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Impact of drought stress on growth and quality of miscanthus for biofuel production

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Abstract

Miscanthus has a high potential as a biomass feedstock for biofuel production. Drought tolerance is an important breeding goal in miscanthus as water deficit is a common abiotic stress and crop irrigation is in most cases uneconomical. Drought may not only severely reduce biomass yields, but also affect biomass quality for biofuel production as cell wall remodeling is a common plant response to abiotic stresses. The quality and plant weight of 50 diverse miscanthus genotypes were evaluated under control and drought conditions (28 days no water) in a glasshouse experiment. Overall, drought treatment decreased plant weight by 45%. Drought tolerance – as defined by maintenance of plant weight – varied extensively among the tested miscanthus genotypes and ranged from 30% to 110%. Biomass composition was drastically altered due to drought stress, with large reductions in cell wall and cellulose content and a substantial increase in hemicellulosic polysaccharides. Stress had only a small effect on lignin content. Cell wall structural rigidity was also affected by drought conditions; substantially higher cellulose conversion rates were observed upon enzymatic saccharification of drought-treated samples with respect to controls. Both cell wall composition and the extent of cell wall plasticity under drought varied extensively among all genotypes, but only weak correlations were found with the level of drought tolerance, suggesting their independent genetic control. High drought tolerance and biomass quality can thus potentially be advanced simultaneously. The extensive genotypic variation found for most traits in the evaluated miscanthus germplasm provides ample scope for breeding of drought-tolerant varieties that are able to produce substantial yields of high-quality biomass under water deficit conditions. The higher degradability of drought-treated samples makes miscanthus an interesting crop for the production of second-generation biofuels in marginal soils.

Keywords: cell wall composition, cellulose, drought tolerance, hemicellulose, lignin, miscanthus, saccharification efficiency

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Introduction

Perennial biomass crops, such as miscanthus, are being developed for the production of biofuels to replace our fossil fuel-based energy supply chain with a renewable and more sustainable biomass-based alternative. Miscanthus is a leading candidate crop for biomass production owing to its rapid biomass accumulation and high nutrient and water-use efficiencies (Jones & Walsh, 2001; Heaton *et al*., 2010; Van Der Weijde *et al*., 2013). In addition, miscanthus biomass typically has a high quality for biofuel production as it is characterized by low moisture and high cell wall and carbohydrate contents, which are traits that contribute favorably to the yield of fermentable sugars to be used for the production of cellulosic ethanol (Wyman, 2007; Himmel & Picataggio, 2008; Zhao *et al*., 2012).

A consistent and predictable supply of high-quality lignocellulosic feedstocks is crucial to the success of cellulosic biorefineries (Perlack *et al*., 2005; Van Der Weijde *et al*., 2013). To achieve this, crops must be high yielding and have stable performance across diverse environments. Drought is one of the most widespread abiotic stresses (Chaves *et al*., 2003; Farooq *et al*., 2009), and the incidence of local and regional drought events is increasing worldwide due to climate change (Sheffield & Wood, 2008; Dai, 2013). In addition, miscanthus is seen as a crop with a high potential for production on

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marginal land, minimizing competition with food crops for arable land (Quinn *et al.*, 2015). Plants growing on marginal soils, such as eroded soils or bare lands, will regularly encounter periods with a water deficit. Unlike most food products, lignocellulosic feedstocks are considered low-value, high-volume commodities and crops such as miscanthus must be produced under low-input regimes. Under these provisions, irrigation is likely to be uneconomical and/or unsustainable for the production of miscanthus biomass (Bullard, 2001). In most miscanthus crop production scenarios, particularly those involving the production of biomass on marginal soils, periods of drought stress may regularly occur (Quinn *et al.*, 2015).

Attractive characteristics of miscanthus with regard to drought tolerance include (i) that its C4 photosynthesis system is characterized by a higher water-use efficiency compared to C3 photosynthetic plants and (ii) that its perennial growth habit and extensive root system enable better exploitation of soil water reserves present in deeper soil layers than annual plants (Heaton *et al.*, 2010; Byrt *et al.*, 2011; Van Der Weijde *et al.*, 2013). Moreover, the genus *Miscanthus* harbors extensive genetic diversity as it is adapted to a wide range of geographical conditions across East Asia (Clifton-Brown *et al.*, 2002, 2008). These features provide scope for the selection and breeding of stress-tolerant miscanthus varieties.

Aside from the adverse effects of drought on plant growth, drought influences virtually all plant physiological processes, including cell wall biosynthesis. These effects are important if miscanthus grown on marginal soils are to be used for biofuel production, as the composition and structural rigidity of the cell wall are key factors determining the techno-economic efficiency of biofuel production (Wyman, 2007; Himmel & Picataggio, 2008; Zhao *et al.*, 2012; Torres *et al.*, 2016). The contents of the two main cell wall polysaccharides, cellulose, and hemicellulose determine the maximum theoretically extractable content of fermentable sugars. The relative contents of the major cell wall components – particularly the content of lignin – as well as the extent of cross-linking between them are important parameters determining the efficiency of converting cell wall polysaccharides into fermentable sugars (Wyman, 2007; Himmel & Picataggio, 2008; Zhao *et al.*, 2012). One of the consequences of drought is a loss of cell turgor (Farooq *et al.*, 2009). A primary plant response to the loss of turgor is stiffening of cell walls to provide structural resistance and arrest cell extension (Moore *et al.*, 2008; Tenhaken, 2015). Longer exposure to drought stress challenges plants to modify their cell walls to sustain growth under conditions with reduced water potential (Moore *et al.*, 2008). Drought stress is thus likely to affect the biomass quality of the feedstock (Iraki *et al.*, 1989a; Moore *et al.*, 2008; Moura *et al.*, 2010; Pauly & Keegstra, 2010; Frei, 2013; Emerson *et al.*, 2014; Tenhaken, 2015).

Although the cell wall is clearly affected by drought stress, surprisingly little is known about drought-induced changes in cell wall composition (Tenhaken, 2015). Transcriptome studies often report cell wall-related genes to be differentially expressed upon drought stress, but actual biochemical changes in cell wall components are sparsely investigated. Studies that have investigated biochemical changes in cell wall composition consistently report a decrease in cellulose content upon drought stress (Frei, 2013). However, both increases and decreases in contents of lignin and hemicellulosic polysaccharides upon drought stress are reported in different crops and plant tissues (Guenni *et al.*, 2002; Vincent *et al.*, 2005; Al-Hakimi, 2006; Moore *et al.*, 2008; Hu *et al.*, 2009; Jiang *et al.*, 2012; Meibaum *et al.*, 2012; Emerson *et al.*, 2014; Rakszegi *et al.*, 2014). Therefore, it is yet largely unknown how water deficits affect biomass quality of bioenergy crops.

