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Chilling tolerance and early vigour-related characteristics evaluated in two *Miscanthus* genotypes

S. FONTEYNE[*,**], P. LOOTENS[*,+], H. MUYLLE[*], W. VAN DEN ENDE[***], T. DE SWAEF[*],
D. REHEUL[**], and I. ROLDAN-RUIZ[*]

Institute for Agricultural and Fisheries Research (ILVO), Plant Sciences Unit, 9090, Melle, Belgium[*]
Ghent University, Department of Plant Production, 9000 Ghent, Belgium[**]
Katholic University Leuven, Laboratory of Molecular Plant Biology, 3000 Leuven, Belgium[***]

Abstract

A long growing season, mediated by the ability to grow at low temperatures early in the season, can result in higher yields in biomass of crop *Miscanthus*. In this paper, the chilling tolerance of two highly productive *Miscanthus* genotypes, the widely planted *Miscanthus* × *giganteus* and the *Miscanthus sinensis* genotype 'Goliath', was studied. Measurements in the field as well as under controlled conditions were combined with the main purpose to create basic comparison tools in order to investigate chilling tolerance in *Miscanthus* in relation to its field performance. Under field conditions, *M.* × *giganteus* was higher yielding and had a faster growth rate early in the growing season. Correspondingly, *M.* × *giganteus* displayed a less drastic reduction of the leaf elongation rate and of net photosynthesis under continuous chilling stress conditions in the growth chamber. This was accompanied by higher photochemical quenching and lower nonphotochemical quenching in *M.* × *giganteus* than that in *M. sinensis* 'Goliath' when exposed to chilling temperatures. No evidence of impaired stomatal conductance or increased use of alternative electron sinks was observed under chilling stress. Soluble sugar content markedly increased in both genotypes when grown at 12°C compared to 20°C. The concentration of raffinose showed the largest relative increase at 12°C, possibly serving as a protection against chilling stress. Overall, both genotypes showed high chilling tolerance for C_4 plants, but *M.* × *giganteus* performed better than *M. sinensis* 'Goliath'. This was not due to its capacity to resume growth earlier in the season but rather due to a higher growth rate and higher photosynthetic efficiency at low temperatures.

Additional key words: bioenergy crop; chlorophyll fluorescence; cold tolerance; leaf growth analysis; low temperature stress.

Introduction

Miscanthus (*Miscanthus sp.*), a perennial C_4 grass, is increasingly used as biomass crop due to its high dry matter yield with limited input of fertilizers, pesticides, and labour (Lewandowski *et al.* 2000, Anderson *et al.* 2011). At present, the vast majority of commercial *Miscanthus* fields are planted with *M.* × *giganteus*, a naturally occurring sterile *M. sinensis* × *sacchariflorus* hybrid (Greef and Deuter 1993). Dry matter yields of *M.* × *giganteus* in temperate and continental regions have been reported as ranging between 10 and 25 t(dry matter, DM) ha^{-1} (Lewandowski *et al.* 2003, Zub and Brancourt-Hulmel 2010). Comparisons of *M.* × *giganteus* with other species and analysis of the variability present within the genus *Miscanthus* have highlighted the relevance of a long growing season as a contributor to the observed high yields. For example, Dohleman and Long (2009) concluded that *M.* × *giganteus* produces 59% more biomass than maize in the American Midwest because its growing season is

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[+] Corresponding author; e-mail: peter.lootens@ilvo.vlaanderen.be

Abbreviations: Chl – chlorophyll; DOY – day of year; F_v/F_m – maximal quantum yield of PSII photochemistry; F_v'/F_m' – quantum yield of open PSII reaction centers; g_s – stomatal conductance; LED – leaf elongation duration; $LED_{10–90\%}$ – duration of leaf elongation from 10 to 90% of a final length; LER – leaf elongation rate; LER_{max} – maximum leaf elongation rate; L_m – maximum leaf length; NPQ – nonphotochemical quenching; P_N – net photosynthetic rate; q_P – photochemical quenching coefficient; T_a – air temperature; WSC – water soluble carbohydrates; Φ_{CO2} – quantum yield of photosynthesis; Φ_{PSII} – effective quantum yield of PSII photochemistry.

