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Physiological basis of chilling tolerance and early-season growth in miscanthus

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• **Background and Aims** The high productivity of *Miscanthus* × *giganteus* has been at least partly ascribed to its high chilling tolerance compared with related C_4 crops, allowing for a longer productive growing season in temperate climates. However, the chilling tolerance of *M.* × *giganteus* has been predominantly studied under controlled environmental conditions. The understanding of the underlying mechanisms contributing to chilling tolerance in the field and their variation in different miscanthus genotypes is largely unexplored.
• **Methods** Five miscanthus genotypes with different sensitivities to chilling were grown in the field and scored for a comprehensive set of physiological traits throughout the spring season. Chlorophyll fluorescence was measured as an indication of photosynthesis, and leaf samples were analysed for biochemical traits related to photosynthetic activity (chlorophyll content and pyruvate, Pi dikinase activity), redox homeostasis (malondialdehyde, glutathione and ascorbate contents, and catalase activity) and water-soluble carbohydrate content.
• **Key Results** Chilling-tolerant genotypes were characterized by higher levels of malondialdehyde, raffinose and sucrose, and higher catalase activity, while the chilling-sensitive genotypes were characterized by higher concentrations of glucose and fructose, and higher pyruvate, Pi dikinase activity later in the growing season. On the early sampling dates, the biochemical responses of *M.* × *giganteus* were similar to those of the chilling-tolerant genotypes, but later in the season they became more similar to those of the chilling-sensitive genotypes.
• **Conclusions** The overall physiological response of chilling-tolerant genotypes was distinguishable from that of chilling-sensitive genotypes, while *M.* × *giganteus* was intermediate between the two. There appears to be a trade-off between high and efficient photosynthesis and chilling stress tolerance. *Miscanthus* × *giganteus* is able to overcome this trade-off and, while it is more similar to the chilling-sensitive genotypes in early spring, its photosynthetic capacity is similar to that of the chilling-tolerant genotypes later on.

Key words: PPDK, water-soluble carbohydrates, antioxidants, chlorophyll fluorescence, *M. sinensis*, *M.* × *giganteus*, *M. sinensis* × *sacchariflorus*, chilling stress, oxidative stress, miscanthus, early season growth

INTRODUCTION

Miscanthus is a genus of perennial C_4 grasses increasingly used for the production of lignocellulosic biomass in temperate regions of the world (van der Weijde *et al.*, 2013). Improvement of early canopy development is a breeding goal in miscanthus, as this allows the plants to take advantage of long daylengths in spring, potentially leading to the production of more biomass during the entire growing season (Dohleman and Long, 2009; Robson *et al.*, 2013; Clifton-Brown *et al.*, 2015). However, in temperate regions of Europe, earlier canopy development requires the capacity to photosynthesize and grow at low temperature due to risk of chilling stress in early spring (Sage *et al.*, 2015; Fonteyne *et al.*, 2016*c*). The relevance of chilling tolerance was shown, for example, in a field trial conducted in Denmark which showed a significant correlation between growth rate in the early growing season and high photosynthesis at low temperature in a miscanthus germplasm collection of 14 genotypes from four species (Jiao *et al.*, 2016).

Miscanthus × *giganteus*, the most commonly planted miscanthus type, has been reported to be more chilling tolerant than other phylogenetically related C_4 species such as maize (*Zea mays*), sorghum (*Sorghum bicolor*) or sugarcane (*Saccharum officinarum*) (Long and Spence, 2013; Sage *et al.*, 2015). Compared with these crops, *M.* × *giganteus* is capable of higher photosynthetic activity at lower temperatures (Naidu *et al.*, 2003; Głowacka *et al.*, 2014). In contrast to maize, which can be severely damaged by chilling stress (Kaiser and Sacks, 2015; Sobkowiak *et al.*, 2016), most miscanthus genotypes investigated in field trials do not show irreversible damage at low, above-zero temperatures (Long and Spence, 2013; Friesen *et al.*, 2014; Kaiser and Sacks, 2015; Fonteyne *et al.*, 2016*b*). Chilling stress does decrease photosynthetic efficiency and causes a temporal growth reduction in miscanthus (Clifton-Brown and Jones, 1997; Głowacka *et al.*, 2014; Jiao *et al.*, 2016).

Furthermore, considerable genotypic variation in photosynthetic capacity and growth rate at low temperature has been

reported in miscanthus germplasm (Clifton-Brown and Jones, 1997; Purdy *et al.*, 2013; Friesen *et al.*, 2014; Głowacka *et al.*, 2014, 2015; Fonteyne *et al.*, 2016*a*), and genotypes that perform even better than *M.* × *giganteus* have been identified (Głowacka *et al.*, 2014, 2015) which offers prospects for breeding. There is, however, little known about the biochemical processes underlying these adaptations, as available studies on biochemical aspects have mostly involved only one or a few genotypes, and have focused on only a limited set of parameters. For example, in *M.* × *giganteus* exposed to chilling stress, the transcript abundance and content of key photosynthetic enzymes such as RuBisCo (ribulose-1,5-bisphosphate carboxylase/oxygenase) and PPDK (pyruvate, Pi dikinase) has been shown to increase (Naidu *et al.*, 2003; Wang, *et al.*, 2008*b*; Spence *et al.*, 2014). The increased concentration of these enzymes probably counters their reduced enzymatic kinetics at lower temperature and prevents a reduction of photosynthetic activity. Similarly, Friesen and Sage (2015) observed a reduction in RuBisCo and PPDK activity in a chilling-sensitive hybrid miscanthus variety but not in the more chilling-tolerant *M.* × *giganteus* when exposed to chilling temperatures. This agrees with the observation of a lower decrease in chlorophyll content and photosynthesis activity in *M.* × *giganteus* than in the more chilling-sensitive *M. sinensis* 'Goliath' at low temperatures (Fonteyne *et al.*, 2016*a*).

Under field conditions, low temperatures combined with high light intensities induce photobleaching in chilling-sensitive miscanthus genotypes, indicating oxidative stress (Fonteyne *et al.*, 2016*b*). In the few miscanthus genotypes in which this has been investigated, the ratio of the quantum efficiency of photosystem II (Φ_{PSII}) to the quantum efficiency of CO_2 fixation (Φ_{CO2}) seems to remain constant until temperatures drop below 12 °C (Naidu and Long, 2004; Friesen and Sage, 2015) but at 10 °C this ratio increases, indicating the channelling of electrons to alternative electron sinks such as the Mehler reaction (Farage *et al.*, 2006), which can lead to increased oxidative stress. Differences in chilling sensitivity among miscanthus genotypes could thus be a result of differences in the capacity to cope with oxidative stress, as is the case in maize, where tolerant genotypes display a larger increase in reactive oxygen species- (ROS) scavenging enzymes and molecular antioxidants when exposed to chilling temperatures (Leipner *et al.*, 1999; Aroca *et al.*, 2001). As far as we know, no data are available on the oxidative stress response of miscanthus genotypes that differ in chilling sensitivity.

Water-soluble carbohydrates (WSCs) have also received some attention in miscanthus, as they not only are indicative of photosynthesis and growth, but also provide protection against damage by chilling stress and serve as stress signalling molecules (Janská *et al.*, 2010; Purdy *et al.*, 2013; Tarkowski and Van den Ende, 2015). In a comparison of four miscanthus genotypes, Purdy *et al.* (2013) reported differences in the increase in glucose, fructose and sucrose content in leaves after a chilling shock, with the most chilling-tolerant genotype (*M.* × *giganteus*) showing the highest total carbohydrate content under chilling conditions.

Although the findings summarized above are certainly relevant, current knowledge of chilling response mechanisms in miscanthus is still rather fragmentary, as different genotypes and growth conditions have been used to investigate different aspects. This prevents generalization and the characterization of global physiological responses of miscanthus genotypes to low temperature, which possibly involve separate mechanisms simultaneously and in interaction. In addition, all studies on chilling tolerance in miscanthus thus far have been carried out in growth chambers, and frequently with plants exposed to sudden chilling shock (Naidu and Long, 2004; Farage *et al.*, 2006; Wang, *et al.*, 2008*a*; Purdy *et al.*, 2013; Głowacka *et al.*, 2014). However, leaves developed under chilling stress are metabolically different from leaves developed at warmer conditions and then exposed to chilling temperatures (Gray and Heath, 2005). Furthermore, conditions in the field are more variable, not only with day–night temperature and light changes as simulated in growth chambers, but also with fluctuations throughout the day and the night, and over the entire growth period. Investigation of the response to miscanthus to low temperatures in the field is therefore a required complement to insights gained in the growth chamber experiments summarized above.

In this study, photosynthesis and several biochemical traits putatively related to chilling tolerance were investigated under field conditions in a diverse set of five miscanthus genotypes, including *M.* × *giganteus*. The main objectives were (1) to characterize the response of these five genotypes to changes in temperature throughout the early growing season and (2) to identify traits and responses that distinguish genotypes classified as chilling tolerant and chilling sensitive. Traits indicative of photosynthesis, redox homeostasis and carbohydrate metabolism responses were investigated. The overall physiological response of chilling-tolerant genotypes was clearly distinct from that of chilling-sensitive genotypes. Whereas chilling-tolerant genotypes accumulated protective monosaccharides such as raffinose and sucrose, and displayed high catalase activity at low temperatures, chilling-sensitive genotypes were characterized by higher concentrations of glucose and fructose and higher PPDK activity. *Miscanthus* × *giganteus* responses were similar to those of the tolerant genotypes in spring, but resembled those of chilling-sensitive genotypes later in the season. The high productivity reported for *M.* × *giganteus* might thus be related to this remarkable behaviour, which allows a good protection against chilling temperatures and high assimilation capacity when temperature rises.