Increasing our understanding of drought-induced cell wall modifications and their impact on biomass quality is of major importance for developing miscanthus varieties for biomass production under low-input conditions and/or on marginal soils. In this study, plant growth and the compositional quality of stem and leaf material were analyzed in 50 diverse miscanthus genotypes, comprising *Miscanthus sinensis*, *Miscanthus sacchariflorus*, and interspecific hybrids, cultivated under drought and control growing conditions. To our knowledge, this is the first study to explore the magnitude of available variation in plant growth and biomass quality under drought stress in the germplasm pool of bioenergy feedstock miscanthus.

Materials and methods

Plant material

The experiment comprised 50 miscanthus genotypes including 35 *M. sinensis*, 8 *M. sacchariflorus*, and 7 *M. sinensis* × *M. sacchariflorus* species. All genotypes used in this study were supplied by Wageningen University and Aberystwyth University, in a collaboration that is part of the EU Seventh Framework Programme OPTIMISC (www.optimisc-project.eu). Like-sized tillers were split from clonal stock plants into eight separate parts and transferred to prelined 1-meter pipes filled with John Innes number-3 soil (Fig. 1a, b). Plants were allowed to grow with sufficient watering for 84 days prior to the start of treatment.

Drought experiment

The experiment was designed to evaluate genotypic responses to total water withdrawal. A total of 50 miscanthus genotypes

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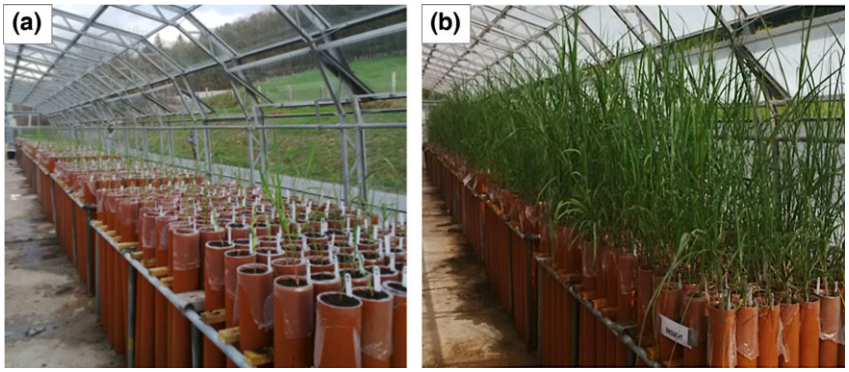

Fig. 1 Establishment of 50 miscanthus genotypes (n = 500) in 1-m pipes between March (a) and June 2014 (b) prior to screening.

were planted in a randomized split-plot block design with four blocks. Each block was randomly split in two segments, each containing the full set of genotypes, which received one of two treatments: well-watered vs. complete water withdrawal for 28 days, commencing June 2014. In total, four replicates per genotype per treatment were evaluated (n = 400). The experiment was conducted in a glasshouse at IBERS (52°43′N, 04°02′W).

After 28 days of treatment, all five replicate plants per genotype per treatment were harvested. Using secateurs, plant tillers were cut just above soil level. Stem (with panicle) and leaf material were separated and oven-dried to a constant dry weight (DW) at 60 °C for 72 h to determine stem, leaf, and plant weights in gram dry matter per plant, as well as the stem: leaf ratio (g g^{-1}). Plant weight as defined here refers to the aboveground biomass (stem + leaf) of the plants. Drought tolerance was calculated as the percentage of maintained biomass under water stress [average plant weight under drought stress (n = 4)/average plant weight under control treatment (n = 4) × 100%]. One genotype, OPM-17, yielded insufficient material for analysis and was excluded from the study. To obtain enough material for biochemical analyses, the samples were pooled for stem and leaf samples independently, by randomly combining two of the four replicate samples per genotype per treatment into one of two pools. All pooled leaf and stem samples [n = 400 (50 genotypes × 2 treatments × 2 pools × 2 tissue types)] were ground using a hammer mill with a 1-mm screen prior to biochemical analysis.

Biochemical analysis of the cell wall

Contents of neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin contents (ADL) of stem and leaf dry matter were determined according to protocols developed by ANKOM Technology that are essentially based on the work of Goering and Van Soest (Van Soest, 1967; Goering & Van Soest, 1970). Neutral and acid detergent extractions were performed using an ANKOM 2000 Fiber Analyzer (ANKOM Technology Corporation, Fairpoint, NY, USA). Acid detergent lignin was determined after 3-h hydrolysis of the ADF residue in 72% H_2SO_4 with continuous shaking. All analyses were performed in triplicate. The weight fractions of detergent fiber residues in dry matter were subsequently used to estimate the content of cell wall in dry matter (NDF% dm) and to obtain the contents of cellulose [(ADF% dm − ADL% dm)/NDF% dm × 100%], hemicellulosic polysaccharides [(NDF% dm − ADF% dm)/NDF% dm × 100%], and lignin (ADL% dm/NDF% dm × 100%) relative to the cell wall content.

Analysis of saccharification efficiency

Saccharification efficiency of the samples was assessed by the conversion of cellulose into glucose by mild alkaline pretreatment and enzymatic saccharification reactions. Reactions were carried out in triplicate using 500 mg subsamples per stem or leaf sample. All subsamples were incubated for 13 min with thermostable α-amylase (ANKOM Technology Corporation), followed by three-five-minute incubations with warm deionized water (60 °C) to remove interfering soluble sugars. The remaining biomass was then subjected to a mild alkaline pretreatment, carried out in 50-ml plastic centrifuge tubes with 15 ml 2% NaOH at 50 °C with constant shaking (160 RPM) for two hours in an incubator shaker (Innova 42; New Brunswick Scientific, Enfield, CT, USA). In this study, the objective of the pretreatment was not to maximize cellulose conversion but to treat samples to better allow discrimination of genotypic differences in cellulose conversion efficiency. Pretreated samples were washed twice with deionized water (5 min, 50 °C) and once with 0.1 M sodium citrate buffer (pH 4.6, 5 min, 50 °C).

Saccharification reactions were subsequently carried out according to the NREL Laboratory Analytical Procedure 'Enzymatic saccharification of lignocellulosic biomass' (Selig *et al.*, 2008). Pretreated samples were hydrolyzed for 48 h with 300 μl (25.80 mg of enzyme) of the commercial enzyme cocktail Accellerase 1500 (DuPont Industrial Biosciences, Leiden, the Netherlands) supplemented with 15 μl (0.12 mg of enzyme) endo-1,4-β-xylanase M1 (EC 3.2.1.8; Megazyme International Ireland, Bray, Ireland) in an incubator shaker (Innova 42; New Brunswick Scientific) set at 50 °C and constant shaking (160 RPM). This enzyme mixture has the following reported specific activities: endoglucanase 2200–2800 CMC U g^{-1}, beta-glucosidase 450–775 pNPG U g^{-1}, and endoxylanase 230 U mg^{-1}. Reactions were carried out in 44 ml 0.1 M sodium citrate buffer (pH

4.6), containing 0.4 ml 2% sodium azide to prevent microbial contamination.

