessary to be avoided, such as lodging, which is considered to correlate with the lignin content, and decreasing underground biomass. Breeding for Miscanthus is slow because of its perennial characteristic, thus we need to think about it (van der Weijde, 2016).

The breeding program for *Miscanthus sinensis* in Wageningen University & Research has been established for almost 10 years and the *M. sinensis* and *M. sinensis* hybrids were developed from a 50 plants recurrent family 20 years ago.

One selection cycle is 3-5years while one year for producing the seed and 2-4 years for evaluation. The rule for assessment is to look for parents that have well behavior and could be used for producing hybrid seeds and for next selection cycle. The chosen parents are cloned to maintain for producing hybrid seeds. The selection now is mostly based on the pair-wise crosses using an isolation cage to produce intraspecies hybrids, in which selected plants are planted pair-wise (Figure 1.).

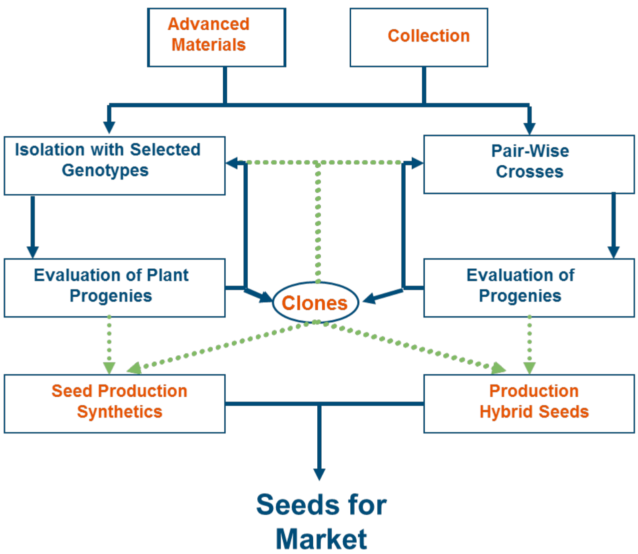

Figure 1. The *M. sinensis* hybrids breeding program in WUR breeding group (Figure from O. Dolstra)

Objectives

What could be the research objectives are:

1. To characterized the cell wall quality of the Miscanthus hybrid lines in WUR Miscanthus breeding program for multiple biobased products
2. To identify the phenotypic variation among hybrids lines and realize the parents-offspring relationship in WUR Miscanthus breeding program
3. To update and promote the NIRS model

Materials and methods

Plant materials

In this project, we analyzed a collection of *M. sinensis* * *M. sinensis* hybrids and their parents (when available). These hybrids have been generated and tested in diverse fields trials over a period of 10 years (2007-2017). Therefore, extensive phenotypic variation can be revealed within certain cell wall compositional characteristics and materials from different trial can show some insights of the variation among the *M. sinensis* hybrid lines. The plants in trial OD0505 are the seed parent of the trial AV0701 and the heritability of different cell wall quality traits can be estimated by developing the parents-offspring regression in this experiment. The experimental *Miscanthus sinensis* hybrids and their parents used in this experiment are shown in Appendix 1. All Harvested materials from these trials have been chopped and dried at 70℃. These materials were ground by a hammer mill with a 1mm sieve to prepare for subsequent biochemical measurements.

NIRS Scanning

Near infrared spectroscopy (NIRS) is a high-throughput technique which can be used for predicting biomass biochemical quality traits (including saccharification efficiency). All 574 ground biomass samples were scanned with the NIRS DS2500 (FOSS, Denmark). Next, an evaluation was conducted to see how well these samples can be predicted with the *in-house* prediction model for biomass quality traits. This prediction model can be used to predict the cellulose, hemicellulose and lignin content of Misanthus biomass samples, as well as their saccharification efficiency following mild alkaline pretreatment.