MATERIALS AND METHODS

Growth conditions

Chlorophyll fluorescence, plant growth measurements and biomass sampling were performed in April and May 2015 in a field trial established in Merelbeke, Belgium (50°58'24.7"N, 3°46'50.9"E, sandy loam soil, temperate maritime climate). Weather data were collected in a meteorological station located at about 1 km from the trial. This field trial was planted in May 2013 as a complete randomized block design including six blocks containing plants of 114 miscanthus genotypes as described in Fonteyne *et al.* (2016*b*).

Selection of plant material

Five genotypes were chosen based on species and contrasting behaviour during the 2014 growing season for aspects

indicative of chilling tolerance and early-season growth: (1) cold stress symptoms after a cold spell in March 2014; and (2) early-season shoot growth in 2014. Cold stress symptoms were determined visually on field-grown plants on 28 March 2014, 2 d after the end of a cold spell, using a score ranging from 1 (sensitive) to 9 (very tolerant), as described in Fonteyne *et al.* (2016*b*). The shoot growth analysis was based on frequent measurements (twice a week) of shoot length, number of leaves and number of shoots taken from 18 March 2014 to 27 May 2014. Every time measurements were taken, the number of shoots per plant was counted and the longest shoot of each plant was identified. The length of this shoot was then measured from soil level to leaf tip, and the number of visible leaves was counted. Shoot length measurements were used to calculate the absolute growth rate (AGR) and the accumulated thermal time to reach a length of 30 cm (L30) and 50 cm (L50), using linear regression. The accumulated thermal time until the fourth leaf appeared on the longest shoot (Leaf4) was chosen as the indicator of canopy formation. A linear regression of leaf count vs. thermal time was used to calculate Leaf4 for each plant. An overview of the overall median, maximum and minimum values of these parameters for the whole collection and for the five genotypes chosen for this study is shown in Table 1. Two genotypes were chosen due to indications of chilling tolerance: *M. sinensis* 'OPM66' and *M. sinensis* × *sacchariflorus* hybrid 'OPM06'. Two genotypes were chosen as more chilling sensitive: *M. sinensis* 'OPM51' and *M. sinensis* × *sacchariflorus* hybrid 'OPM35'. *Miscanthus* × *giganteus* 'OPM09' was included in the study because it is the most widely used, both in scientific research and in commercial production. All genotypes were obtained through the OPTIMISC project (Lewandowski *et al.*, 2016; van der Weijde *et al.*, 2017). OPM06, OPM09 and OPM35 were supplied by Aberystwyth University, and OPM51 and OPM66 were supplied by Wageningen University.

Growth measurements and sampling in 2015

From 18 February 2015 to 28 May 2015, the length of the longest shoot, the number of leaves on that shoot and the number of shoots per plant were recorded twice weekly to determine growth parameters as described above. Calculations based on both day of the year (DOY) and thermal time (T_{base} = 7 °C) were carried out. In miscanthus research, a base temperature of 10 °C is often used (Clifton-Brown *et al.*, 2000; Hastings *et al.*, 2009). However, we had already observed plant growth in the field at temperatures below 10 °C, indicating that a lower base temperature is probably more appropriate for the genotypes investigated here. Other authors have described base temperatures between 6 and 8.5 °C depending on the miscanthus genotype (Farrell *et al.*, 2006; Zub *et al.*, 2012). Since we are working with different genotypes for which T_{base} has not been characterized, we assumed that T_{base} = 7 °C was a good choice. The trial was harvested on 12 January 2016 and the total above-ground part of each individual plant was cut and weighed individually. A sub-sample of approx. 300 g was weighed, dried in an oven at 70 °C for 48 h and weighed again. This information was used to determine moisture content and dry weight per plant.

Leaf samples for biochemical analyses were taken on five dates in the early 2015 growing season (28 April, 7 May, 12 May, 27 May and 9 June, referred to as T1–T5) (Table 2). On each sampling day, 5–10 young, fully expanded leaves from the upper part of the canopy were harvested per plant on six plants per genotype. The central leaf veins were removed upon harvest, and leaves of the same plant were bulked in one single sample. Half of each sample was freeze-dried for the determination of chlorophyll, carotenoids and soluble sugar content (glucose, fructose, sucrose, raffinose, maltose and total carbohydrate contents) and the other half was stored at –80 °C for the analysis of malondialdehyde (MDA), glutathione (GSH) and ascorbate contents, and catalase activity. Additionally, several leaf discs

TABLE 1. *Early-season growth parameters of five miscanthus genotypes (OPM06, OPM66, OPM09, OPM35 and OPM51) in 2014 and 2015*

Genotype	Year	Stress score	AGR (mm GDD^{-1})	L30 (GDD)	L50 (GDD)	Leaf4 (GDD)
Median	2014	*5.9*	*3.5*	*122*	*179*	*141*
Maximum		*8.0*	*5.5*	*223*	*328*	*220*
Minimum		*2.8*	*1.5*	*70*	*123*	*71*
OPM06	2014	*6.7* ± 0.2	*4.3* ± 0.1	*118* ± 2	*165* ± 2	*130* ± 3
OPM66		*7.5* ± 0.2	*3.5* ± 0.1	*70* ± 7	*127* ± 7	*95* ± 10
OPM09		*4.7* ± 0.6	*3.9* ± 0.3	*107* ± 5	*161* ± 4	*120* ± 9
OPM35		*4.8* ± 0.7	*4.3* ± 0.1	*129* ± 3	*175* ± 2	*142* ± 12
OPM51		*4.5* ± 0.3	*3.8* ± 0.4	*137* ± 8	*194* ± 16	*135* ± 8
OPM06	2015	–	3.4 ± 0.2	94 ± 1	155 ± 5	104 ± 18
OPM66		–	6.6 ± 0.6	41 ± 3	73 ± 5	54 ± 7
OPM09		–	3.8 ± 0.2	99 ± 2	151 ± 2	72 ± 12
OPM35		–	4.1 ± 0.2	110 ± 2	164 ± 5	61 ± 3
OPM51		–	3.8 ± 0.1	108 ± 3	157 ± 5	73 ± 6

n = 6, values ± s.e. Growth parameters for these five genotypes and overall median, maximum and minimum values in 2014 for a larger collection of genotypes are shown.

Data for 2015 refer to the season in which the investigation presented here was carried out.

GDD, growing degree-day or thermal time (calculated with a 7 °C base temperature); stress score, damage to plants scored after a cold spell; AGR absolute growth rate; L30, thermal time at which a length of 30 cm was reached; L50, thermal time at which a length of 50 cm was reached; Leaf4, thermal time at which the fourth leaf on the longest shoot emerged.

TABLE 2. *Mean, minimum and maximum air temperatures in the 24 h before leaf sampling for biochemical analyses and chlorophyll fluorescence measurements*

Code	Leaf sampling						Φ_{PSII}			
	T1	T2	T3	T4	T5		t1	t2	t3	t4
Mean (°C)	7.1	11.3	17.0	12.5	12.2	Mean (°C)	8.6	15.0	12.6	11.8
Minimum (°C)	1.7	7.8	12.6	6.2	7.4	Minimum (°C)	2.6	10.2	6.5	5.9
Maximum (°C)	12.2	15.3	24.4	18.9	18.3	Maximum (°C)	14.4	20.4	20.0	17.7
At 16.00 h (°C)	12.1	14.7	18.5	18.9	15.3	At 08.00 h (°C)	4.9	11.6	8.5	7.9

The temperature at sampling (16.00 h) or at the start of the chlorophyll fluorescence measurements (08.00 h) is also provided.

Calculations are based on data recorded by a weather station located at approx. 1 km from the field trial. (T1, 28 April; T2, 7 May; T3, 12 May; T4, 27 May; T5, 9 June; t1, 29 April; t2, 8 May; t3, 13 May; t4, 21 May).

of 1.1 cm diameter were taken per plant at each sampling date and stored in Eppendorf tubes at –80 °C for PPDK activity determination. All samples were taken between 15.00 and 17.00 h.

Chlorophyll fluorescence measurements

The quantum efficiency of photosystem II (Φ_{PSII}) was measured on four dates close to the sampling dates (29 April, 8 May, 13 May and 21 May 2015, referred to as t1–t4). The measurements were always started at sunrise and involved three plants per genotype and three leaves per plant. On each of these 45 leaves, three chlorophyll fluorescence measurements were made using a PAM 2100 portable fluorescence meter (Walz GmbH, Effeltrich, Germany). These three measurements on the same leaf were considered technical replications and were averaged. This sequence of measurements (45 leaves × 3 measurements per leaf) was repeated sequentially over a period of 3 h, rendering a total of approximately five measurement points per leaf at slightly differing light and temperature conditions on each measurement date. Leaves were measured without bending or changing the natural orientation of the leaves. Measurements were carried out using natural irradiance, which was recorded by the PAM 2100 with each measurement.

Biochemical analyses

An overview of the traits investigated is provided in Table 3. Freeze-dried samples were ground using a Retsch Tissuelyser II (Retsch, Haan, Germany). The samples stored at –80 °C were ground into a fine powder in liquid nitrogen and aliquoted for the different assays as described below. All spectrophotometric measurements were made using a CLARIOstar microplate reader (BMG labtech GmbH, Ortenberg, Germany) unless mentioned otherwise. Each sample was analyzed in three technical replicates, after which the average was calculated per sample after exclusion of outliers.

Chlorophyll a + b and carotenoid content. A 40 mg aliquot of freeze-dried leaf powder was weighed in a 2 mL Eppendorf tube, then 1600 µL of 80 % acetone was added and mixed with the sample. The samples were then incubated at 4 °C for 24 h in the dark and turned around periodically. Subsequently the samples were centrifuged at 10 000 rpm at 4 °C for 10 min. A 200 µL aliquot of twice-diluted supernatant was then pipetted in triplicate in a microtitre plate. Chlorophyll was then estimated by measuring the absorption at 663, 647 and 470 nm, and calculated using the formulae reported in Lichtenthaler and Buschmann (2001).