Glucose contents in the enzymatic saccharification liquors were determined in duplicate using the enzyme-linked D-glucose assay kit (R-Biopharm, Darmstadt, Germany). This assay was adapted to a 96-well microplate format, and the increases in sample absorption following enzyme-mediated conversion reactions were spectrophotometrically determined at 340 nm using a Bio-Rad Microplate Reader (Bio-Rad, Richmond, CA, USA). All sample absorbance measurements were corrected using blanks, containing water instead of sample solution. Glucose release was determined by calculating the glucose content in the saccharification liquor from absorbance measurements using Eqn (1).

$$\text{Glucose release (mg)} = \frac{V \times \text{MW}}{\varepsilon \times d \times v \times 1000} \times \text{df} \times \Delta\text{Abs} \quad (1)$$

where V = final well volume (= 3.02 ml); MW = molecular weight of glucose (= 180.16 g mol^{-1}); ε = the molar extinction coefficient of NADPH (= 6.3 l × mol^{-1} × cm^{-1}); d = light path-length (= 1.016 cm); v = sample volume (ml); df = dilution factor (= 10); and ΔAbs = increase in sample absorbance, corrected for the increase in blank absorbance. Cellulose conversion was calculated from the release of glucose relative to the cellulose content in the sample, as detailed in Eqn (2).

$$\text{Cellulose conversion (\%)} = \frac{\text{Glucose release (mg)}}{\text{CC} \times 1.111} \times 100\% \quad (2)$$

where CC = cellulose content in the sample (in mg), and 1.111 = the mass conversion factor that converts cellulose to equivalent glucose (the molecular weight ratio of 180.16–162.16 g mol^{-1} for glucose and anhydro-glucose, Dien, 2010).

Analysis of miscanthus biomass using near-infrared spectroscopy (NIRS)

Multivariate prediction models based on near-infrared (NIR) spectral data were developed to allow high-throughput prediction of biomass quality traits. Near-infrared absorbance spectra of stem and leaf samples were obtained using a Foss DS2500 near-infrared spectrometer (Foss, Hillerød, Denmark). Averaged spectra were obtained consisting of eight consecutive scans from 400 to 2500 nm with an interval of 2 nm using ISI-Scan software (Foss). Obtained spectra were further processed by weighted multiplicative scatter correction and mathematical derivatization and smoothing treatments using WINISI 4.9 statistical software (Foss). These statistical transformations of spectra help to minimize effects resulting from light scatter and differences in particle size. Parameters for derivatization and smoothing were set at 2-6-4-1, in which the first number of this mathematical procedure refers to order of derivatization, the second number to the gap in the data points over which the derivation is applied, and the third number and fourth number refer to the number of data points used in the smoothing of the first and second derivative.

For the creation of prediction models, a calibration set of 110 samples was randomly selected from the complete set of samples, but with an approximate 1 : 1 representation of leaf and stem samples. The biochemical reference data and near-infrared spectra of the calibration samples were used for the development and validation of prediction models using WINISI version 4.9 (Foss). The prediction equations were generated using modified partial least-squares regression analyses (Shenk & Westerhaus, 1991), and obtained calibration statistics are reported in Table 1. Another 20 of the remaining samples were randomly selected as an external validation set to evaluate the quality of the generated prediction models. The prediction models were validated using the squared Pearson coefficient of correlation (r^2) between predicted and biochemical data of the external validation set (n = 20) and by evaluating for these samples the standard error of prediction (SEP) and its comparison to the standard error of laboratory (SEL) for each of the traits (Table 2). The prediction models were used to determine the cell wall, cellulose, hemicelluloses, and lignin contents, as well as the cellulose conversion rate of all leaf and stem samples.

Statistical analysis

General analyses of variance (ANOVA) were performed to determine the significance ($P < 0.05$) of genotype, treatment, and interaction sources of variation. For growth-related traits, ANOVAS were performed following the completely randomized split-plot block design of the experiment. The four original biological replicates per genotype per treatment were used as a fixed block effect with a nested split-plot on which treatment was applied. Variance analyses for biomass quality-related traits were performed considering that the four biological replicates were combined into two pools. For variance analyses, these two pools were considered as two independent replicates per genotype per treatment and used as a block effect. As these pools were not actual blocks in the original experimental

Table 1 Summary of calibration statistics of mPLS models used for the prediction of biomass quality traits

Trait	Samples	Mean chemical analysis	Mean NIRS prediction	r^2	SEC	SECV
Cell wall content (% dm)	104	67.38	67.14	0.99	0.56	1.25
Cellulose (% ndf)	106	45.82	45.75	0.96	0.77	1.15
Hemicellulose (% ndf)	107	47.28	47.33	0.96	0.86	1.40
Lignin (% ndf)	105	6.89	6.85	0.81	0.40	0.59
Cellulose conversion (%)	103	49.99	49.34	0.61	4.42	4.68

r^2, coefficient of determination; SEC, standard error of calibration; SECV, standard error of cross validation.

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Table 2 Summary of validation statistics of mPLS models used for the prediction of biomass quality traits

Trait	Samples	Slope	Intercept	r^2	SEP	SEL
Cell wall content (% dm)	19	0.78	0.13	0.86	2.36	0.51
Cellulose (% ndf)	19	0.92	0.59	0.82	1.53	0.39
Hemicellulose (% ndf)	19	0.93	−0.54	0.86	1.55	0.34
Lignin (% ndf)	19	1.01	−0.09	0.74	0.43	0.26
Cellulose conversion (%)	19	1.33	−1.63	0.73	4.62	2.99

r^2, coefficient of determination; SEP, standard error of prediction; SEL, standard error of laboratory.

design, they could not be used as a fixed block effect, but instead were used as a random block effect. The analyses were performed for stem and leaf samples separately following a mixed effect model (3):

$$Y_{ijk} = \mu + G_{ij} + T_k + GT_{ijk} + B_j + e_{ij} \quad (3)$$

where Y_{ijk} is the response variable, μ is the grand mean, G_{ij} is the genotype effect, T_k is the treatment effect, GT_{ijk} is the interaction term between genotype and treatment, B_j is the block effect, and e_{ijk} is the residual error.

Multiple comparisons analyses were performed to distinguish significant ($P < 0.05$) genotypic differences within each treatment using Fisher's protected least significant difference (LSD) test on genotype means. The significance of differences ($P = 0.05$) in trait means between two groups of genotypes that were formed based on tolerance level was evaluated using unpaired two-sample *t*-tests. Correlation analyses were performed on genotype means to identify the significance, strength, and direction of correlations among traits using Pearson's correlation coefficients. All statistical analyses were performed using the statistical software package GENSTAT, 16th edition (VSN International, Hemel Hempstead, UK).