Biomass Biochemical Analyses

Total 101 samples were randomly selected within all 574 samples by WinISI program

version 4.9 (Foss, Hillerød, Denmark) and their cell wall compositions were measured in the laboratory. The samples selected for calibration of the model were analysed using the Van Soest method (Goering and Van Soest, 1970) by Ankom 2000 Fiber Analyzer (ANKOM Technology, USA). The neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were measured for the samples.
NDF, ADF and ADL content were calculated using the equation [1]:

$$\%\mathrm{NDF}(\%\mathrm{ADF}\text{ or }\%\mathrm{ADL}) = \frac{W_2-(W_1*C_1)}{W_3} * 100\% \qquad [1]$$

as
W_1 = Bag weight
W_2 = Total Dry weight of bag and fiber after treatment
W_3 = Dry weight of fiber before treatment
C_1 = Blank bag correction
Meanwhile, the cell wall components were calculated as following:
NDF = Lignocellulose
Hemicellulose = NDF – ADF
Cellulose = ADF – ADL
Lignin = ADL

Updating NIRS model, Prediction and Data Analysis

After gathering the data from the biochemical analysis, a representative calibration set of 81 samples was used to update the NIRS prediction model and a validation set of 20 samples was used to validate and do comparison between lab data and prediction data in different model versions to look at the reliability and the performance of the updated model. The new model was used to predict the biochemical quality traits for all hybrid samples and their parents (when available). This information was used to evaluate the extent of biochemical diversity available in the WUR *M. sinensis* breeding program among different trials.
General analyses of variance (ANOVA) were executed to find out the variation within different cell wall compositional traits. Estimated heritability of half-sib family can be calculated using parents-offspring regression with the equation [2] presented by Nguyen & Sleper (1983). The $\sigma_{P_{PO}}$ represents the phenotypic covariance between

parent and offspring and σ_P^2 is the parental phenotypic variance, while the ratio is called the linear regression coefficient (slope). The narrow-sense heritability of half-sib family can be calculated by double the regression coefficient shown in clarify as equation [3] (Nguyen & Sleper,1983):

$$h^2 = 2\frac{\sigma_{P_{PO}}}{\sigma_P^2} \quad [2]$$

$$h^2 = 2 * slope \quad [3]$$

Results and Discussion

Updating of NIRs prediction model for *M. sinensis* hybrids

Since the experimental hybrids lines measured in this experiment were from a large extent of period and not part of the experiments existing in the earlier model database, there is a risk that the former models cannot capture the real data of the cell wall composition. In order to obtain more accurate predicted statistics of cell wall quality traits in WUR *M. sinesis* breeding program, the NIRs prediction model (v8, version 8) was built based on the models from previous years (v6, version 6 from Van Der Weijde T. and v7, version 7 from Bogers R.).

The model v8 was conducted based on an 81 samples calibration set while a validation set of 20 samples were used to compare with the prediction statistics to improve the accuracy and reliability of the model. The updated prediction model had 11 principal component terms with a variance of 98.70%.

The prediction model v8 has the best coefficient of determination (*r* value or R^2) in both ADL%DM and ADF%DM with 0.912 and 0.968, respectively and hold the second position in NDF%DM (Figure 2.). In the ADL%DM, all the three models performed well while there were no large differences presented among the three models and the possible reason is that most of the data selected in the calibration set was included in the previous database. Meanwhile, one remarkable point to be reminded is that the model v6 displayed a higher coefficient of determination and bias in NDF%DM than other models but most of the prediction data of model v6 was high above the lab data line instead of distributing on two sides. On the other hand, model v8 presented a high correlation with more accurate values at the lower aspect and promoted the bias compared to model v7. Here shows an alert that because the variation of cell wall compositional characteristics is reported in a certain range, for example, lignin from ~5 to ~21% (Van der weijde et al., 2016), sometimes a higher correlation is not completely related to a better model performance in the trait. Therefore, comparison of the correlation and bias between lab data and predicted statistics is an estimated method to show the improvement and accuracy of the model, however, the range of the data need to be considered.