PPDK activity. The protocol for PPDK activity analysis was adapted from Wang *et al.* (2008*b*). Two buffers were used. The extraction buffer contained 50 mM HEPES-NaOH, pH 8.0, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 1 mM EDTA, 1 % casein, 1 % polyvinylpyrrolidone (PVP), 0.05 % Triton X-100, 20 mM NaF, 2 µM orthovanadate and one protease inhibitor cocktail tablet per 10 mL of buffer. The assay buffer contained 100 mM HEPES-NaOH, pH 8.0, 15 mM $MgCl_2$, 0.15 mM EDTA, 5 mM $NaHCO_3$, 0.3 mM NADH, 5 mM NH_4Cl, 2.5 mM K_2PO_4, 5 mM DTT, 1 mM glucose-6-phosphate, 1.5 mM ATP and 10 U mL^{-1} malate dehydrogenase. Two leaf discs were ground in a Retsch tissue lyser for 15 s at 20 Hz in a Eppendorf tube with one 5 mm stainless steel bead. To each tube, 500 µL of the extraction buffer was then added and the sample was mixed with the extraction buffer. The tubes were then centrifuged for 10 min at 15 000 rpm at 4 °C. The supernatant was pipetted into a new tube and kept on ice until needed. For the measurement, 10 µL of the extract was mixed with 240 µL of the assay buffer in PCR strips. This was done four times (three technical repeats and one blank). The samples were then incubated at 30 °C for 5 min in an Eppendorf thermomixer block. Then, 5 µL of enzyme mix

TABLE 3. *Overview of biochemical traits determined in this study*

Trait	Unit	Reference
Chlorophyll *a* + *b* content	mg g^{-1} d. wt	Lichtenthaler and Buschmann (2001)
Carotenoid content	mg g^{-1} d. wt	Lichtenthaler and Buschmann (2001)
PPDK activity	µmol m^{-2} s^{-1}	Wang *et al.* (2008*b*)
MDA content	nmol g^{-1} f. wt	Hodges *et al.* (1999)
Catalase activity	µmol H_2O_2 mg^{-1} protein	Acbi (1984)
Glutathione content	nmol g^{-1} f. wt	Queval *et al.* (2007)
Ascorbate content	µmol g^{-1} f. wt	Queval *et al.* (2007)
Glucose content	mg g^{-1} d. wt	Zhang *et al.* (2015)
Fructose content	mg g^{-1} d. wt	Zhang *et al.* (2015)
Sucrose content	mg g^{-1} d. wt	Zhang *et al.* (2015)
Raffinose content	mg g^{-1} d. wt	Zhang *et al.* (2015)
Maltose content	mg g^{-1} d. wt	Zhang *et al.* (2015)
Total carbohydrate content	mg g^{-1} d. wt	Zhang *et al.* (2015)

[0.75 µL (1 U µL L^{-1}) of mPEPc, 3.125 µL of pyruvate (100 mM) and 1.125 µL of assay buffer] was added to three of the four strips. The reaction was mixed, centrifuged and transferred into a UV plate (96-well flat bottom) and measured every 12 s during 10 min at 340 nm at 30 °C. The PPDK activity was calculated using the extinction coefficient of 6.221 µL µmol^{-1} cm^{-1} (Wang *et al.*, 2008*b*).

Malondialdehyde (MDA) content. The protocol of Hodges *et al.* (1999) was followed, which reduces the chance of error by adding buthylated hydroxytoluene (BTH) to the reagent in order to make the reaction more specific and by additionally measuring light absorbance at 400 and 600 nm to correct for interfering components. Frozen leaf powder (100 mg) was homogenized in 1 mL of 80 % (w/v) ethanol solution with 0.02 % BHT. The homogenate was centrifuged at 12 000 rpm for 10 min at 4 °C, then 400 µL of the supernatant was added to 800 µL of TBA^- solution [20 % trichloroacetic acid (TCA)] and another 400 µL of the supernatant was added to 800 µL of TBA^+ solution [20 % TCA and 0.65 % (w/v) 2-thiobarbituric acid (TBA)] in vials. The mixture was incubated in boiling water for 30 min, and the reaction was stopped by placing the vials in an ice bath. Vials were briefly vortexed and tubes were centrifuged at 12 000 rpm for 10 min at 4 °C. Aliquots of 200 µL from each tube were placed in triplicate in 96-well flat-bottom plates. The absorbance of the supernatant was read at 440, 532 and 600 nm. The amount of MDA equivalents was calculated using the formula of Hodges *et al.* (1999).

Catalase activity. Catalase activity was estimated using a protocol based on Aebi (1984). In 1.5 mL reaction tubes, 100 mg of fresh leaf powder was weighed. The samples were mixed with 1000 µL of extraction buffer [60 mM Tris; pH 6.9, 10 mM DTT, 20 % glycerol and 1 mM phenylmethylsulphonyl chloride (PMSF)] on ice. The tubes were centrifuged for 15 min at 14 000 rpm and 4 °C, and the supernatant was transferred to a fresh 1.5 mL reaction tube. A 5-fold diluted sub-sample of the extract was used for determination of the protein content according to Bradford (1976). In total, 5 µL of the diluted extract was added to 25 µL of Milli-Q water and 270 µL of Coomassie Brilliant Blue solution. Absorption at 595 nm was then measured using an iMARK spectrophotometer (Biorad, Hercules, CA, USA). The concentration of protein was determined using a bovine serum albumin (BSA) standard curve. A total of 250 µL of phosphate buffer (50 mM pH 7.0)/protein extract (containing 30 µg protein mL^{-1}) was pipetted in triplicate into 24 wells of a flat-bottom microtitre plate. The plate was incubated at 30 °C for 5 min in the CLARIOstar. Then 6 µL of H_2O_2 (3.75%) was added and, after mixing by pipetting, absorption at 240 nm was measured for 3–4 min at 30 °C. Catalase activity was calculated using the extinction coefficient of 0.0436 ml µmol^{-1} cm^{-1}.

Glutathione and ascorbate contents. Analysis was performed according to Queval *et al.* (2007). A 1 µL aliquot of 0.2 M HCl was added to 100 mg of frozen leaf sample and homogenized in liquid nitrogen. The mixture was centrifuged for 10 min at 4 °C at 14 000 rpm. A 500 µL aliquot of supernatant was neutralized by adding 50 µL of sodium phosphate buffer 0.2 M (pH 5.6) and 420 µL of 0.2 M NaOH to a final pH of 5. For measurement of glutathione, 20 µL of the neutralized supernatant and 50 µL of water were added in triplicate to wells in a microtitre plate. A mixture of 100 µL of 0.2 M sodium phosphate buffer (pH 7.5, 10 mM EDTA), 10 µL of 10 mM NADPH, 10 µL of 12 mM DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] and 10 µL of glutathione reductase (20 U mL^{-1}) was added to each well to start the reaction. The plate was shaken for 5 s before each cycle and the reaction was monitored for 20 cycles of 20 s at 415 nm. On each plate, glutathione standards with consecutive dilutions of 0, 0.2, 0.4 and 1 nM were run. Ascorbate was measured after reduction of dehydroascorbare to ascorbate. A 100 µL aliquot of neutralized supernatant was mixed with 140 µL of sodium phosphate buffer (0.12 M, pH 7.5) and 10 µL of 25 mM DDT then incubated at room temperature for 30 min. Each sample was then measured in triplicate using 50 µL of DTT-treated neutralized extract with the procedure outlined above.

Soluble carbohydrate contents. A 40 mg sample of freeze-dried leaves was weighed and mixed with 1.6 mL of Milli-Q water in a 2 mL reaction tube. Samples were then heated for 15 min in a warm water bath at 100 °C and centrifuged for 15 min at 20 °C and 14 000 rpm. The supernatant (200 µL) was pipetted onto Dowex anion exchange columns to remove charged ions. These columns were rinsed six times with 200 µL of Milli-Q water; the water was collected together with the sample. The soluble sugar content of the samples was then analysed for contents of fructose, glucose, sucrose, maltose and raffinose using high-performance anion-exchange chromatography with pulsed amperometric detection (Thermo-Fischer Scientific, Waltham, MA, USA) as reported in Zhang *et al.* (2015).

Data analysis

The chlorophyll fluorescence measurements were analysed using generalized linear models with the 'glm' function of the 'stats' package in R 3.1.0 (R Core Team, Vienna, Austria). The following model was fit:

$$Y = \mu + G_i + D_j + L_k + T_l + G_i \times D_j + G_i \times L_k + G_i \times T_l + L_k \times T_l + B_m + e_{ijklm} \quad (1)$$

where Y is the quantum efficiency of photosystem II (Φ_{PSII}), μ the overall mean, G_i the effect of genotype i, D_j the effect of measuring date j, L the effect of light intensity k, T_l the effect of temperature l, B the effect of block m, and e_{ijklm} the first residual term. Light intensity (L), temperature (T) and block (B) were considered random effects. The significance of the differences between genotypes at a given time point and between time points was determined by post-hoc least square means calculation using the 'lsmeans' function of the 'lsmeans' package. Differences among genotypes and sampling dates for biochemical traits were analysed using generalized linear models with the 'glm' function of the 'stats' package. Data were analysed according to the model:

$$Y = \mu + G_i + D_j + G_i \times D_j + B_k + e_{ijk} \quad (2)$$

where Y is a biochemical trait, μ the overall mean, G_i the effect of genotype i, D_j the effect of sampling date j, B the effect of block k, and e_{ijk} the first residual term. Samples of six plants of

each genotype were analysed for all traits, except for glutathione content, which was only determined on four plants.