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substantially longer and it thus absorbs more radiation. *Miscanthus* can grow at lower temperatures than most other C_4 crops (Farage *et al.* 2006, Friesen *et al.* 2014, Głowacka *et al.* 2014) and it is able to actively assimilate carbon at temperatures well below those tolerated by most other C_4 species (Long and Spence 2013). For example, the base temperatures for shoot emergence as determined by Farrell *et al.* (2006) on four *Miscanthus* genotypes ranged between 6.0 and 8.6°C (8.5°C in *M.* × *giganteus*). Furthermore, their model of a yield potential for southern Germany revealed that a lower base temperature, combined with early emergence and leaf frost tolerance, could lead to 25% higher biomass yields (Farrell *et al.* 2006). Similarly, after a comparison of 16 long-term field trials in Europe, spanning more than 12 years, it was concluded that trials at lower latitudes are higher yielding due to longer growing seasons and higher temperatures (Lesur *et al.* 2013). Miguez *et al.* (2008) reached the same conclusion in a meta-analysis of 31 field trials. In contrast, the meta-analysis of Heaton *et al.* (2004) showed no correlation between the accumulated thermal time in a growing season and *M.* × *giganteus* yields. At the intragenus level, a study of 244 *Miscanthus* genotypes in Wales, including *M.* × *giganteus*, showed that early emerging and/or late flowering genotypes were higher yielding (Robson *et al.* 2013b). However, a field trial of 21 genotypes in northern France pinpointed late emergence and high growth rates as the main contributors to higher yields (Zub *et al.* 2011). Different methodologies to determine the beginning of the growing season and the different sets of the genotypes investigated might at least partially explain these contrasting results (Robson *et al.* 2013b). Taken together, the results summarized above support the view that the long growing season can contribute to the high yields observed in *Miscanthus* in general, and *M.* × *giganteus* in particular. In temperate and continental climates, a critical aspect to achieve a long growing season is early emergence combined with good chilling tolerance and early vigour. Several studies have investigated the genotypic variation available for chilling tolerance in the genus *Miscanthus* on the basis of growth rates (Clifton-Brown and Jones 1997, Farrell *et al.* 2006, Purdy *et al.* 2013, Głowacka *et al.* 2014), photosynthesis-related characteristics (Purdy *et al.* 2013, Friesen *et al.* 2014, Głowacka *et al.* 2014), and/or soluble sugar contents (Purdy *et al.* 2013). With the exception of the study of Yan *et al.* (2011), who analysed plant growth in field trials at different locations, most studies have mainly focused on the comparison of plants grown at optimal conditions with plants at low temperatures in controlled environments. In addition, ecophysiology studies have mainly investigated the effects of short-term chilling stress. This might not be representative of the field situation, however, net yield gains due to increased chilling tolerance are only to be expected in genotypes able to keep growing during longer periods of exposure to low temperatures (but still above the critical point of irreversible tissue damage). Furthermore, it should be noted that the *M.* × *giganteus* genotype might comprise clones from different sources with slightly different responses to chilling stress, making difficult extrapolation of results among studies and the comparison of field and growth chamber results of different studies. This ambiguity migth explain some of the apparent contradictory conclusions about the chilling tolerance of *M. x giganteus* in literature, as the link between field performance of a particular genotype and physiological aspects that might be responsible for chilling tolerance has rarely been explored using the same source material. Notable exceptions are the studies by Friesen *et al.* (2014) and Głowacka *et al.* (2015), who compared photosynthesis under a controlled environment with measurements of the quantum efficiency of PSII (F_v/F_m) of clonal replicates in the field, but only with a rather limited set of field measurements. In the experiments presented here, we used clonal replicates of two high yielding *Miscanthus* genotypes to deepen our understanding of the relationship between chilling tolerance characteristics and biomass accumulation in the field. A thorough comparison of the field performance and the physiological and growth response to chilling temperatures was carried out using one *M.* × *giganteus* clone and one *M. sinensis* Goliath clone. *M.* × *giganteus* was chosen because it is the most planted and studied *Miscanthus* genotype, while *M. sinensis* Goliath has been included in several field trials (Robson *et al.* 2011, Van Hulle *et al.* 2012, Zub *et al.* 2012a, Larsen *et al.* 2013) and physiological studies (Clifton-Brown and Jones 1997, Vargas *et al.* 2002, Zub *et al.* 2012b, Domon *et al.* 2013, Purdy *et al.* 2013). Similar to a report from Denmark (Larsen *et al.* 2013), *M.* × *giganteus* was consistently higher yielding than *M. sinensis* Goliath in a field trial established in Melle, Belgium in 2007 (Muylle *et al.* 2015). *M.* × *giganteus* has been reported to display a relatively smaller decline in leaf elongation rate (LER) when transferred from 28 to 12°C than that of *M. sinensis* Goliath, and a higher photosynthetic rate at 28 and 12°C (Purdy *et al.* 2013). This indicates a higher tolerance to chilling in *M.* × *giganteus*, as also shown by Clifton-Brown and Jones (1997). The main purpose of the present work was to create basic comparison tools to investigate chilling tolerance in *Miscanthus* in relation to field performance. In a later stage, this may enable the screening of a large collection of genotypes. The following specific questions were investigated: (*1*) How do shoot formation and shoot elongation rates early in the season relate to leaf growth measurements in the growth chamber? (*2*) Do these two high yielding genotypes use similar strategies to cope with chilling stress?

Materials and methods

Experiment 1, field trial: Field measurements were performed on a field trial established in Melle, Belgium (51°0'N, 3°48'E, light sandy loam soil, temperate maritime climate) in 2007; the results have been partly reported previously (Van Hulle *et al.* 2012, Muylle *et al.* 2015). The trial consisted of two parts. In the first part, in which the yield was determined, the crops *Miscanthus* (one *M.* × *giganteus* clone and one *M. sinensis* Goliath clone), switchgrass, willow, maize, sorghum, and several forage grasses were planted according to a randomized block design with three replicates. The *M. sinensis* Goliath plants (‘Goliath’ in what follows) were obtained from *Bruckeveld* (Belgium) and the *M.* × *giganteus* plants (‘Giganteus’ in what follows) were obtained from *Agrimiscanthus* (The Netherlands). The *Miscanthus* plots (3.6 × 7 m) were harvested once a year from 2008 to 2013, with a cutter bar (*Agria-Werke GmbH*, Möckmühl, Germany) by the end of February/ beginning of March. For further details see Muylle *et al.* (2015). In the second part, plant growth measurements were performed. This part of the field trial was a complete randomized block containing 26 *Miscanthus* genotypes with three repetitions per genotype. Each repetition consisted of one row of ten plants (distance between rows was 1 m, distance between plants within the row was 0.6 m). In each plot of Giganteus and Goliath, one plant was marked for measurements in March 2013. Prior to the beginning of the growing season (26 March 2013), all plants were cut to 5 cm above ground level. Three times per week, the length of five marked shoots per plant was measured from soil level to the tip of the highest leaf using a ruler, and the number of shoots longer than 5 cm was counted. Average daily air temperature was recorded in a weather station approximately 100 m from the field trial.