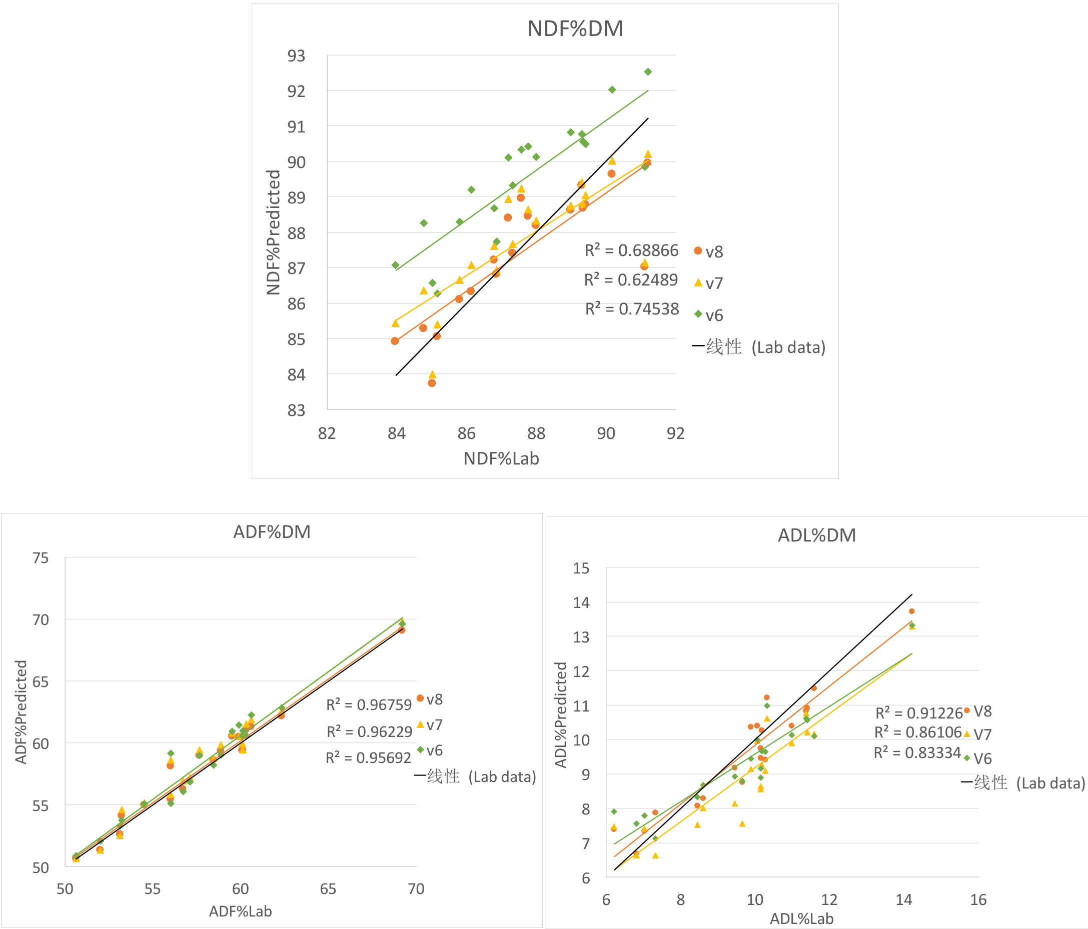

Figure 2. Predicted validation samples for model v8, v7 and v6 compared with the data from biochemical analysis. *The black line represents the data from biochemical analysis*.

At last, the performance of prediction model v8 showed the usability of NIRS and improved predicting ability in range and coefficient of determination. Compared to other NIRS cell wall quality traits prediction equations, which had some difficulties with predicting ADL, the model performed better and most likely because of the extensive database from different experiments of Van der Weijde (2016). Combined with the advantages of reducing the effort and cost of biochemical analysis, NIRS can be more reliable and considerable based on a continually updating sample set from outlier samples and used for breeders to get a quick knowledge of the plant cell wall traits behavior.

Variation within cell wall quality traits

Ground *M. sinensis* hybrids stem materials were measured for cell wall component characteristics. A large variation was observed within cell wall quality traits and indicated a wide difference in the evaluated experimental hybrid lines during the decennium as shown by the summarized values in Table 1.

Table 1. Variation and significance within cell wall quality traits

	n	Min	Mean	Max	Range	F pr.	SD	CV%
NDF (%DM)	101	81.3	87.1	91.2	9.9	<.001	2.3	2.6
Cellulose (%DM)	101	41.0	48.5	55.7	14.6	<.001	3.0	6.2
Hemicellulose (%DM)	101	15.7	28.3	34.8	19.1	<.001	3.2	11.2
Lignin (%DM)	101	6.2	10.3	14.4	8.2	<.001	1.6	15.9

DM, dry matter; F pr. probability from ANOVA; SD, standard deviation; CV%, coefficient of variation.