Results

Drought stress affects plant weight and morphology

Growth, composition, and bioconversion efficiency of 50 miscanthus genotypes were evaluated using the leaf and stem tissues of plants grown under drought stress and control conditions. The drought treatment had a significant impact on almost all evaluated traits (Tables 3 and 4). The results showed that both final plant weight and the stem: leaf ratio were significantly affected by treatment. The set of genotypes showed significant differences in genotype performance with a low residual error (Table 3).

The mean and the range in genotype performance for plant weight and stem: leaf ratio in control and drought conditions are displayed in Table 5. Mean plant weight under control conditions was 20.10 g per plant ($n = 200$), whereas under drought stress, plant weight was on average 11.10 g per plant ($n = 200$). Drought

Table 3 Tables of analyses of variance for yield and stem-to-leaf ratio of 50 miscanthus genotypes grown under drought stress compared to control conditions

Trait	Source of variation	Degrees of freedom	Mean squares	*F*-prob.
Plant weight (g dm per plant)	*Wplot stratum*	3	115.33	
	Wplot.SplitPlot stratum			
	Treatment	1	8259.40	<0.001
	Residual	3	22.70	
	Wplot.SplitPlot.Unit stratum			
	Genotype	48	164.88	<0.001
	Genotype × treatment	48	42.48	<0.001
	Residual	257	17.92	
Stem : leaf ratio (g g^{-1})	*Wplot stratum*	4	0.91	
	Wplot.SplitPlot stratum			
	Treatment	1	1.53	0.135
	Residual	3	0.37	
	Wplot.SplitPlot.Unit stratum			
	Genotype	48	0.40	<0.001
	Genotype × treatment	48	0.06	0.393
	Residual	257	0.06	

Wplot, whole blocks in the experiment containing two split-plots to which treatment was applied; SplitPlot, split-plots in the experiment containing all genotypes.

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Table 4 Tables of analyses of variance for stem and leaf biomass quality traits of 50 miscanthus genotypes grown under drought stress compared to control conditions

		Stem			Leaf		
Trait	Source of variation	df	m.s.	*F*-prob.	df	m.s.	*F*-prob.
Cell wall content (% dm)	Treatment	1	3603.55	0.004	1	2608.88	<0.001
	Residual	2	14.94		2	2.58	
	Genotype	48	82.06	<0.001	48	30.57	<0.001
	Treatment × genotype	48	10.43	<0.001	48	4.42	0.036
	Residual	94	2.90		95	2.87	
Cellulose (% ndf)	Treatment	1	1154.58	0.009	1	39.82	0.033
	Residual	2	10.10		2	1.40	
	Genotype	48	9.83	<0.001	48	4.24	<0.001
	Treatment × genotype	48	3.24	0.020	48	1.81	0.009
	Residual	94	1.98		95	1.03	
Hemicellulose (% ndf)	Treatment	1	1239.44	0.009	1	81.03	0.008
	Residual	2	11.19		2	0.64	
	Genotype	48	12.85	<0.001	48	6.35	<0.001
	Treatment × genotype	48	4.17	0.018	48	2.03	0.068
	Residual	94	2.52		95	1.42	
Lignin (% ndf)	Treatment	1	8.96	0.015	1	0.01	0.522
	Residual	2	0.14		2	0.02	
	Genotype	48	1.67	<0.001	48	0.61	<0.001
	Treatment × genotype	48	0.35	0.027	48	0.22	0.002
	Residual	94	0.22		95	0.11	
Cellulose conversion (%)	Treatment	1	3486.63	0.003	1	689.99	0.001
	Residual	2	9.53		2	1.04	
	Genotype	48	54.22	<0.001	48	7.03	<0.001
	Treatment × genotype	48	7.85	<0.001	48	1.60	0.020
	Residual	94	2.66		95	0.98	

df, degrees of freedom; m.s., mean squares.

treatment in this experiment thus reduced plant weight on average by 45%. Moreover, drought-treated plants, with on average a stem : leaf ratio of 0.77, were generally more leafy than the corresponding control plants, which had on average a stem : leaf ratio of 0.91. Variation in plant weight and stem : leaf ratio among genotypes was extremely large under both stress and control conditions. Final mean plant weight of genotypes under control conditions ranged from 5.80 to 35.63 g, while the range under drought stress was from 2.78 to 20.38 g per plant (Fig. 2). Under both drought and control conditions, leaf biomass contributed on average more to total plant weight than stem biomass (Table 5), but for some genotypes, stems comprised the largest weight fraction of total plant weight.

Genotypes responded very differently to the drought treatment, as shown by the variation in plant weight and drought tolerance (Fig. 2) and by the significance of the genotype-by-treatment interaction term (Table 3). For example, two genotypes, OPM-6 and OPM-19, are both high-yielding genotypes, but differed considerably in drought tolerance. OPM-6 was the genotype with the highest plant weight under drought stress (20.38 g per plant), which was even higher than the average plant weight (20.10 g per plant) over all genotypes under control conditions. This particular genotype had a plant weight of 26.98 g per plant under control conditions, leading to a drought tolerance of 75.53% (only a 25% reduction in plant weight due to the drought treatment). OPM-19 was the genotype with the highest plant weight under control conditions (35.63 g per plant), but was more severely affected by drought stress. Its plant weight under stress conditions was 17.75 g per plant, leading to a drought tolerance of 49.82% (a 50% reduction in plant weight due to the drought treatment). Variation in drought tolerance among all genotypes ranged from 29.60% to 109.90%. The two genotypes with a tolerance value above 100% had a higher plant weight under drought conditions than under control conditions, although the difference in mean plant weight was smaller than the variation between replicates and both genotypes were low biomass types. On the other side of the tolerance spectrum, genotypes displayed large reductions (up to

Table 5 Genotypic variation in plant weight, stem: leaf ratio, and quality traits across 50 miscanthus genotypes under control and drought conditions