Experiment 2, controlled environment: Rhizomes of the two genotypes investigated were harvested in February 2012 in the field trial described above (from plants not used for measurements) and stored at 3°C in plastic trays covered with potting soil until used. To generate plantlets, rhizomes were cut into pieces of approximately 10 cm long, planted in 3L containers in potting soil (*Saniflor Beroepspotgrond*, *Van Israel NV*, Geraardsbergen, Belgium) and allowed to form shoots in the greenhouse [20°C, minimum 150 µmol(photon) m^{-2} s^{-1} PAR, 16-h photoperiod]. Ten plants per genotype were moved to a growth chamber when three leaves had formed on one of the shoots, while ten other plants per genotype remained in the greenhouse. To avoid border effects, both in the growth chamber and greenhouse the plants used for measurements were surrounded by one line of plants of the same genotype. Conditions in the growth chamber (*Weiss Umwelttechnik GmbH*, Reiskirchen, Germany) were 12°C, 70% of relative humidity, 150 µmol(photon) m^{-2} s^{-1} PAR, 16-h photoperiod. Plants were watered weekly using rainwater, no fertilizers were added.

Leaf growth analysis: The length of the fourth emerging leaf on one shoot per plant was measured five times per week with a ruler. A sigmoid function was fitted to the data using the *LEAF-E Excel* macro developed by Voorend *et al.* (2014). The derivative of the sigmoid function, representing the leaf elongation rate (LER), was also calculated using this tool. In these calculations, t_0 was set to the start of the experiment. A good fit of the sigmoid curves to the leaf-length measurements was obtained, with $R^2 > 0.97$ for all plants of both genotypes and temperatures. For representation purposes, average growth curves per genotype were calculated based on the average values of the model parameters in *STATISTICA* (*StatSoft Inc.*, Tulsa, OK, USA) and as described in Voorend *et al.* (2014).

Photosynthesis and chlorophyll (Chl) fluorescence: Photosynthesis measurements were conducted using a *Li-COR 6400XT* (*Li-COR Biosciences*, Lincoln, NE, USA) in a temperature-controlled growth chamber (*Weiss Umwelttechnik GmbH*, Reiskirchen, Germany). Net photosynthesis (P_N) and Chl fluorescence were measured through light-response curves. Six plants per temperature and per genotype were monitored. Plants were measured at the temperature they were grown. Basic fluorescence (F_0) was measured after a dark-adaptation period of 30 min. A saturation pulse was then given to determine maximum fluorescence (F_m). Actinic light was then set to an intensity of 1,000 µmol(photon) m^{-2} s^{-1} PAR. After 30 min under actinic irradiance, a saturation pulse was given again. Thereafter every 3 min a saturation pulse was given, and after each saturation pulse the light intensity was lowered subsequently to 750, 500, 250, 100, 50, and 25 µmol(photon) m^{-2} s^{-1} PAR. Actinic light was then switched off and three extra measurements were made with 3 min intervals. Leaf light absorptance could not be measured; instead the standard settings of the *Licor 6400XT* were used (absorptance of blue light 0.92, absorptance of red light 0.87).

Chl measurements: The Chl content was estimated after each photosynthesis measurement using a *CCM-200* Chl meter (*Opti-Sciences Inc*., Hudson, NH, USA). The output was expressed in a Chl concentration index (CCI), defined as the ratio of transmission at 931 to 653 nm through a leaf (*Opti-Sciences Inc*., USA). For each leaf three Chl content measurements were performed next to the area where photosynthesis had been measured, and the average was calculated.

Sugar content: Leaf samples were taken after the completion of the growth measurements and after 10 h of light. Three mature leaves per plant were cut, stored in paper envelopes, and immediately frozen in liquid nitrogen. The leaves were then freeze dried, vacuum sealed, and stored at room temperature. The samples were ground using a *Retsch Tissuelyser II* (*Retsch*, Haan, Germany). A 40 mg subsample was weighed and mixed with 1.6 mL of MQ water in a 2 mL Eppendorf tube. Samples were then heated for 15 min in a warm water bath at 90°C and centrifuged for 15 min at 20°C and 14,000 rpm. The supernatant (200 µL) was pipetted onto *Dowex* columns to remove charged ions. These columns were rinsed six times with 200 µL of MQ water; the water was collected together with the sample. The soluble sugar content of the samples was then analysed using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC IPAD) (*Thermo-Fischer Scientific*, Waltham, MA, USA) as described in Zhang *et al.* (2015).

Statistical analyses: Differences in leaf growth parameters between treatments or between genotypes were analysed using *t*-tests. Differences in photosynthesis, Chl fluorescence parameters, and sugar contents were analysed independently for each light intensity using analysis of variance (*ANOVA*). The effect of genotype and temperature on Φ_{PSII}/Φ_{CO2} was analysed through multiple linear regression with dummy variables coding for temperature and genotype. All analyses were performed in *STATISTICA v. 12* (*StatSoft Inc*., USA).