Genotype by sampling date interactions were significant for all traits except glutathione and ascorbate contents. Therefore, the data were analysed per genotype and per sampling date separately. The significance of the differences between genotypes at a given time point and between time points was determined by post-hoc least square means calculation using the 'lsmeans' function of the 'lsmeans' package. Principal component analysis (PCA) of a data set comprising all biochemical traits was performed using the 'PCA' function from the 'FactoMineR' package. Only T1, T3 and T5 were considered, as not all traits were determined at T2 and T4. The analysis was thus based on average trait values per plant and sampling date (5 genotypes × 6 plants × 3 sampling dates). The correlation between trait values and the first two principal components was determined using the 'dimdesc' function from the 'PCA' package.

RESULTS

Air temperature evolution during the study period

Chilling stress in miscanthus is generally studied in growth chamber experiments at temperatures of 10–15 °C, with control treatments grown at around 20 °C. The plants in this study were grown under more realistic conditions in the field. For characterization of the five miscanthus genotypes, we therefore had to rely on the climatological characteristics of the season investigated. An overview of the evolution of the maximum, minimum and mean daily air temperature during the study period is provided in Fig. 1. Table 2 summarizes the main characteristics of the dates chosen for leaf sampling (T1–T5) and chlorophyll fluorescence (t1–t4) measurements. On the first sampling dates (T1 and T2) the air temperature did not surpass 15 °C (the highest temperature considered as 'chilling' in growth chamber experiments). On T1 and, to a lesser extent, T2, the plants were thus sampled under chilling stress. At T3–T5 they were most probably not experiencing chilling stress at the time of sampling (16.00 h). Air temperature was the lowest in the 24 h period before T1 and highest before T3 (Table 2; Fig. 1). The highest temperatures during the whole sampling period were reached in June, but in the days before sampling on 9 June (T5) temperatures were slightly lower. T5 was thus not the warmest sampling point, but rather T3. The lowest temperature recorded during the entire sampling period was 1.7 °C at T1 and the highest was 32 °C on 5 June. Regarding the chlorophyll fluorescence measurements, the coldest time point was t1 and the warmest t2, but note that 7 May (the day preceding the t2 chlorophyll fluorescence measurements) had a maximum temperature of only 15.3 °C.

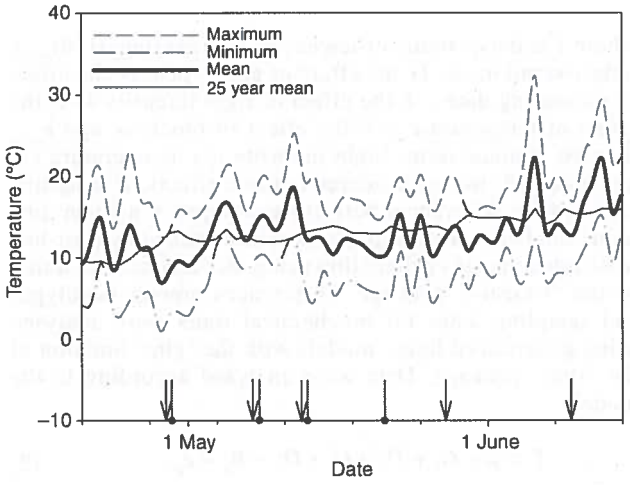

FIG. 1. Maximum, minimum and mean daily air temperature during the study period. The thin solid line shows the 25 year mean. Arrows indicate the leaf sampling dates; vertical lines indicate days when chlorophyll fluorescence was measured.

Growth characteristics of the five genotypes in 2015

In general, the early-season growth characteristics of the five genotypes determined in 2014 (see the Materials and Methods) were confirmed in 2015. No clear signs of low temperature stress ('stress score') were observed on the five tested genotypes during spring 2015. OPM66 was the first genotype to emerge, and remained taller than the other genotypes until June (Fig. 2A). OPM06 also emerged about 2 weeks earlier than other genotypes, but did not grow quickly in early spring and was overtaken in height by OPM09, OPM35 and OPM51 by mid-April. OPM66 reached a length of 50 cm on 17 April, while OPM06 and OPM09 reached this length on 3 May and OPM35 and OPM51 on 5 May. By the beginning of June, *M.* × *giganteus* OPM09 had overtaken the other genotypes in height and remained the tallest genotype throughout the rest of the growing season (data not shown). When these data were plotted vs. thermal time (T_{base} = 7 °C) (Fig. 2B), early-season growth was more proportional to thermal time than to DOY. Again OPM66 emerged earlier and grew faster than the other genotypes at the beginning of the season, while OPM09 started to differentiate from the remaining genotypes at around 240 °Cd and, at approx. 300 °Cd, it even outperformed OPM66. The six earlier length measurements of OPM66 plot very close to each other and seem to be realized with ample change in thermal time (Fig. 2B), suggesting that for this genotype the chosen thermal time of 7 °C might still be too high.

At harvest in January 2016, the highest yielding genotype was OPM09 with 4.1 kg dry matter per plant, followed by OPM35 and OPM51 with 1.3 kg dry matter per plant and by OPM06 and OPM66 with 1.0 and 0.8 kg dry matter per plant, respectively.

Traits related to photosynthetic activity

The Φ_{PSII} was measured at temperatures and light intensities ranging from 2.9 to 21.4 °C and from 4 to 1040 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR) on four dates (Supplementary Data Figs S1 and S2). There were significant interactions between measurement date, temperature and light intensity, complicating data interpretation. When genotypes were compared per measuring date or measuring dates per genotype (Fig. 3), and considering temperature and light

intensity (Supplementary Data Figs S1 and S2), no striking inter-genotype differences were observed, with the exception of OPM35 and OPM51 displaying generally slightly higher Φ_{PSII} values than the other genotypes. Φ_{PSII} was significantly lower in all genotypes on t1 due to the low temperatures registered that day. Due to this strong reduction in Φ_{PSII}, no significant differences were detected among genotypes at t1. Φ_{PSII} was significantly higher for all genotypes at t2, which was the warmest date when measuring took place. At t3 and t4, Φ_{PSII} inter-genotype differences became larger, with OPM35 and OP51 performing better than the other three genotypes. This indicates that, while the efficiency of PSII in these two genotypes was not significantly lower than that of other genotypes during cold days, it became significantly higher as temperatures increase.

Overall, the chlorophyll content was lower on T1 than at other sampling dates (Fig. 4A). OPM66 and OPM09 had significantly lower chlorophyll contents than OPM06, OPM35 and OPM51 throughout the measuring period, except at T5. The concentration of carotenoids in leaves was in general slightly higher at T1, the coldest date, than at other sampling times (Fig. 4B). With some exceptions, inter-genotype differences were not significant on any single date. However, while the concentration of carotenoids did not change significantly over sampling dates for genotypes OPM06 and OPM66, it did show significant differences over time in OPM09, OP35 and OPM51, with the highest values at T1 (Fig. 4B).

The PPDK activity was not significantly higher on T1 than on subsequent dates for any of the genotypes (Fig. 5). PPDK activity was on average lower on T1 (the coldest sampling date) than on T3 (the warmest sampling date). The temporal changes were different for the different genotypes, however. While a decreasing tendency was observed in OPM06 and OPM66, with the highest PPDK activities recorded at the coldest date (T1), an increasing tendency was observed in OPM09, and a peak at T3 for OPM35 and OPM51. This could indicate that while PPDK activity in OPM06 and OPM66 follows changes in air temperature, it is independent of this factor in OPM35 and OPM51, or that these two latter genotypes react to chilling temperatures by reducing their photosynthetic activity [reflected in a relatively lower PPDK activity; similar to the findings of Friesen and Sage (2015); see also the lower Φ_{PSII} for T1]. OPM51 had the highest PPDK activity on all sampling dates, indicating the highest photosynthetic activity for this genotype.

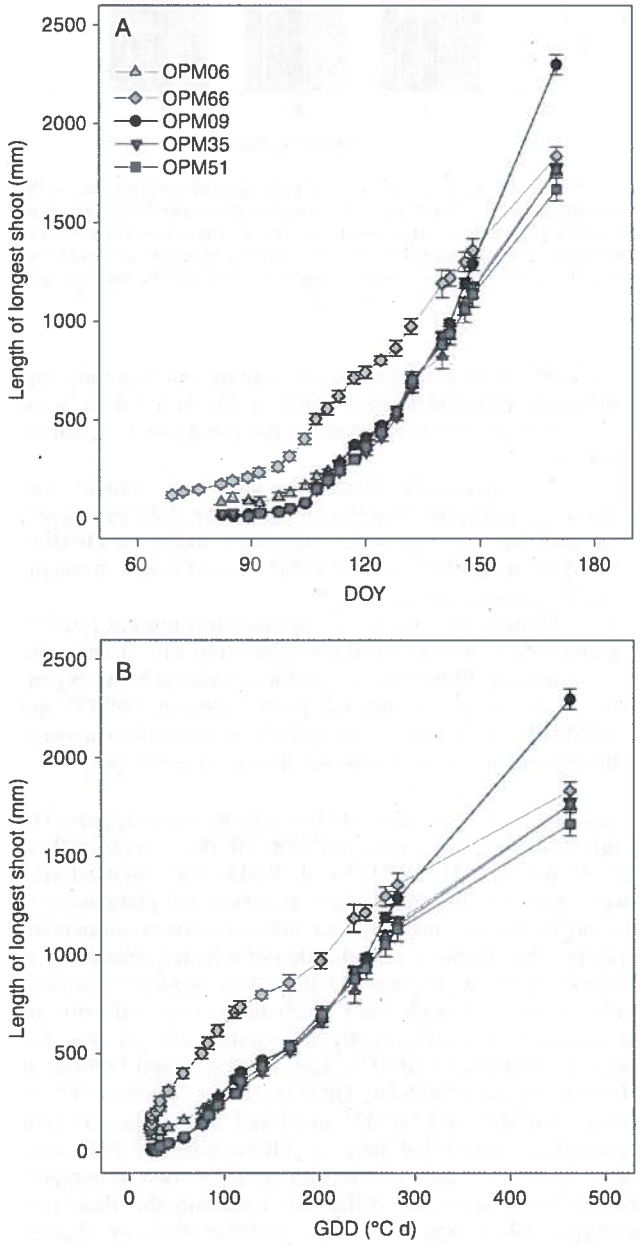

FIG. 2. Evolution of the length of the longest shoot per genotype in spring 2015: (A) vs. day of the year (DOY) and (B) vs. growing degree days (GDD; T_{base} = 7 °C). Symbols show the mean value per genotype; bars represent the s.e. (n = 6).