	Trait	Unit	Treatment	Average	Min	Max	Range	CV (%)	LSD
Plant growth	Plant weight	g dm per plant	Control	20.10	5.80	35.63	29.83	25.12	3.19
			Drought	11.10	2.78	20.38	17.60	30.51	4.24
	Stem: leaf ratio	g g^{-1}	Control	0.91	0.55	1.49	0.94	20.35	0.12
			Drought	0.77	0.36	1.39	1.03	36.45	0.18
	Drought tolerance	%	–	57.75	29.60	109.90	80.32	–	–
Stem composition	Cell wall content	% dm	Control	73.06	62.23	78.71	16.49	1.99	2.92
			Drought	64.57	51.28	73.66	22.38	3.00	3.90
	Cellulose	% ndf	Control	51.06	47.57	53.78	6.22	2.33	2.39
			Drought	46.25	39.33	49.49	10.16	3.47	3.23
	Hemicellulose	% ndf	Control	41.57	37.97	45.33	7.36	3.18	2.66
			Drought	46.56	42.60	52.55	9.95	3.92	3.68
	Lignin	% ndf	Control	7.38	6.26	9.50	3.24	5.94	0.88
			Drought	6.93	5.67	8.67	3.00	7.15	1.00
	Cellulose conversion	%	Control	42.22	37.69	50.75	13.06	2.89	2.45
			Drought	50.57	43.45	62.18	18.73	3.91	3.98
Leaf composition	Cell wall content	% dm	Control	70.25	64.74	75.72	10.98	1.87	2.64
			Drought	62.92	57.10	70.46	13.36	3.18	4.03
	Cellulose	% ndf	Control	43.37	40.29	45.89	5.60	2.22	1.93
			Drought	42.50	40.48	44.58	4.11	2.51	2.14
	Hemicellulose	% ndf	Control	49.93	46.63	54.14	7.51	2.21	2.22
			Drought	51.18	48.64	54.06	5.42	2.50	2.57
	Lignin	% ndf	Control	6.35	5.40	7.12	1.72	5.65	0.72
			Drought	6.37	5.64	7.35	1.71	4.65	0.60
	Cellulose conversion	%	Control	50.37	46.16	52.65	6.50	1.78	1.80
			Drought	54.13	51.36	57.24	5.87	1.99	2.17

CV (%) = coefficient of variation (root-mean-squared error/average × 100%); LSD = least significant difference (0.05).

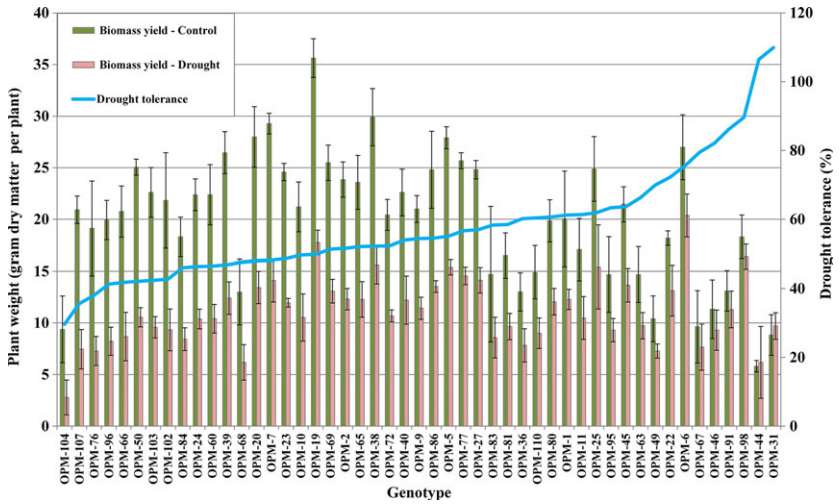

Fig. 2 Plant yield of 49 miscanthus genotypes (expressed in gram dry matter per plant) with varying drought tolerance grown under drought and control conditions. Error bars indicate the standard error of a genotype mean (average of four replicates per genotype per treatment).

70%) in plant weight due to drought treatment. Yields in 39 of the 49 genotypes were reduced by 30–60% following drought treatment, with the majority of the genotypes with a high plant weight under control conditions showing yield reductions of at least 40% under drought (Fig. 2).

Drought stress affects biomass quality in miscanthus

Drought treatment significantly affected most of the biomass quality traits of miscanthus, including cell wall content, cellulosic, and hemicellulosic polysaccharide contents and the efficiency of cellulose conversion (Tables 4 and 5). Stem lignin content, on the other hand, was only moderately affected ($P = 0.015$), and drought stress had no significant effect on lignin in leaf tissues ($P = 0.522$). Significant differences among the set of genotypes were found for all biomass quality characteristics. Furthermore, the effects of drought on biomass quality were more apparent in some genotypes than in others, as indicated by the presence of significant genotype-by-treatment interactions for most traits (Table 4).

Overall, the biochemical composition of the stem samples of drought-treated plants compared to their respective control plants was considerably changed (Table 5). Average cell wall content decreased from 73% to 65% of stem dry matter and from 70% to 63% in leaf dry matter. Average cellulose content decreased in stem tissue, from 51% to 46%, but in leaf tissue remained 43%. In contrast, average content of hemicelluloses increased, from 42% to 47% in stem and from 50% to 51% in leaf tissue. Lignin content was not substantially different between drought-treated and control plants (Table 4), remaining at 6% in leaf and 7% in stem tissue (Table 5).

Genotypic variation for cell wall composition and cellulose conversion was extensive. Generally, genotypic variation in cell wall composition was larger in drought-treated plants compared to control plants and compositional variation between genotypes larger for stem than for leaf tissue. In drought-treated plants, mean cell wall content ranged from 51% to 74% of stem dry matter and 57–70% of leaf dry matter among genotypes (Table 5). Similarly, cellulose content ranged from 39% to 49% in stem and 40% to 45% in leaf, the content of hemicellulosic polysaccharides ranged from 43% to 53% in stem and 49% to 54% in leaf, and lignin content ranged from 5.7% to 8.7% in stem and 5.6% to 7.4% in leaf materials.

Saccharification efficiency was significantly affected by drought treatment. In both stem and leaf materials, considerably higher cellulose conversion efficiencies were achieved in drought-treated plants compared to their respective control plants. Stem cellulose conversion increased from 42% (under control conditions) to 51% (under drought treatment) (Table 5). Similarly, leaf cellulose conversion increased from 50% to 54%. Extensive variation among genotypes was found for cellulose conversion efficiency in both drought-treated and control plants. Stem cellulose conversion ranged from 43% to 62% under drought and from 38% to 51% under control conditions (Table 5, Fig. 3). Less variation was observed in leaf cellulose conversion, but significant genotypic differences were detected (Tables 4 and 5).