Results and discussion

Growth dynamics in the field and under controlled conditions: Giganteus consistently yielded more biomass per hectare than Goliath over a course of seven years (19.1 ± 1.5 t ha^{-1} for Giganteus and 10.1 ± 3.0 t ha^{-1} for Goliath; Table 1). Both genotypes reached maturity after three years (Muylle *et al.* 2015), after which the yield was relatively stable. Giganteus was thus higher yielding compared to Goliath in Flanders, which is consistent with the findings of Larsen *et al.* (2013) for Denmark. The higher chilling tolerance of Giganteus is unlikely to be the only factor of its higher yield, as the genotypes also differ in their morphology: Giganteus has taller and thicker stems, which is another factor correlated with the high yield in *Miscanthus* (Zub *et al.* 2012a, Robson *et al.* 2013a, Arnoult *et al.* 2015). Moreover, the end of Goliath's growing season occurred earlier because it flowered earlier than Giganteus, which had not even flowered every year under Flemish growth conditions. In the field, both genotypes started growing shortly when the mean weekly temperature rose above 8°C (Fig. 1*A*). Giganteus resumed its growth from underground rhizome buds, while the growth of Goliath was partly the result of the elongation of shoots formed the year before and of newly formed shoots. As a consequence, emerging shoots appeared above ground later in Giganteus than those in Goliath (Fig. 1*B*). Goliath reached an average height of 5 cm at day of the year (DOY) 110 (20 April), while Giganteus only reached this height at 115 DOY (25 April); both genotypes reached an average height of 10 cm at 120 DOY (30 April), after which Giganteus surpassed Goliath. After DOY 120, when the average temperature was around 12°C, Giganteus had an average growth rate of 2.7 ± 0.3 cm per day, while Goliath had an average growth rate of 1.9 ± 0.4 cm per day. Thus, early in the season, when temperature varied between 8 and 12°C, Giganteus displayed the higher growth rate than that of Goliath. Shoots of Giganteus emerged later than those of Goliath, but had a higher growth rate afterwards. This conferred Giganteus an advantage over Goliath at the start of the growing season. Under controlled conditions, Goliath produced significantly longer leaves at 20°C than Giganteus did, but when grown at 12°C, the final leaf length (L_m) was reduced more in Goliath (24%) than that in Giganteus (13%) (Table 2). Leaf length reductions caused by chilling stress have also been reported for maize (Rymen *et al.* 2007). If chilling stress also causes Goliath leaves in the field to be significantly shorter than under optimal growth temperatures (not tested in this study), this could potentially reduce the total leaf area of the plant and thereby affect the plant growth rate as the photosynthetically active leaf area is affected. Whole plant leaf area at a given moment is affected by leaf elongation rate (LER) and leaf elongation duration (LED) (Arredondo and Schnyder 2003, Bultynck *et al.* 2004) and has been used to describe the influence of environmental factors, such as temperature (Sadok *et al.* 2007) or drought (Chenu *et al.* 2008) on plant growth. Similar to reports on maize (Bhosale *et al.* 2007), it is possible to describe cold tolerance in the early stages of development in *Miscanthus* by comparison of LER under optimal conditions and at low temperatures. While LER_{max} was lower at 20°C for Giganteus more than for Goliath (4.5 and 5.4 cm per day, respectively), the opposite was true at 12°C (2.2 and 1.9 cm per day, respectively) (Fig. 2). This is in agreement with the higher shoot elongation rate early in the season

Table 1. Average yield [t ha^{-1}] with standard deviation of Giganteus and Goliath in the field trial in Melle, Belgium installed in 2007. Plots were harvested in February–March each year.

Genotype	2008	2009	2010	2011	2012	2013	2014
Giganteus	3.3 ± 0.7	15.4 ± 0.6	25.7 ± 1.0	19.8 ± 1.3	28.0 ± 4.6	17.9 ± 1.2	23.9 ± 1.2
Goliath	0.5 ± 0.1	4.1 ± 0.5	14.0 ± 3.3	14.3 ± 3.3	13.2 ± 4.7	12.8 ± 4.7	11.8 ± 4.4