Percentage of neutral detergent fiber of dry matter content (NDF%DM) and other cell wall traits (cellulose %DM, hemicellulose %DM and lignin %DM) are determined in the biochemical laboratory with an overall number of 101 samples. The cell wall composition of the whole dry matter content showed a low coefficient of variation of 2.6% and a small range of 9.9. Hemicellulose content indicated the highest range of 19.1 and meanwhile, the lignin content displayed the highest CV% as 15.9. Approximately, an average of 87% of dry biomass consisted of cell wall component, 49% consisted of cellulose and 28% and 10% for hemicellulose and lignin, respectively. As the expectation of cell wall composition compared to the data of other researches, the contents of cellulose was from ~26 to ~54%, in hemicellulose from ~25 to ~43% and in lignin from ~5 to ~21% (Van de Weijde et al. 2013; Zhao et al., 2014). The large variation in lignin content in this experiment was because some of the experimental trials were focused on the combustion quality at the starting point instead of biobased end-use (Dolstra, 1/Dec/2017, Pers. Comm.).

The significant variation (*$p<0.001$*) existed within cell wall compositional characters of the *M. sinensis* hybrids accessions and an extensive variation in lignin content (15.9%) was found, which is the one of the main focuses of low lignocellulose recalcitrance and producing ideal bioethanol (Van der Weijde et al, 2016). Coefficient variations (%) of technical error in two experimental replications of NDF%DM, CEL%DM, HEM%DM and ADL%DM were not significant (5%) as 2.26, 3.02, 3.18 and 1.63, respectively, and therefore, revealed that the significances in this experiment were not due to the error of the experiments.

The wide variation can be explained that the hybrids breeding program was established from more than 10 years ago. Different environmental conditions, harvest years, experimental designs influenced both harvested materials and breeding program (Hodgson, 2010). Meanwhile, the number of the samples selected are from different trials, which means they have diverse impacts on the results.

Overall, the variation in *M. sinensis* cell wall quality traits revealed the large extent of biodiversity in WUR breeding program and the future opportunities of breeding for optimal varieties for multiple purposes.

Variation of different trials

Because the *M. sinensis* hybrids breeding program in WUR plant breeding group was established over a decade, environment variation various among different trials, thus bring the uncertainty to the results. To determine the trial differences, an assessment of different trials is displayed in Figure 3. Compared to other trials, the trials with the number of OD0505, which are the same trial harvested from two different years 2007 and 2008, are similar in different cell wall traits. Meanwhile, their offspring trials AV0701 harvested in 2010 and 2012 vary greatly and one possible reason is that the plants were at a juvenile level since 2010 was the second year after the establishment of this trial (Dolstra, 19/Feb/2018, Pers. Comm.). Miscanthus is a perennial plant, which typically matured in three years, and shows plant age dependence (Clifton-Brown et al., 2008). After two years in 2012, the lignin content of *M. sinesis* hybrids plants increased and hemicellulose content decreased in trial AV0701. It is necessary to take into account the performance differences between cultivation years when breeding for Miscanthus and not to predict the mature phenotype by young plants which has been shown the unreliability (Clifton-Brown et al., 2008).

Desired traits in certain genotype can be obtained from different trails. The various variation among different trials showed the possibilities of selecting certain plant with desired trait and reduce breeding effort for multiple biobased end-use. The trial TW1201 is disparate with a larger range of the biomass quality value compared to other trials. This is because that some other species of *Miscanthus*, such as *M. sinensis*, *M. x giganteus* and *M. sacchariflorus*, were mixed in the trial. (Dolstra, 19/Feb/2018, Pers. Comm.)