Cell wall composition does not play a major role in drought tolerance

To evaluate whether differences existed in response to drought between tolerant genotypes and susceptible genotypes, the top six drought-tolerant (OPM-31, 44, 46, 67, 91, and 98) and top six drought-susceptible (OPM-50, 66, 76, 96, 104, and 107) genotypes were grouped together to compare changes in plant weight and

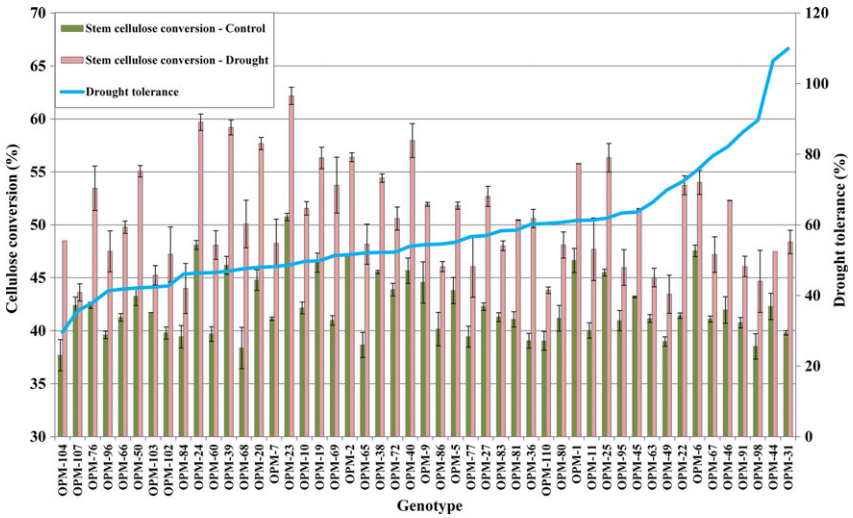

Fig. 3 Cellulose conversion of stem tissues of 49 miscanthus genotypes with varying drought tolerance grown under drought and control conditions. Error bars indicate the standard error of a genotype mean (average of two replicates per genotype per treatment).

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biomass quality between the two groups (Fig. 4a, b). Difference in trait means between drought-treated plants and their corresponding control plants is presented and further referred to as the trait name preceded by a 'Δ' symbol. These differences are a measure of the plasticity in cell wall components, with a larger difference in cell wall composition between drought-treated and control plants indicative of greater plasticity. In the tolerant group, hardly any reduction in leaf and stem biomass was observed, whereas the weight of both plant fractions was highly reduced in the susceptible group. The differences in cell wall content and biomass quality between the two groups contrasting for drought tolerance were small. The only significant difference between the two groups was a significantly larger increase in leaf Δlignin (0.71 vs. 0.02) in the tolerant group compared to the susceptible group (Fig. 4a). Between these two extreme groups, cell wall plasticity was found to be highly similar (Fig. 4a, b).

To further investigate interrelations between drought tolerance and cell wall characters, a correlation analysis was performed on the whole set of genotype means of all traits. The primary objective was to investigate whether cell wall composition and cell wall plasticity play a role in tolerance to drought. A few significant trait associations (with low coefficients of determination) were observed between drought tolerance and biomass quality traits, including correlations with leaf cellulose and hemicelluloses content (Fig. 5a, $r^2 = 0.13$ and −0.11, respectively) and leaf Δcellulose, leaf Δlignin, and stem Δlignin (Fig. 5b, $r^2 = -0.08$, 0.21 and 0.10, respectively). No significant correlations were found between drought tolerance and cellulose conversion. The increase in cellulose conversion in stems of drought-treated plants was highly correlated to Δhemicellulose (Fig. 5c) and to cell wall content (Fig. 5d).

Discussion

Variation in drought tolerance in miscanthus

The extensive variation observed among the evaluated genotypes regarding plant weight under drought stress (2.78–20.38 g plant^{-1}) indicates large genotype differences in vegetative growth vigor under dry cultivation conditions. The average loss in plant weight under drought stress compared to control conditions was considerable (45%); however, the range of variation in drought tolerance among the evaluated genotypes (30–110%) was comprehensive and is evidence of the suitability of this test panel for the experiments that were conducted. This indicates that the genotypes tested may interesting candidates for investigation of mechanisms underlying drought tolerance and could possibly be used in breeding programs.

Some plant defense strategies against the injurious effects of drought, such as dehydration avoidance, are rarely compatible with high biomass yields (Blum, 2005). Drought tolerance and plant yield of the genotypes included in this study were evaluated (Fig. 2). Plants that achieved higher plant weights in drought conditions than in control conditions (drought tolerance >100%) were quite small and had low plant weights in both control and drought conditions. The applied drought treatment was potentially less harsh for small

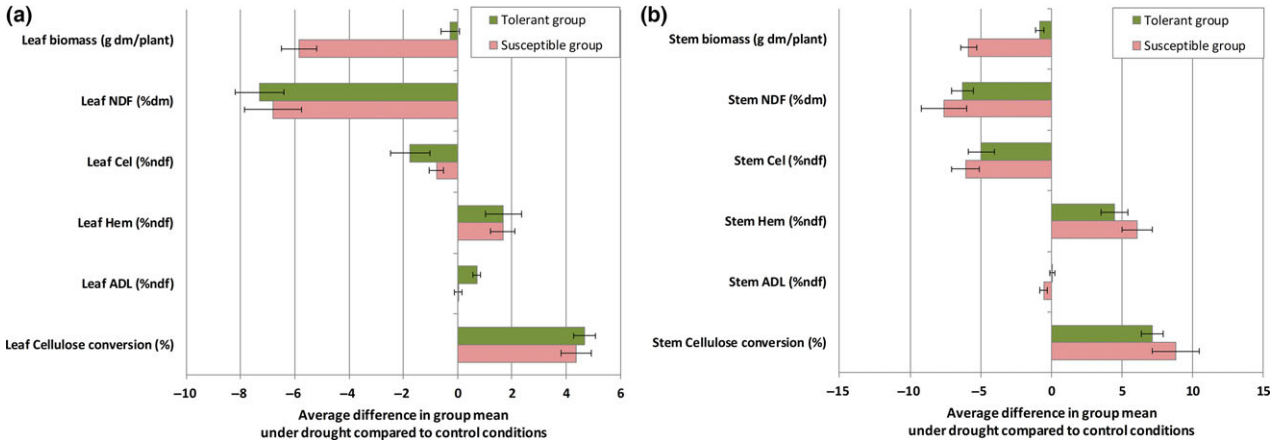

Fig. 4 Change in leaf (a) and stem (b) weight per plant and composition of drought-stressed plants relative to the control plants grouped by tolerance/susceptibility to drought. Unit on *x*-axis is determined by the unit of the trait on the *y*-axis. Error bars indicate standard errors on group means ($n = 6$ for tolerant and $n = 6$ for susceptible group). The significance of differences in group means per trait was evaluated by unpaired two-sample *t*-tests. Group means per trait that have a different suffix are significantly different ($P < 0.05$).