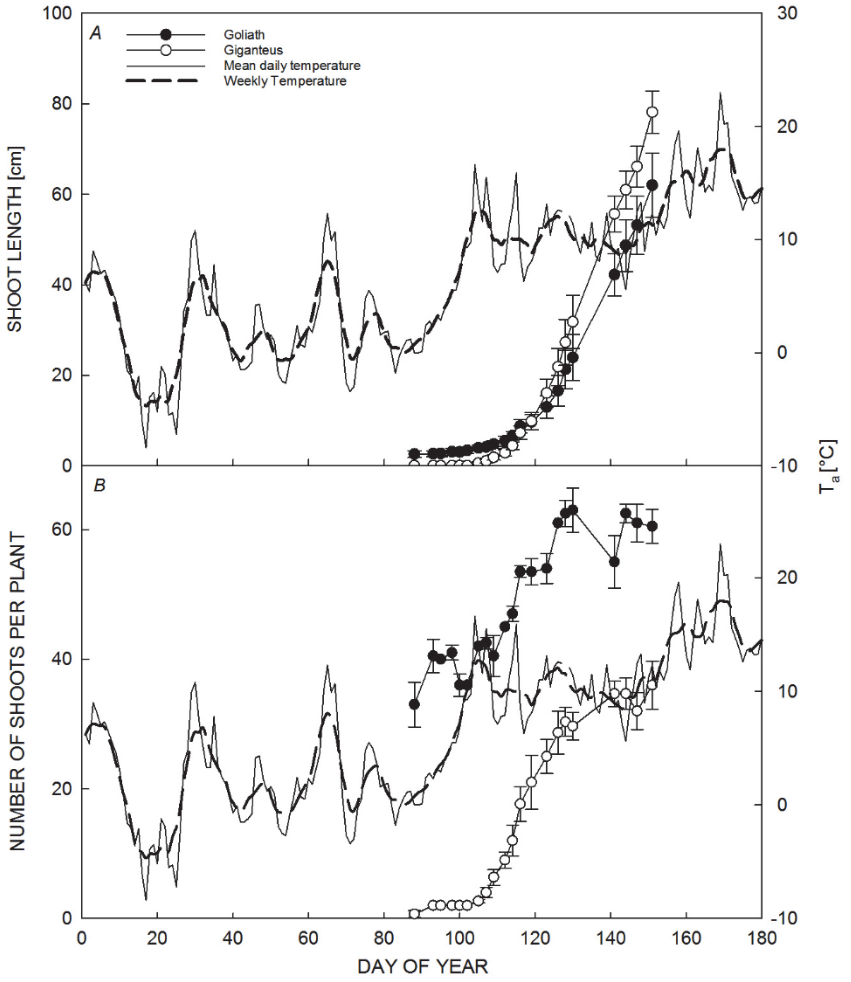

Fig. 1. Average shoot length (*A*) and a number of shoots per plant (*B*) of Giganteus and Goliath in the field trial and mean daily and weekly air temperature (T_a) in the spring of 2013. Error bars show standard errors ($n = 15$).

Table 2. Leaf growth parameters of Giganteus and Goliath calculated by *LEAF-E* as a function of time. L_m – maximum leaf length, $\text{LER}_{\max}$ – maximum leaf elongation rate, $\text{LED}_{10–90\%}$ – duration of leaf elongation from 10 to 90% of maximum leaf length. Parameters marked with ^{a} are significantly different (*t*-test, $p<0.05$) between the two genotypes at the same temperature, while parameters marked with ^{b} show significant differences of one genotype between the two temperature levels.

Parameter	Giganteus 20°C	12°C	% change	Goliath 20°C	12°C	% change
L_m [cm]	92.3 ± 2.6^a	80.2 ± 5.9	–13	113.7 ± 4.1^{ab}	86.2 ± 5.5^b	–24
$\text{LER}_{\max}$ [cm d^{-1}]	4.5 ± 0.2^{ab}	2.2 ± 0.1^{ab}	–50	5.4 ± 0.2^{ab}	1.9 ± 0.1^{ab}	–64
$\text{LED}_{10–90\%}$ [d]	20.0 ± 1.2^b	33.7 ± 1.8^b	68	19.4 ± 0.6^b	40.6 ± 3.0^b	110

under the field conditions. The moment at which $\text{LER}_{\max}$ was reached, $\text{LER}_{\max}$ was not affected by chilling treatment in Giganteus, while in Goliath a delay of 37% was observed (data not shown). The duration of leaf elongation ($\text{LED}_{10–90\%}$) was about 20 d for both genotypes at 20°C, but at 12°C, a lower value was obtained for Giganteus (33.7 d) than that for Goliath (40.5 d). The growth curves were also fitted in a function of accumulated thermal time in order to test whether the plants had the same growth rate per unit of thermal time at both temperatures. However, the best base temperature to calculate thermal time is not known in *Miscanthus* and can vary strongly between genotypes (Farrel *et al.* 2006), making accurate calculation of thermal time difficult. For example, using a base temperature of 8°C, the growth curves of Giganteus at 12 and 20°C overlapped, while those of Goliath did not (data not shown). This could either mean that Goliath is relatively more chilling stressed, or that it has a lower base temperature than Giganteus. Overall, the higher growth rates of Giganteus under the field conditions in the spring can be linked to a relatively smaller decline in leaf growth rate under chilling stress. Relative to 20°C, $\text{LER}_{\max}$ and LED at 12°C were less affected in Giganteus than those in Goliath. Similar results have been reported by Głowacka