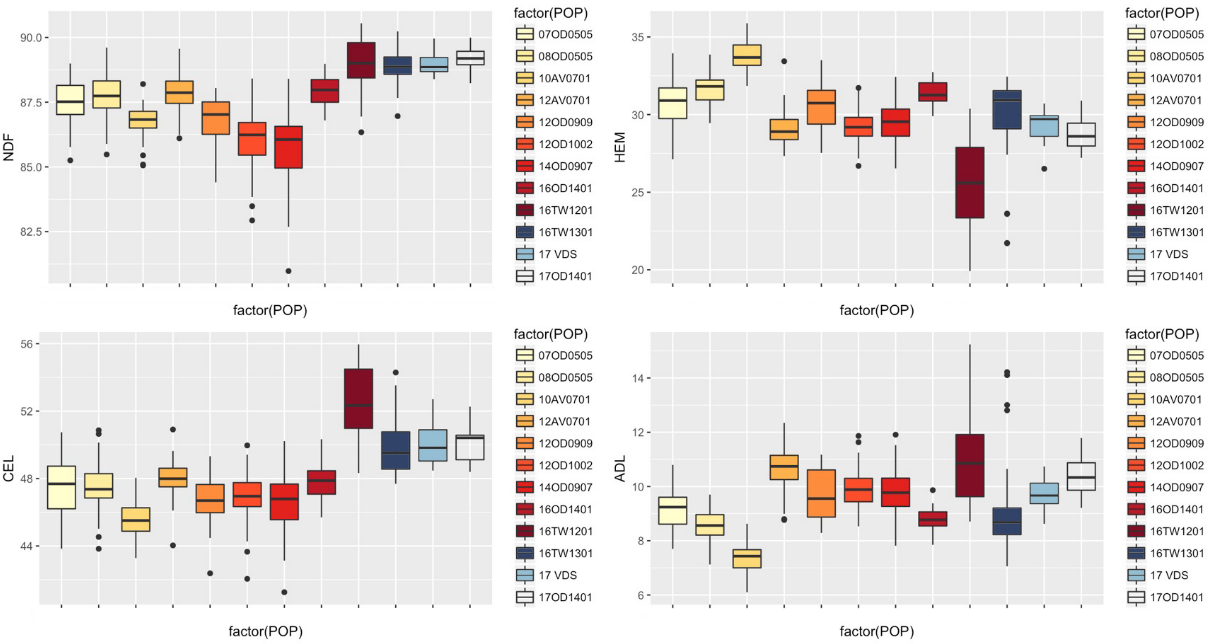

Figure 3. Boxplots displaying variation in the cell wall quality traits among different trials