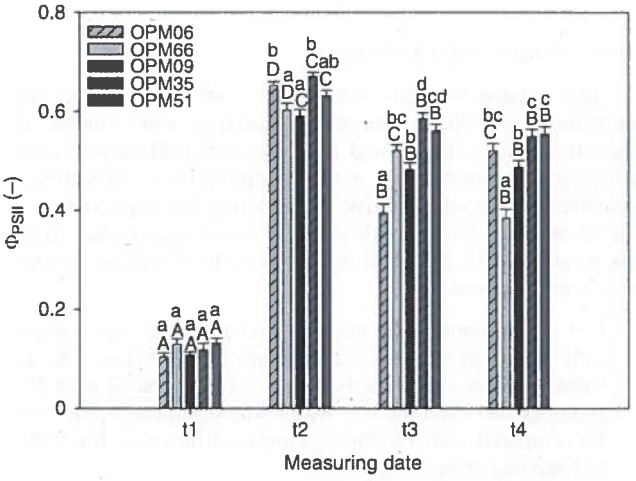

FIG. 3. Bar plots of mean Φ_{PSII} per genotype and measuring date. Error bars show the s.e.. Upper case letters refer to homogenous groups of sampling dates for a specific genotype and lower case letters refer to homogenous groups of genotypes for a specific sampling date ($P < 0.05$).

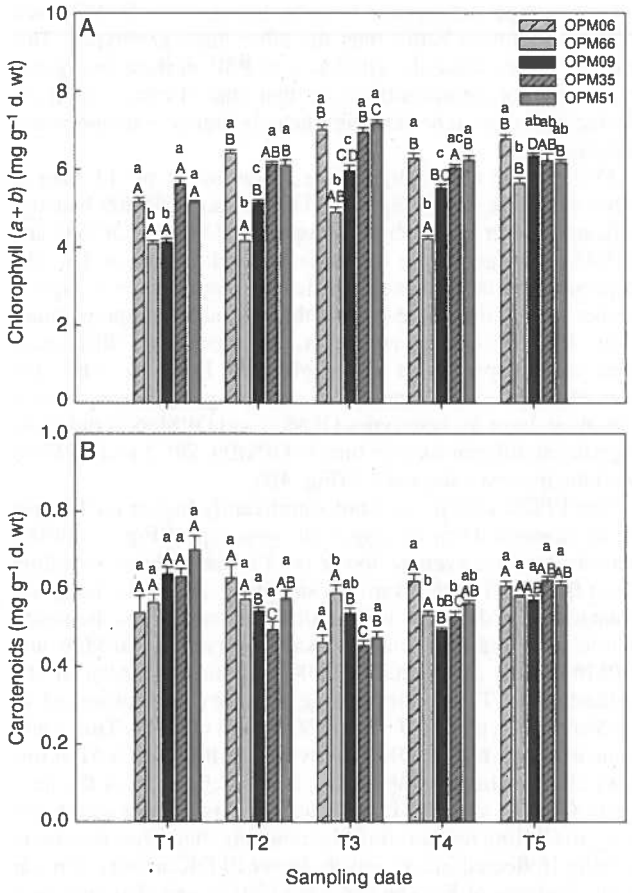

FIG. 4. (A) Content of chlorophyll *a* and *b* in the leaves (mg chlorophyll g^{-1} d. wt). (B) Leaf carotenoid content (mg carotenoids g^{-1} d. wt), sampled on T1, T2, T3, T4 and T5. Each bar depicts the mean value per genotype and sampling point. Error bars show the s.e. ($n = 6$). Upper case letters refer to homogenous groups of sampling dates for a specific genotype and lower case letters refer to homogenous groups of genotypes for a specific sampling date ($P < 0.05$).

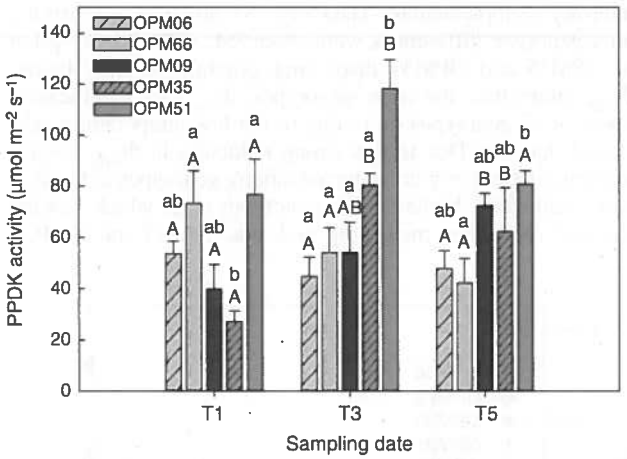

FIG. 5. PPDK activity (μmol m^{-2} s^{-1}) per genotype and sampling date in the leaves sampled on T1, T3 and T5. Each bar depicts the mean value per genotype and sampling point. Error bars show the s.e. ($n = 6$). Upper case letters refer to homogenous groups of sampling dates for a specific genotype and lower case letters refer to homogenous groups of genotypes for a specific sampling date ($P < 0.05$).

Traits related to redox homeostasis

Traits related to redox homeostasis (MDA, ascorbate and glutathione contents, and catalase activity) were studied at three time points (T1, T3 and T5). No striking differences were found among genotypes or across sampling dates, indicating a possible absence of oxidative stress during the experiment, or the occurrence of only subtle changes whose significance could not be statistically established. However, the following tendencies were observed.

1. MDA content did not vary over time and was consistently higher in OPM06, OPM66 and OPM09 (Fig. 6A). In OPM09 it was significantly lower at T1 compared with T3, and in OPM35 and OPM51 MDA was significantly lower at T3 compared with T5. Inter-genotype differences for MDA became less pronounced at T5.
2. OPM06 and OPM66 had higher catalase activity than OPM35 and OPM51 at T3 (Fig. 6B). At T1, OPM66 had a higher catalase activity than OPM35 and OPM51, but OPM06 was not significantly different from OPM35. OPM09 had an average catalase activity and was only significantly different from OPM66 at T1. At T5 there were no significant differences among the genotypes for catalase activity.
3. No significant change in ascorbate content was observed during the sampling period in any of the genotypes, but ascorbate content was significantly higher in OPM66, OPM09 and OPM06 than in OPM35 and OPM51 throughout the growing season (Fig. 6C)
4. Glutathione content was significantly higher at T1 compared with T3 and T5 in all genotypes (Fig. 6D). There were no significant differences in glutathione content between genotypes at any of the sampling points, although OPM35 and OPM51 tended to have lower glutathione contents on average throughout the measuring period than other genotypes.

In general, parameters related to redox homeostasis appeared to be different between the group OPM06, OPM66 and OPM09 vs. OPM35 and OPM51. OPM35 and OPM51 had comparatively lower contents of the antioxidants ascorbate and glutathione at T1 (and T3, but less pronounced), indicating lower antioxidant capacity. This might be an indication of a higher sensitivity to oxidative stress or the fact that these two genotypes avoided oxidative stress through other mechanisms (e.g. reduction of light capture). Correspondingly, the catalase activity was also lower in OPM35 and OPM51 than in OPM06 and OPM66 at T3 and lower in OPM35 and OPM51 than in OPM66 at T1. In contrast, OPM35 and OPM51 displayed fewer signs of lipid peroxidation (quantified here as MDA content), indicating lower damage of cell membranes in these two genotypes. Whether this is a genuine difference, indicating that these two genotypes indeed experienced less oxidative stress, or whether this occurred due to the correction applied to account for the possible presence of interfering compounds that also absorb at 532 nm, as proposed by Hodges *et al.* (1999), could not be established.

Carbohydrate concentrations

Different WSC profiles were observed in the five genotypes (Fig 7A–F). OPM35 and OPM51 were characterized by significantly higher levels of glucose and fructose compared with OPM06 and OPM66 throughout the measuring period (Fig. 7A, B). Interestingly, *M.* × *giganteus* OPM09 was similar to OPM06 and OPM66 in the beginning of the growing season, with relatively low glucose and fructose contents. After T3, OPM09 was similar to OPM35 and OPM51, with relatively high glucose and fructose contents.

Sucrose content remained relatively stable in OPM06, OPM35 and OPM51, but varied strongly in OPM09 and OPM66 (Fig. 7C). In OPM66, the sucrose content was higher than in OPM06, OPM35 and OPM51 throughout the growing season, except at T5. Raffinose was significantly lower in all genotypes on the warmest day, T3 (Fig. 7D), indicating an effect of temperature on raffinose content. OPM06 and OPM09 had significantly higher raffinose contents than the other genotypes on all days, except T5. All genotypes had higher maltose contents at T1 compared with the other sampling days, after which the concentration of maltose decreased strongly in all genotypes except in OPM66 (Fig. 7E).