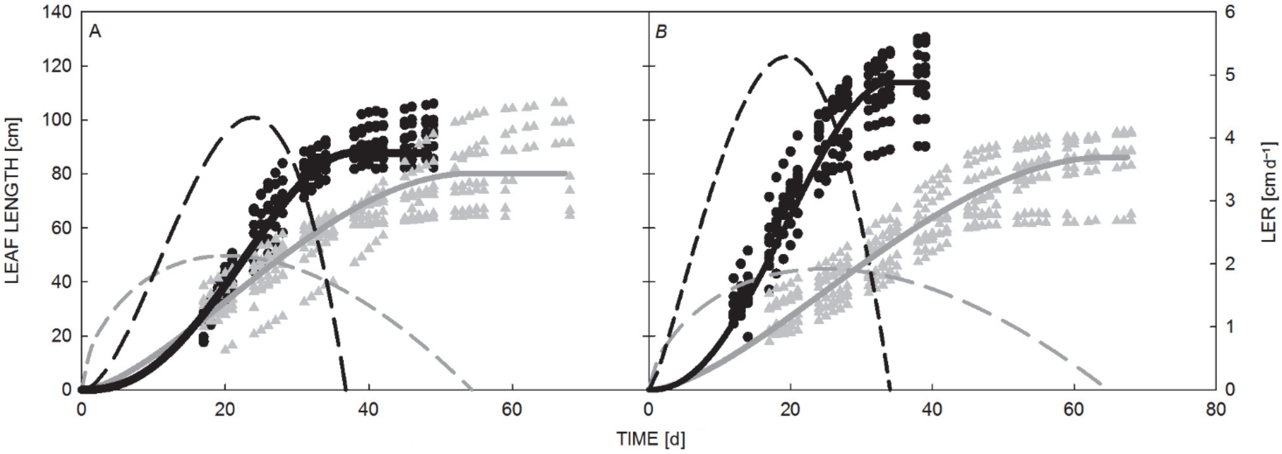

Fig. 2. Growth of the fourth leaf of Giganteus (*A*) and Goliath (*B*) at 20°C (*black*) and at 12°C (*grey*). *Full lines* show the average growth curve per treatment ($n = 10$), calculated using *LEAF-E*. Leaf elongation rates (LER) are shown in *dashed lines*. The actual measurements are represented by symbols.

et al. (2014) in a comparison of a larger set of genotypes. They found that Giganteus was among the genotypes that retained the highest growth rates under chilling stress. On the contrary, Clifton-Brown and Jones (1997) reported a similar temperature response for Goliath and one of the Giganteus accessions investigated, but a relatively higher growth reduction at low temperature for the other Giganteus genotype investigated. The length of the period investigated might lay at the basis of these discrepancies; Clifton-Brown and Jones (1997) investigated the response over a period of 72 h, while Głowacka *et al.* (2014) reported the response over a period of 14 d, which is more similar to the comparisons presented here. It is possible that the initial response of Giganteus to a decrease in temperature is stronger than that of Goliath, but a more realistic representation of the field situation is that the relative response of these genotypes is reversed on the longer term if the low temperature is maintained.

Capacity for carbon assimilation under chilling stress: Giganteus showed slightly higher P_N than Goliath when both grew at 20 and 12°C (Fig. 3*A*). The relative decrease due to a lower temperature at 1,000 µmol(photon) m^{-2} s^{-1} was 67 and 73%, respectively, indicating that Goliath was slightly more affected by the lower temperature. This is in accordance with Purdy *et al.* (2013) who found that when Giganteus and Goliath were transferred from 28 to 12°C P_N declined by 65% in both genotypes over the course of 12 h, but Giganteus also retained a higher P_N than Goliath at both temperatures. In the second experiment, where Giganteus and Goliath were grown and measured at 12 and 20°C (data not shown), similar results were obtained. Moreover, while Goliath had a significantly higher Chl content index per leaf area at 20°C than that of Giganteus (40.4 ± 2.3 and 28.6 ± 1.8, respectively), at 12°C, the Chl content in Goliath became lower and intergenotype differences disappeared (28.4 ± 2.4 and 27.5 ± 1.8, respectively). Lower values of Chl in susceptible *Miscanthus* genotypes under chilling stress have also been reported by Kao *et al.* (1998). This suggests that photosynthesis of Giganteus is better adapted to chilling temperatures and metabolically more active after prolonged chilling stress. This is in agreement with the abovementioned reports. It has been shown that when *M.* × *giganteus* is exposed to prolonged chilling stress, the expression of genes coding for photosynthetic proteins and proteins protecting PSII is increased (Wang *et al.* 2008, Spence *et al.* 2014). It is in contrast with maize, where the expression of these genes decreases under chilling stress. The higher expression allows *M.* × *giganteus* to counteract the lower activity and stability of these enzymes at lower temperatures and to maintain a high photosynthesis under chilling stress, whereas most other C_4 plants, such as maize, show a marked decline in photosynthesis under chilling stress (Wang *et al.* 2008, Spence *et al.* 2014). However, there are no reports concerning this effect in other *Miscanthus* genotypes and we can only speculate about this effect in Goliath. Stomatal conductance (g_s) for both species was the same at 20°C. At a growth temperature of 12°C, Giganteus was able to maintain a higher g_S in comparison to Goliath, 0.064 ± 0.003 and 0.025 ± 0.002 mol(H_2O) m^{-2} s^{-1}, respectively. These values are in the same range as those measured by Głowacka *et al.* (2015) on several *Miscanthus* genotypes at 15°C. The lower g_S found for Goliath grown at 12°C compared to Giganteus was not probably the cause of the lower photosynthesis in the plants. The g_S decreased with temperature but the internal CO_2 concentration was mostly around 200 µmol(CO_2) mol^{-1}, the concentration which is saturating for photosynthesis in *Miscanthus* (Głowacka *et al.* 2015). Głowacka *et al.* (2014) also concluded that stomata close at low temperature in order to adjust for the reduced need for CO_2 due to decreasing photosynthesis; they observed no impairment of stomatal functioning in *Miscanthus* under