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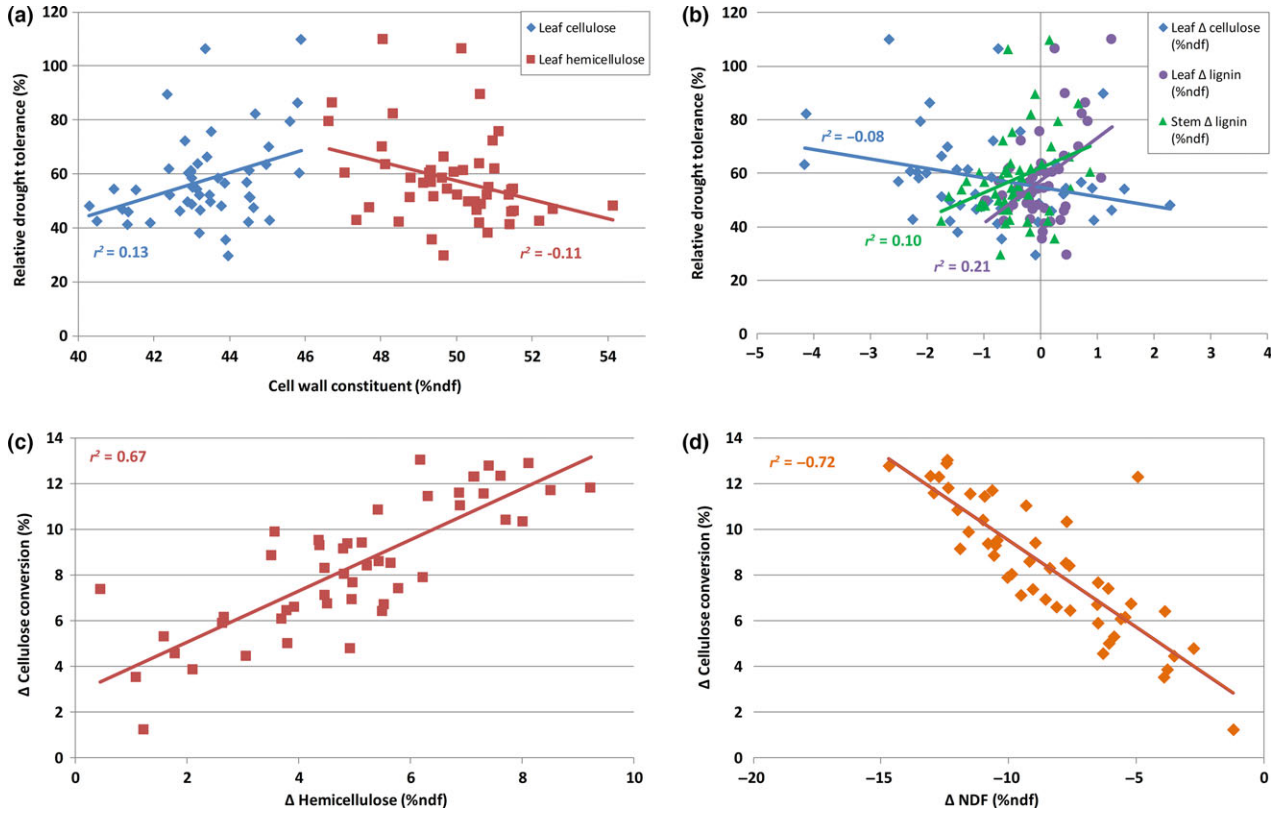

Fig. 5 Correlations between drought tolerance and cell wall composition (a), between drought tolerance and leaf Δcellulose, leaf Δlignin and stem Δlignin (b), between stem Δcellulose conversion and stem Δhemicellulose (c), and between stem Δcellulose conversion and stem ΔNDF (d), where Δ = genotype difference between drought and control conditions.

plants than for large plants; small plants need less water and are less likely to lose water due to a proportionally small leaf surface area (Blum, 2005). However, some genotypes exhibited both a relatively high plant weight and a relatively high drought tolerance, indicating that some genotypes utilize drought tolerance strategies that to some extent could be compatible with high yield. For example, one of the more drought-tolerant (75%) genotypes, OPM-6, in this experiment achieved a plant weight in drought conditions that was similar to the plant weight of *M.* × *giganteus* (OPM-9) in control conditions (Fig. 2). Extrapolations of the reported plant weights to estimate yield potential under field conditions should be approached with care. The current experiment is more suitable for investigating early vegetative growth than yield potential. Moreover, genotypes that had a relatively low plant weight in this experiment, might still achieve substantial yields under field conditions, perhaps by optimizing planting density. The findings reported here suggest the availability of drought-tolerant varieties in miscanthus germplasm resources that may achieve substantial biomass yields, even under dry cultivation conditions.

Drought reduces cell wall and cellulose content, while increasing hemicellulosic polysaccharides

A key objective of this study was to determine the effects of drought stress on biomass composition and conversion properties, to evaluate whether growing miscanthus under water deficit conditions affects its biomass quality for biofuel production. Biomass composition was substantially affected by drought, with significant reductions in cell wall and cellulose content and a significant increase in hemicellulosic polysaccharides observed in plants grown under drought compared to control conditions (Table 5, Fig. 4a, b). One of the most striking effects of drought was a large decrease in average cell wall content (11.62% in stem and 10.43% in leaf tissue). A drastic reduction in cell wall content was also reported in field-grown *M.* × *giganteus*, after evaluation of its biomass composition in a year with low precipitation as compared to a year with average precipitation (Emerson *et al.*, 2014).

It was previously shown that cultured tobacco cells subjected to osmotic stress were reduced in size and had thinner cell walls compared to untreated cells (Iraki

et al., 1989b). Under normal conditions, cells expand using turgor pressure and cell walls thicken when they no longer need to be elastic to accommodate cell expansion (Lam *et al*., 2013; Da Costa *et al*., 2014). During drought, plants have to act to maintain turgor, leading to a stop or slowing down of cell division and cell expansion, which reduces average cell size (Farooq *et al*., 2009). However, if water would become available again, cell walls need to be able to accommodate cell expansion. Therefore, it is unlikely that the small-sized cells of drought-stressed plants will undergo extensive premature cell wall thickening. Such physiological and developmental processes could explain the lower cell wall content found in drought-stressed plants compared to the control plants.

A physiological explanation for the reduction in cellulose during drought stress may be found in the formation of osmolytes (such as soluble sugars and proline). Osmolytes are solutes formed to aid the maintenance of osmotic equilibrium in the cell under dry growing conditions, and plant stress due to water deficit is associated with a disturbance of the osmotic equilibrium of cells. The production of osmolytes at the expense of cellulose biosynthesis (or financed by cellulose deconstruction) is well reported in the literature (Guenni *et al*., 2002; Piro *et al*., 2003; Vincent *et al*., 2005; Al-Hakimi, 2006; Moore *et al*., 2008; Hu *et al*., 2009; Jiang *et al*., 2012; Meibaum *et al*., 2012; Emerson *et al*., 2014; Rakszegi *et al*., 2014). The fact that in stem tissue, the reduction in cellulose is much more apparent than in leaf tissue may indicate that in most plants, the production of osmolytes in stems is more associated with a concomitant decrease in cell wall cellulose than in leaves.