The ratio of glucose to sucrose was <0.4 in OPM06 and OPM66 on all dates, but was as high as 0.6 in OPM35 and OPM51 at T1 and T2 (Supplementary Data Fig. S3). It could be argued that OPM35 and OPM51 invest more in growth and less in storage. In OPM09, this ratio was low in the beginning of the growing season, similar to OPM06 and OPM66, and high after T3, similar to OPM35 and OPM51. This could indicate a metabolic change in this genotype as temperature increased during the season. While the relative proportions of WSCs changed over time, the total WSC content did not show a clear trend over time (Fig. 7F). In OPM09 and OPM66, total WSC contents were significantly higher at T3 compared with the other days, while this was not the case in OPM06, OPM35 and OPM51. OPM06 had the lowest total WSC concentration on every sampling date. At T1, OPM35 and OPM51 had significantly higher total WSC concentrations than other genotypes, but these differences were in the same range as the inter-genotypic differences on other sampling dates and are therefore not necessarily an indication of increased WSC accumulation due to low temperature.

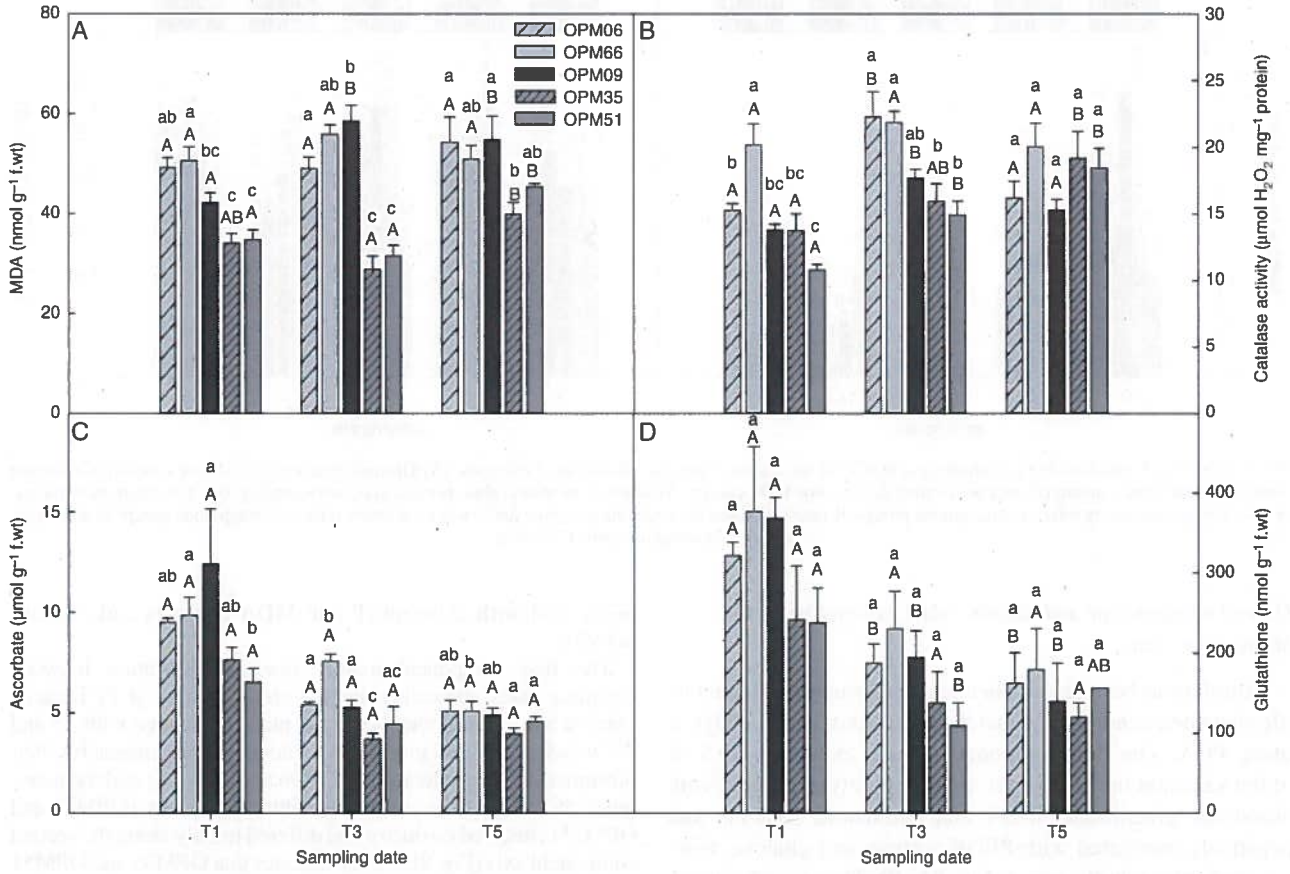

FIG. 6. Contents of malondialdehyde and antioxidants in the leaves of the five miscanthus genotypes sampled. (A) Malondialdehyde content; (B) catalase activity; (C) ascorbate content; and (D) glutathione content. Each bar depicts the mean value per genotype and sampling point. Error bars show the s.e. ($n = 6$ for MDA, catalase activity and ascorbate; $n = 4$ for glutathione). Upper case letters refer to homogenous groups of sampling dates for a specific genotype and lower case letters refer to homogenous groups of genotypes for a specific sampling date ($P < 0.05$).

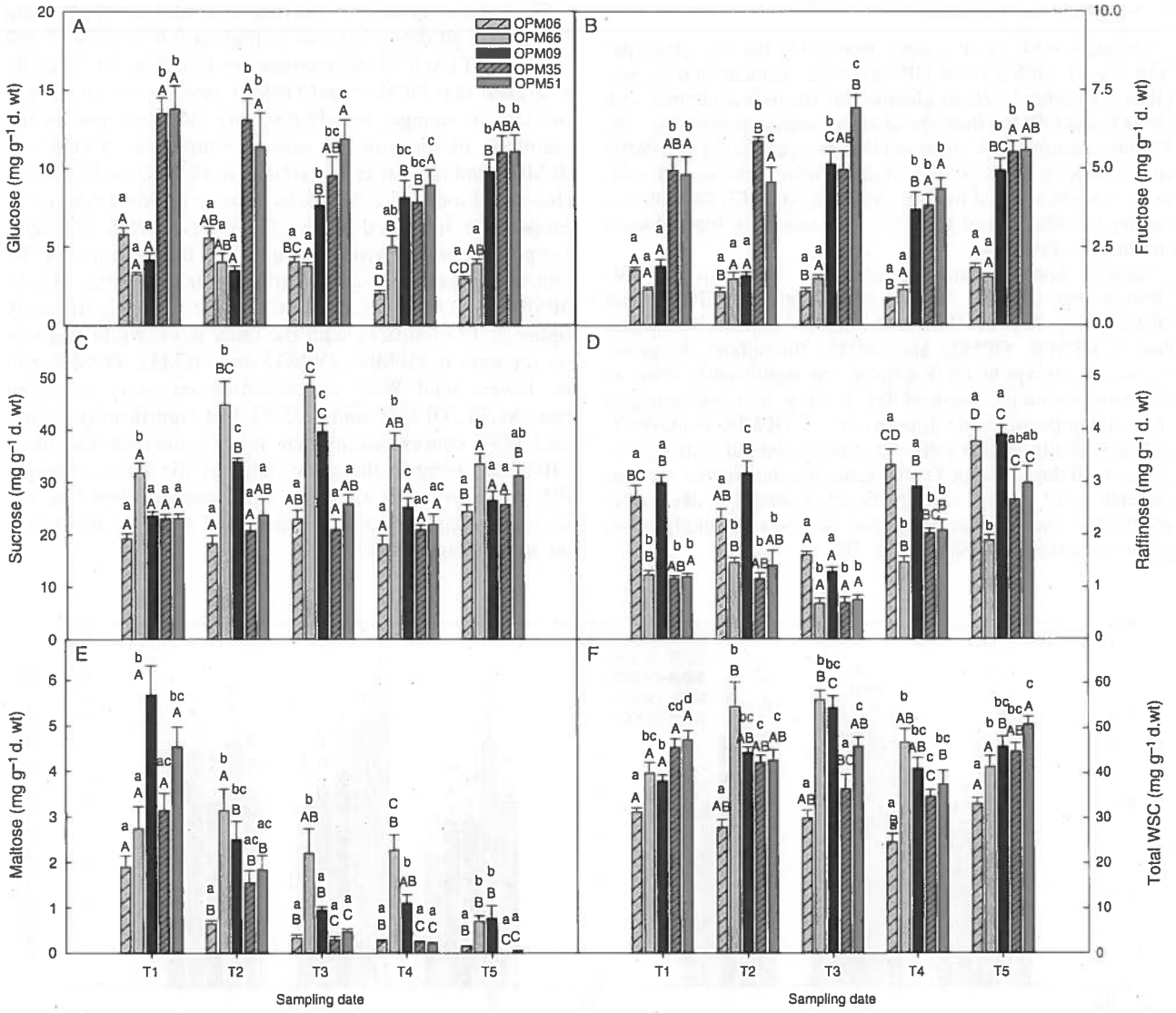

FIG. 7. Content of water-soluble carbohydrates (WSCs) in the leaves of the five miscanthus genotypes. (A) Glucose content; (B) fructose content; (C) sucrose content; (D) raffinose content; (E) maltose content and (F) total WSC content. Symbols show mean values per genotype and sampling date. Error bars show the s.e. ($n = 6$). Upper case letters refer to homogenous groups of sampling dates for a specific genotype and lower case letters refer to homogenous groups of genotypes for a specific sampling date ($P < 0.05$).