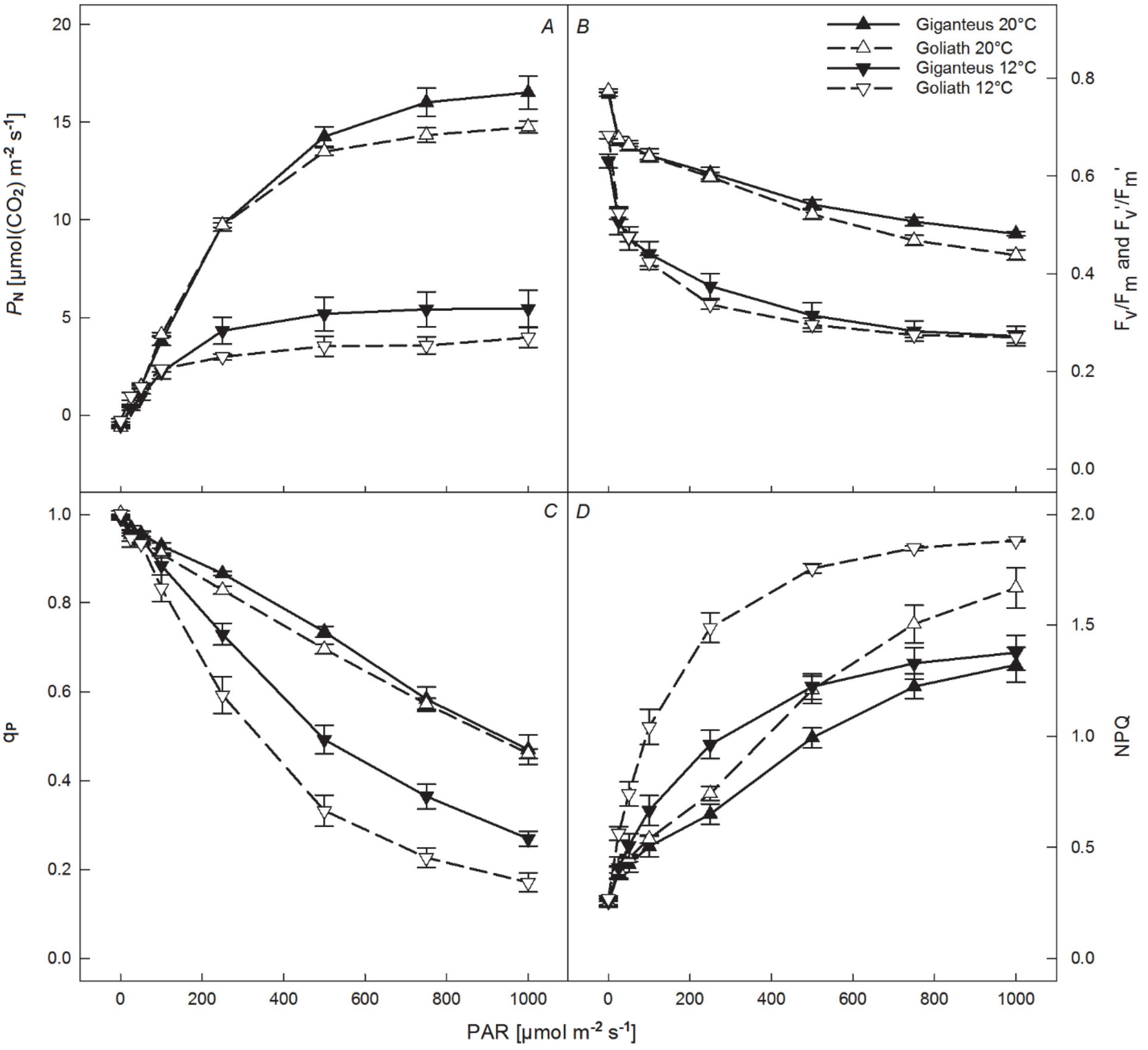

Fig. 3. Net photosynthetic rate (P_N) (*A*), maximum (F_v/F_m, at PAR of 0 µmol m^{-2} s^{-1}) and quantum yield of open PSII reaction centers (*B*), photochemical quenching (q$_P$) (*C*), and nonphotochemical quenching (NPQ) (*D*) of *Miscanthus* × *giganteus* and *M. sinensis* Goliath grown and measured at 20°C and at 12°C at different photosynthetic active radiation levels (PAR). Error bars show standard errors ($n = 6$).

chilling stress. Taken together, the photosynthesis measurements demonstrated that Giganteus was capable of higher P_N at optimal temperatures and displayed a lesser decline after exposure to chilling stress.