Lignin content in leaves of drought-treated plants was not significantly different from that of control plants and in stem tissue only a slight decrease in lignin content was observed. Previously a large reduction in lignin content was reported to be one of the side-effects of drought on biomass composition of field-grown *M.* × *giganteus* (Emerson *et al*., 2014). However, there is no consistency among studies in different crops and tissues regarding the effect of drought on lignin content, with some studies reporting an increase in lignin (Guenni *et al*., 2002; Hu *et al*., 2009; Jiang *et al*., 2012; Meibaum *et al*., 2012) and some reporting a decrease in lignin content (Vincent *et al*., 2005; Al-Hakimi, 2006). The associations between drought stress and lignin are complex and perhaps influenced by yet uncharacterized factors that may explain discrepancies between studies. The small effect of drought on cell wall lignin content and the large effect on cell wall and cellulose content reported here were consistently observed for a diverse set of genotypes comprising three miscanthus species.

Similarly, inconsistent effects of drought on hemicellulose content were previously reported (Al-Hakimi, 2006; Moore *et al*., 2008; Jiang *et al*., 2012; Emerson *et al*., 2014; Rakszegi *et al*., 2014), whereas in this study, drought-treated plants of all genotypes consistently had a significantly higher content of hemicellulosic polysaccharides compared to their respective control plants. Some of the discrepancies may also be explained by a difference in the duration of the applied drought treatment. A long-term exposure to drought, such as the treatment applied in this study, challenges plants to alter their cell wall structure to sustain cell expansion with reduced water potential. Hemicelluloses contribute to cell wall rigidity by reinforcing the cell wall matrix through cross-linking to lignin and to cellulose fibers (Le Gall *et al*., 2015). Lignin also provides cell wall rigidity, but is mostly deposited in mature cells that no longer require the flexibility to accommodate cell expansion (Lam *et al*., 2013; Da Costa *et al*., 2014). Compared to lignin, hemicellulose cross-links are more easily broken to ensure cell wall plasticity. An increase in the relative proportion of hemicelluloses might enable cell walls of drought-treated plants to uphold their structural rigidity without compromising plasticity (Le Gall *et al*., 2015; Tenhaken, 2015).

In this experiment, the effects of drought were evaluated in a controlled glasshouse environment, in which environmental factors other than those related to the drought treatment were reduced to a minimum. Compared to the often contradictory results reported in previous studies regarding the effects of drought on biomass quality, in this study the observed effects were highly consistent for a diverse set of genotypes.

Drought improves saccharification efficiency in miscanthus

In addition to cell wall composition, drought treatment was shown to significantly affect cell wall degradability. Cellulose conversion was substantially increased in biomass samples of drought-stressed plants compared to those of control plants, indicating that available cell wall polysaccharides were more easily released as fermentable sugars by pretreatment and enzymatic saccharification reactions (Fig. 3; Table 5). According to these results, the occurrence of drought during growth of bioenergy feedstocks can have highly beneficial side-effects on the processing efficiency of the biomass for the production of biofuel.

The observed increase in cellulose conversion in drought-treated plants was shown to be highly correlated to an increase in the relative proportion of hemicelluloses (Fig. 5c). It has been reported that the content of hemicelluloses is positively correlated to saccharification

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efficiency (Xu *et al.*, 2012; Torres *et al.*, 2014; Van Der Weijde *et al.*, 2016). The positive effect of hemicelluloses on cell wall digestibility was associated with a reduction in cellulose crystallinity (Xu *et al.*, 2012). Hemicelluloses, unlike cellulose, are highly branched polysaccharides that form an amorphous network through different types of cross-links (Hatfield *et al.*, 1999; Doblin *et al.*, 2010). Hydrolytic enzymes can more efficiently penetrate the cell wall matrix during enzymatic saccharification and have a higher affinity to the cellulose substrate when the ratio of hemicellulose to cellulose in the cell wall matrix is increased (Xu *et al.*, 2012). This explains how a relative increase in cell wall hemicelluloses in response to drought treatment resulted in a reduction in cell wall recalcitrance to deconstruction.

Saccharification efficiency is often negatively correlated to cell wall content (Jung & Casler, 2006; Torres *et al.*, 2014; Van Der Weijde *et al.*, 2016). The reduction in cell wall content observed in drought-treated plants may be another side-effect of drought that contributes positively to saccharification efficiency. As was reported previously, drought treatment of cultured tobacco cells reduced cell wall thickness (Iraki *et al.*, 1989b). Similarly, the reduction in cell wall content observed in drought-treated miscanthus plants could be due to thinner cell walls, as discussed above. Thinner cell walls, in turn, might be more easily penetrated by hydrolytic enzymes due to increased accessible surface area compared to thicker cell walls. This could provide an explanation for the negative correlation found between ΔNDF and Δcellulose conversion (Fig. 5d). However, microscopic investigations of differences in cell wall thickness were beyond the scope of this study.

Overall, growing miscanthus under drought conditions substantially affected biomass composition and saccharification efficiency, with cell walls of plants grown under drought conditions being more readily deconstructed during mild alkaline pretreatment and enzymatic saccharification. Hereby, the occurrence of drought during the growth of miscanthus biomass may contribute beneficially to its compositional quality for biofuel production, through the enhanced efficiency of releasing cell wall polysaccharides as fermentable sugars during processing. Importantly, this effect appears to occur even in genotypes that maintained high biomass yield despite drought.

Implications for breeding drought-tolerant varieties for biofuel production

These results show that genotypic variation for drought tolerance exists within miscanthus germplasm resources and that the development of drought-tolerant varieties that produce substantial biomass yields should be possible. Drought stress significantly reduced cell wall and cellulose content, which reduces the amount of structural sugars available per unit of biomass. This effect was previously reported to have a negative impact on theoretical ethanol yields of *M.* × *giganteus* grown during a year with limited compared to a year with average precipitation (Emerson *et al.*, 2014). However, in the current study, it was shown that drought also substantially increased cellulose conversion, which considerably enhances the techno-economic performance of bioconversion processes (Wyman, 2007; Himmel & Picataggio, 2008; Zhao *et al.*, 2012; Torres *et al.*, 2016). The occurrence of drought during the growth of miscanthus may thus beneficially affect biomass quality, through the substantial increase in cellulose conversion efficiency. The question that remains is whether in terms of the total ethanol yield per hectare, the reductions in cellulose and biomass yield associated with drought are compensated for by an increase in conversion efficiency. However, the selection of drought-tolerant high-yielding genotypes should minimize any penalty.

The absence of strong correlations among drought tolerance and compositional characters in the set of genotypes and the observation that in the tolerant group similar differences in biomass composition were observed as in the susceptible group are strong indicators that biomass quality characteristics and drought tolerance are largely under independent genetic control. Hence, drought tolerance and biomass quality are not mutually exclusive breeding goals and biomass quality can be selected for independently and simultaneously, without adversely affecting drought tolerance and vice versa. The wide range of variation for the evaluated traits observed among this set of miscanthus genotypes provides evidence of ample scope in the miscanthus germplasm pool for breeders to improve both drought tolerance and biomass composition to supply optimized varieties for the biofuel industry.

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