Overall intergenotype and sampling date patterns for biochemical traits

Correlations between biochemical components and similarities between genotypes in metabolic response were analysed using PCA. The first component, which explained 29.5 % of the variation in the data set, was positively correlated with ascorbate, glutathione, MDA and carotenoid contents and negatively correlated with PPDK activity and glucose, fructose and chlorophyll content (Fig. 8A, B). The second component, which explained 19.4 % of the variation, was positively correlated with glucose, fructose, maltose, carotenoids, total WSCs, ascorbate and glutathione contents, and negatively associated with chlorophyll and MDA contents and catalase activity.

The first component mainly described variation between sampling dates, indicating that the chilling stress at T1 induced marked biochemical changes in the plants compared with T3 and T5, which were similar (Fig. 8A). The genotypes chosen for their presumed chilling tolerance (OPM06 and OPM66) and the genotypes chosen for their presumed chilling sensitivity (OPM35 and OPM51) clustered distinctly and differed mainly along the second component axis (Fig. 8B). This indicates that OPM35 and OPM51 were mainly characterized by higher concentrations of glucose, fructose, and total WSCs, and higher PPDK activity, while OPM06 and OPM66 were characterized by high levels of MDA, raffinose and sucrose, and high catalase activity. *Miscanthus* ×

giganteus OPM09 was intermediate to the other genotypes. Other PCA components did not indicate differences between genotypes or sampling dates. The clustering of OPM06 and OPM66 vs. OPM35 and OPM51 also stood out when PCAs were calculated per sampling day (Supplementary Data Figs S4–S6).

DISCUSSION

To our knowledge, this is the first study in which a whole battery of physiological and biochemical traits related to chilling tolerance was analysed in a common set of field-grown miscanthus plants. In contrast to most studies, where young, growth chamber-grown plants were used, we studied plants that had been growing under field conditions for two seasons. These plants had resumed growth after winter; their shoots had emerged and developed at low temperature. They were probably still acclimatized to low temperature during the first sampling date(s), losing this acclimation later on, as evidenced by the separate grouping of T1 in the PCA analysis. Because no control on temperature, water availability or light regime was imposed, greater variation among plants was observed than in a typical growth chamber experiment. The results obtained might thus not be directly comparable with other literature reports, in which often sudden and severe stresses are applied, but the present results should reflect more realistic plant responses that are more representative of field conditions.

Differential responses of chilling-tolerant and chilling-sensitive genotypes

Genotypic variation for photosynthesis and biochemical traits related to chilling tolerance was evaluated in five miscanthus genotypes. Temperatures shortly before sampling dates T1 and T2 were as low as 1.7 and 7.8 °C, respectively, with maxima below or around 15 °C. We can thus assume that on these two dates the plants were experiencing chilling stress at levels similar to the chilling stresses applied in previous growth chamber experiments (mostly 10–15 °C; Naidu and Long, 2004; Farage *et al.*, 2006; Wang *et al.*, 2008*a*; Purdy *et al.*, 2013; Głowacka *et al.*, 2014). At later sampling points, temperatures were higher.

The results at these time points can thus be interpreted as similar to an unstressed control treatment. Significant differences between the genotypes originally selected as chilling tolerant (OPM06 and OPM66) and the genotypes selected as chilling sensitive (OPM35 and OPM51) were detected, mainly in PPDK activity, traits related to redox homeostasis and WSC content. OPM35 and OPM51 seemed to have a more efficient photosynthesis, while OPM06 and OPM66 invested more in stress protection. Genotype OPM66 emerged significantly earlier than the other four genotypes, while the early-season growth of OPM06 was more similar to OPM35 and OPM51 than to OPM66. *Miscanthus* × *giganteus* OPM09 had intermediate behaviour, with characteristics of both chilling-tolerant and chilling-sensitive genotypes. It seemed to avoid a trade-off between stress tolerance and efficient photosynthesis at optimal temperatures. Such a trade-off between chilling tolerance and growth capacity at higher temperature has often been observed in growth chamber experiments, where the miscanthus genotypes with the highest growth rates under chilling stress were less efficient at higher temperatures (Clifton-Brown and Jones, 1997; Farrell *et al.*, 2006; Głowacka *et al.*, 2014) and vice versa. This trade-off was the case for OPM66, the earliest emerging genotype that displayed signs of being the most chilling tolerant, as it had a significantly lower growth rate later during the spring. By the beginning of June, the more chilling-sensitive genotypes had formed more shoots (results not shown) and had reached the same height, despite having emerged later. The capacity of these latter genotypes to grow faster as temperatures rose compensated for the later emergence. These patterns were also reflected in a higher biomass yield by the end of the growing season for OPM09, OPM35 and OPM51 than for OPM06 and OPM66. These observations call into question whether chilling tolerance is a means to increase biomass yield. Regardless, a larger

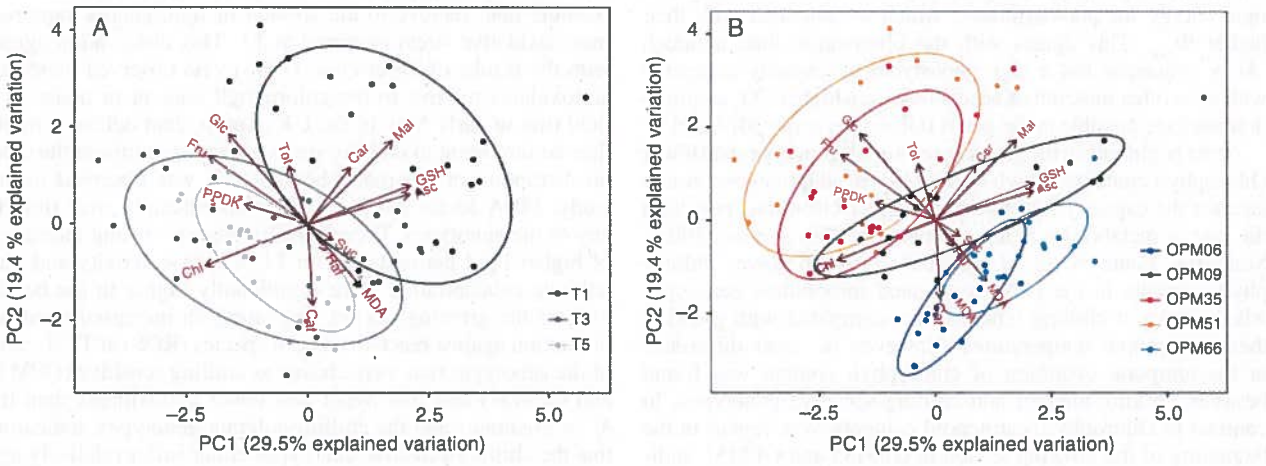

FIG. 8. Principal component analysis of the biochemical parameters at T1, T3 and T5. Dots show values of the first and second component for individual plants. Different colours indicate different sampling dates (A) or genotypes (B). Ellipses show confidence intervals per sampling date.

set of genotypes should be investigated before any definitive conclusions are drawn.

What are the differences at photosynthesis level?

Friesen and Sage (2015) reported that the more chilling-sensitive miscanthus genotype in their study did not suffer damage due to chilling stress, but rather responded to chilling temperatures by decreasing the production of photosynthetic enzymes. Similarly, we did not observe any permanent damage in OPM35 or OPM51 plants (note that the 'stress score' values shown in Table 1 refer to damage by frost temperatures in 2014). The PPDK activity values recorded in this study were of the same order of magnitude as reported in Wang *et al.* (2008*b*) and Friesen and Sage (2015). However, while Wang *et al.* (2008*b*) found higher PPDK activity values in cold-grown than in warm-grown *M.* × *giganteus* in growth chamber experiments, in our field-grown plants the opposite was seen. Interestingly, the chilling-tolerant genotypes OPM06 and OPM66 maintained similar levels of PPDK activity throughout the sampling period, while in the other genotypes PPDK activity was lower early in the growing season. This might be an indication of OPM35 and OPM51 responding to chilling stress by reducing photosynthetic activity to a greater extent than OPM06 and OPM66. Likewise, Friesen and Sage (2015) observed lower PPDK activity in a chilling-sensitive miscanthus hybrid at low temperature. In sugarcane, PPDK activity does not change in chilling-tolerant genotypes after exposure to chilling stress, while in chilling-sensitive genotypes PPDK activity declines markedly (Du *et al.*, 1999), in agreement with the results of this study. The different response in PPDK activity in our study compared with that of Wang *et al.* (2008*b*) could be caused by the different climatic conditions in our field trial [in terms of both temperature, irradiance (intensity and daily light integral) and their continuous changes] compared with the growth chamber (Wang *et al.*, 2008*b*). The *in vitro* activity of photosynthetic enzymes, such as PPDK, is indicative of the *in vivo* carbon assimilation rate (Usuda *et al.*, 1984). The generally higher PPDK activity detected in OPM35 and OMP51 with rising temperatures (T3 and T5) indicates that these genotypes have a higher photosynthetic capacity: they are able to use more light energy for photosynthesis, which is consistent with their higher Φ_{PSII}. This agrees with the observation that, although *M.* × *giganteus* has a high photosynthetic capacity compared with most other miscanthus accessions, even higher CO_2 assimilation rates are possible in the genus (Głowacka *et al.*, 2014, 2015).