Parents-offspring relationship

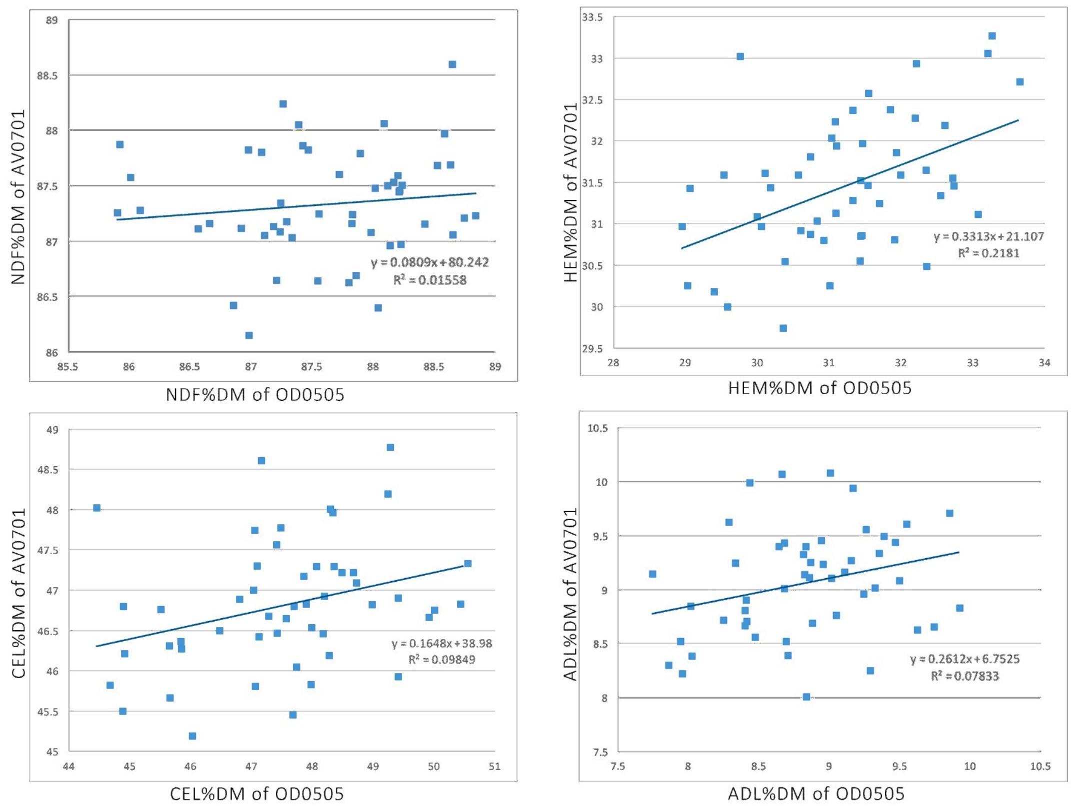

Figure 4. Parents-offspring linear regression of cell wall quality traits

The trials with the number of 07OD0505 and 08OD0505 are the same trial while the difference is that they were harvested from year 2007 and 2008. All genotypes cultivated in this experiment were selected from BIOMIS mapping population and the trial was designed in RBD with 100 units of 1-row plots (10 plants). Besides, the plants of OD0505 were the seed parents of the trial AV0701, which had two replications from different years 2010 and 2012 as well. There were 50 half-sib families existing in this experiment and cultivated in 4-row plots with a plant density of 2 plant/m^2. In order to get the insights of the parents and offspring relationship, the linear regression of average values of neutral fibers in parents and offspring trials were calculated to reveal the correlation and to visualize the estimated heritability (Figure 4).

The HEM%DM displayed the highest *r* value and the slope while the NDF%DM was the lowest. All the cell wall composition traits were positive which proved in some cases that the heritability existed between parents and their offspring. However, the deficiencies are the lack of the information from male parents and the half-sib family heritability was calculated based on the linear regression of the parents-offspring relationship. The method of Nguyen & Sleper (1983) was used and the results are shown in Table 2 below.

Table 2. Estimated heritability by linear parents-offspring regression

	Covariance of parents-offspring	SD of parents	Estimated h^2	*F pr*
NDF	0.046	0.76	0.16	0.388
CEL	0.372	1.52	0.32	0.026
HEM	0.442	1.17	0.65	<.001
ADL	0.075	0.54	0.51	0.046

SD, Standard deviation; F pr , F probability of linear regression analyses

The highest heritability was observed in hemicellulose as 0.65 in this experiment while lignin had a medium heritability as 0.51. The *p* values of linear regression were significant (<0.05) in CEL, HEM and ADL but in NDF, it was not significant, which represented that there was no linear regression observed in this experiment.

As shown in Appendix 2., the correlations of different cell wall quality traits between parents and offsprings were indicated. Parental hemicellulose content had a strong positive impact on offspring hemicellulose content and showed a negative correlation with lignin and cellulose content.

A high content of hemicellulose can replace the function of lignin by the crosslinking within hemicellulosic polysaccharides and with other components and at the same time reduce the cellulose crystallinity, thus increase degradability and saccharification efficiency with a low influence to the plant (Van der Weijde, 2013). The high heritability of hemicellulose shows the possibilities of breeding for ideotype with high cell wall quality.

Conclusions

In conclusion, 1) an extensive phenotypic variation was observed within cell wall quality traits in the *M. sinensis* hybrids breeding program of Wageningen University & Reseach; 2) Heritability existed in some traits such as hemicellulose and lignin; 3) NIRS prediction model was updated depend on traits and when using NIRS to predict samples, it should be considered of the need of accuracy and reliability. However, NIRS is good enough to determine the range and mean, thus gives breeder an insight of the samples. Consequently, M. sinensis hybrids are promising as a lignocellulose feedstock and certain breeding program for multiple unique industrial purpose needs to be established in the future.

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Appendices

Appendix 1. List of *Miscanthus sinensis* hybrids materials

Trial number	Number of samples	Harvest year	Type of sample	Group	Experimental Units
OD0505	50	2007	Stems	BIOMIS	Plant
OD0505	50	2008	Stems	BIOMIS	Plant
AV0701	50	2010	Stems	BIOMIS	Family
AV0701	50	2012	Stems	BIOMIS	Family
OD1002	104	2012	Stems	-	Miniplot
OD0907	108	2014	Stems	Exp hybrids	Miniplot
OD0909	36	2015	Plant	-	Plot
OD1401	22	2016	Stems	Exp hybrids	-
TW1201	45	2016	Stems	Genotypes	Plot
TW1301	32	2016	Stems	Genotypes	Plot
OD1402	22	2017	Stems	-	-
VDS	21	2017	Stems	Exp hybrids	-

Appendix 2. Correlation plots of parents and offspring cell wall quality traits