At the beginning of the growing season, all genotypes had lower chlorophyll contents, which is probably an adaptive response to balance the capacity for photosynthetic electron transport with the rate of metabolism at low temperature (Foyer *et al.*, 2002). Similarly, Fonteyne *et al.* (2016*a*) observed lower chlorophyll contents in the two investigated miscanthus genotypes when grown at chilling temperatures compared with growing them at optimal temperatures. However, no clear difference in the temporal evolution of chlorophyll content was found between chilling-tolerant and chilling-sensitive genotypes. In contrast to chlorophyll, carotenoid contents were higher in the beginning of the growing season in OPM35 and OPM51, indicating a need for increased protection against excessive light energy, while the carotenoid content did not change significantly in OPM06 and OPM66. Farage *et al.* (2006) reported that chlorophyll concentrations become lower and carotenoid concentrations increase with chilling stress in *M.* × *giganteus*, while non-photochemical quenching (the thermal dissipation of energy) increases as temperature rises. Similarly, we found that the ratio of chlorophyll to carotenoids was lower by the beginning of the growing season in *M.* × *giganteus* OPM09. This was also the case for OPM35 and OPM51, but not for OPM06 and OPM66. In addition, under chilling stress, *M.* × *giganteus* has been shown to increase levels of proteins (Naidu *et al.*, 2003) or mRNA coding for the synthesis of photosynthetic proteins and proteins protecting PSII (Wang *et al.*, 2008*b*; Spence *et al.*, 2014). Carotenoids, such as β-carotene, lutein and xanthophyll, protect PSII against excessive light by thermal dissipation of light energy (Huner *et al.*, 1993; Demmig-Adams and Adams, 2006). The higher carotenoid content on T1 and lower carotenoid content on T3 coincide with the coldest and warmest sampling days and suggest that OPM35 and OPM51 adapt the concentration of carotenoids to changing temperatures, while the other genotypes do not.

Are parameters related to redox homeostasis indicators of chilling tolerance in miscanthus?

The chilling-sensitive genotypes were characterized by lower MDA and antioxidant contents than the chilling-tolerant genotypes, which suggests a role for oxidative stress tolerance as a part of chilling tolerance in miscanthus. Although numerous studies have linked chilling tolerance in maize to oxidative stress (Fryer *et al.*, 1998; Leipner *et al.*, 1999; Pastori *et al.*, 2000; Marocco *et al.*, 2005), oxidative stress has not been thoroughly studied in miscanthus. One exception is the study by Ezaki *et al.* (2008), who reported a high tolerance to oxidative stress in miscanthus compared with other plant species (Ezaki *et al.*, 2008). Here we have presented results for several indicators of redox homeostasis for a common set of five miscanthus genotypes. In general, the lower chlorophyll content at the beginning of the growing season indicates that MDA and antioxidant contents were high in the early growing season relative to the chlorophyll content for all genotypes. It is therefore possible that, relative to the amount of light energy captured, more oxidative stress occurred at T1. This observation agrees with the results of Fryer *et al.* (1998) who observed increased antioxidants relative to the chlorophyll content in maize in a field trial in early May in the UK. Antioxidant defences might thus be important to chilling stress tolerance in miscanthus, but no disruption of the redox homeostasis was observed in our study. MDA levels did not change significantly over time in any of the genotypes. There was therefore no strong indication of higher lipid peroxidation on T1. Catalase activity and glutathione concentration were significantly higher in the beginning of the growing season, indicating an increased need for protection against reactive oxygen species (ROS) at T1. Leaves of the genotypes that were chosen as chilling sensitive (OPM35 and OPM51) had less MDA and fewer antioxidants than the *M.* × *giganteus* and the chilling-tolerant genotypes, indicating that the chilling-sensitive genotypes either suffer relatively less oxidative stress or invest less in protection against oxidative stress. The latter agrees with the observation of higher levels of

antioxidants in chilling-tolerant maize genotypes than in sensitive genotypes (Leipner *et al.*, 1999; Aroca *et al.*, 2001).

Is there a link between chilling tolerance and carbohydrate content and composition?

The amount and composition of carbohydrates in leaf tissues varied significantly among genotypes and sampling dates. The chilling-sensitive genotypes (OPM35 and OPM51) were similar in carbohydrate composition and were characterized by high levels of glucose and fructose as well as a high glucose to sucrose ratio. OPM06 and OPM66 were characterized by relatively high sucrose contents and glucose to sucrose ratios <0.4. OPM09 was similar to OPM06 and OPM66 at the beginning of the season and similar to OPM35 and OPM51 later on.

According to available literature, maltose and raffinose are the sugars induced most in plants under chilling stress (Tarkowski and Van den Ende, 2015) and have been shown to act as protective agents for cell membranes (Kaplan and Guy, 2005; Valluru and Van den Ende, 2008), act as antioxidants (Nishizawa *et al.*, 2008; Keunen *et al.*, 2013; Peshev *et al.*, 2013), protect PSII and play a role as stress signalling molecules (Van den Ende and El-Esawe, 2014; Tarkowski and Van den Ende, 2015). In agreement with this view, maltose concentrations were inversely related to temperature, with a clear tendency to decrease throughout the study period in all genotypes except OPM66, for which maltose concentrations were not the highest at T1. These higher concentrations of maltose on T1 could be a protective measure against chilling stress. On the other hand, while raffinose concentrations were the lowest in all genotypes at T3, the warmest sampling point, they increased again at T4 and T5, resulting in no clear overall relationship between raffinose concentration and temperature. This contrasts with the findings of Fonteyne *et al.* (2016*a*), who found higher raffinose concentration in *M.* × *giganteus* when grown at 12 °C than at 20 °C.

Overall, the chilling-tolerant OPM06 and OPM09 had higher concentrations of raffinose than other genotypes throughout the sampling period. Souza *et al.* (2013) also reported high levels of raffinose throughout the growing season in *M.* × *giganteus*, indicating that this might be a characteristic of this genotype. This agrees with knowledge available in rice (*Oryza sativa*), oat (*Avena sativa*) and *Arabidopsis thaliana*, where raffinose contents are higher in chilling-tolerant genotypes (Klotke *et al.*, 2004; Livingston *et al.*, 2006; Morsy *et al.*, 2007). However, raffinose concentrations were not significantly higher in the chilling-tolerant genotype OPM66 at colder sampling dates, indicating other responses in this genotype. Taken together with the results for maltose concentration, these results indicate that, in regard to sugars, the protection mechanisms and metabolic responses of genotype OPM66 is different from those of other relatively chilling-tolerant genotypes such as OPM06 and OPM09.

Overall genotypic responses and implications for breeding

Analysis of the overall response of genotypes and temporal patterns using PCA indicated that sampling date was the main differentiating factor, with T1 clearly separated from warmer sampling moments. The second main source of differentiation was chilling tolerance, with tolerant (OPM06 and OPM66) and sensitive (OPM35 and OPM51) genotypes forming separate clusters. As anticipated by the results obtained for the different parameters separately, OPM09 took an intermediate position in the PCA plot. We can therefore conclude that the genotypes chosen as chilling sensitive and the genotypes chosen as chilling tolerant differ at the metabolic level, even if they might differ for particular types of reactions as illustrated by differing trends for specific biochemical characteristics.

Of particular interest are the results obtained for OPM09: while in the beginning of the growing season this genotype was most similar to the chilling-tolerant genotypes, after the warm weather around 12 May 2014, it was more similar to the chilling-sensitive genotypes, with higher fructose and glucose contents and higher PPDK activity. *Miscanthus* × *giganteus* OPM09 seems to be well adapted to low temperatures at the beginning of the growing season and shows good growth capacity once temperatures rise. The high productivity reported for *M.* × *giganteus* might thus be related to this remarkable adaptability.

Of all investigated traits, WSC analysis was the most informative, as it indicates both chilling tolerance and growth capacity. The analysis of WSCs was also fast and relatively easy to perform. Purdy *et al.* (2015) studied the applicability of WSC as a marker trait for the detection of high-yielding genotypes in breeding programmes. Our results show that WSC analysis does indeed show promise as a marker trait for the detection of chilling-tolerant and/or high-yielding genotypes, but the time of sampling must be chosen carefully, as the WSC profiles change greatly during the growing season. Sampling at midsummer, as done by Purdy *et al.* (2015), is most probably the best timing to detect high-yielding genotypes, while screening for chilling tolerance should be done as early as possible in the growing season. The ratio of glucose to sucrose clearly distinguished the chilling-tolerant and chilling-sensitive genotypes in our study; if this would be confirmed, this ratio could be a good marker trait to select chilling-tolerant genotypes.

Conclusions

The overall physiological response of chilling-tolerant genotypes was clearly distinguishable from that of chilling-sensitive genotypes. Chilling-tolerant genotypes accumulated protective monosaccharides such as raffinose and sucrose and displayed high catalase activity at low temperatures. The chilling-sensitive genotypes were characterized by higher concentrations of glucose and fructose, and higher PPDK activity later in the growing season, indicating a higher photosynthetic activity. Overall, there appeared to be a trade-off between high growth and chilling stress tolerance for the investigated genotypes. *Miscanthus* × *giganteus* seems to be able to overcome this trade-off; its responses were similar to the tolerant genotypes early in spring, but more similar to the chilling-sensitive genotypes later in the season. The high productivity reported for *M.* × *giganteus* might thus be related to this remarkable behaviour, which allows a good protection against chilling temperatures and a high assimilation capacity when temperature rises. Of all the traits measured, WSCs appear to be the most suitable for large-scale screening, since these were both fast to

measure and had a strong relationship to chilling tolerance. It thus appears to be possible to combine both chilling tolerance and strong growth in one genotype.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Figure S1: Φ_{PSII} as a function of temperature plotted per genotype and measuring date. Figure S2: Φ_{PSII} as a function of light intensity plotted per genotype and measuring date. Figure S3: mean ratio of glucose/sucrose content in the leaves per genotype and per sampling date. Figure S4: PCA of biochemcial components on T1. Figure S5: PCA of biochemcial components on T3. Figure S6: PCA of biochemcial components on T5.

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