Efficiency of the photosynthetic apparatus under chilling stress: Chl fluorescence revealed that the plants grown at 12°C suffered from photoinhibition due to chilling stress. The maximum quantum efficiency of PSII (F_v/F_m) was significantly lower under prolonged chilling stress in Giganteus than that in Goliath contrary to the quantum yield of open PSII reaction centers (F_v'/F_m') (Fig. 3*B*). Low F_v/F_m values are indicative of photoinhibition, the reduction in photosynthetic capacity due to damage to PSII that can occur under abiotic stress (Murchie and Lawson 2013). F_v/F_m of Giganteus and Goliath grown at 20°C was 0.768 ± 0.005 and 0.775 ± 0.003, respectively, and thus not significantly different. Plants grown at 12°C had significantly different F_v/F_m values of 0.631 ± 0.014 and 0.684 ± 0.007 for Giganteus and Goliath, respectively, showing that at 12°C plants suffered from chilling stress. This was a significant reduction of 17.8 and 11.7%, respectively, when compared to plants grown at 20°C, indicating Giganteus suffered relatively more from photoinhibition. Chl fluorescence has been successfully used to distinguish chilling tolerant maize genotypes, where cold tolerant genotypes (described as genotypes possessing good early vigour) have higher F_v/F_m, F_v'/F_m', and Φ_{PSII} at low temperatures (Fracheboud *et al.* 1999, Lootens *et al.* 2004, Peter *et al.* 2009). These findings contrast with our study, where Giganteus showed lower F_v/F_m but also higher growth and photosynthesis than Goliath under chilling stress. Similarly, Friesen *et al.* (2014) and Głowacka *et al.* (2015) measured F_v/F_m on several *Miscanthus* genotypes after cold stress in the field. As expected, values tended to be lower in more cold-sensitive genotypes, but in both studies, the genotypes were identified with relatively high F_v/F_m values and relatively low CO_2-assimilation rates. As mentioned by Murchie and Lawson (2013), a low F_v/F_m, which is determined in the dark, does not necessarily meam a lower photosynthetic rate at high light intensities. Furthermore, the range of F_v/F_m values reported in the maize studies mentioned above is considerably larger than the difference observed in our study between Giganteus and Goliath, suggesting that the significant differences in F_v/F_m found between these two genotypes when grown at 12°C were not an indication of a higher susceptibility to chilling stress in Giganteus. Differences between genotypes were more pronounced for the photochemical (q_P, light energy is used for photosynthesis) and nonphotochemical (NPQ, light energy that is dissipated) quenching in the plants grown at 12°C (Fig. 3*C,D*). For Giganteus, higher q_P values and lower NPQ values were found for irradiances higher than 100 μmol(photon) m^{-2} s^{-1}. At 20°C, q_P was similar for both genotypes, but NPQ was again higher in Goliath. Friesen *et al.* (2014) reported lower values of quantum yield of NPQ associated with photoinactivated PSII and higher values of dark-reversible NPQ in *M.* × *giganteus* than in the other hybrids tested in their study, which was accompanied by a higher P_{max} during and after chilling under controlled conditions and a higher F_v/F_m in the field. Farage *et al.* (2006) found also increased NPQ in *M.* × *giganteus* grown at low temperature, which was associated with higher zeaxanthin and carotenoid contents. It should be noted, however, that the calculation of NPQ depends on the dark-adapted F_v/F_m, and plants differing in F_v/F_m therefore cannot be directly compared. However, the differences between Giganteus and Goliath were substantial and were indicative of a difference in dissipation of excess light energy. The role of NPQ in chilling tolerance in *Miscanthus* should be studied more deeply. The relationship between Φ_{PSII} (the fraction of absorbed photons that are used for photochemistry for a light adapted leaf based on the Chl fluorescence measurements) and the quantum yield of photosynthesis (Φ_{CO2}, the quantum yield based on the gas-exchange data) was linear for all measurements (Fig. 4). The slope of the relationship (11.01 ± 0.17) was not different between the genotypes or measuring temperatures. As the Chl content was markedly lower in Goliath at 12°C, this might have influenced light absorptance (not measured in this study) and thus accurate determination of Φ_{CO2}. However, this would not influence the linearity of the relationship between Φ_{CO2} and Φ_{PSII} (Genty *et al.* 1989). The values obtained here were similar to those of cold-stressed maize (Leipner *et al.* 1999, Naidu and Long 2004) and showed no indication of markedly increased transport of electrons to alternative electron sinks, other than to CO_2, such as the Mehler reaction, at lower temperatures. In other studies, alternative electron sinks were observed in *M.* × *giganteus* only when grown at 10°C but not in plants grown at higher temperatures (Naidu and Long 2004, Farage *et al.* 2006). In contrast, maize leaves formed in the field early in the growing season show a higher rate of electron transport through PSII than that is needed for CO_2 assimilation (Fryer *et al.* 1998). However, Naidu and Long (2004) did not observe this in maize grown in a growth chamber at 14/11°C. Overall, the photosynthesis of neither genotype was markedly disturbed at the temperatures measured here. Stomata closed in accordance to CO_2 demand and little light energy was diverted to alternative electron sinks. However, Giganteus exhibited the higher assimilation rate than Goliath, even at 12°C. This seems to be related to

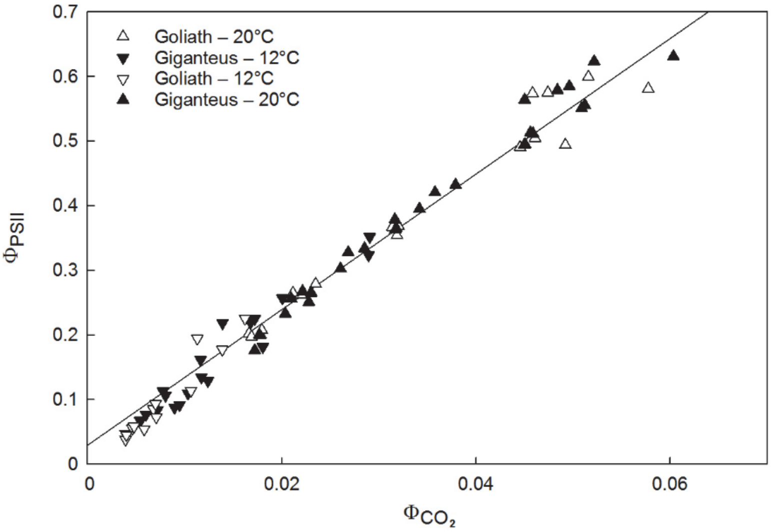

Fig. 4. Relationship between PSII operating efficiency (Φ_{PSII}) and quantum yield of CO_2 (Φ_{CO2}) assimilation in the plants grown at 20°C (*upward triangles*) and 12°C (*downward triangles*) of Giganteus (*black symbols*) and Goliath (*white symbols*). assimilation. Regression line for both genotypes and temperatures ($R^2 = 0.983$).

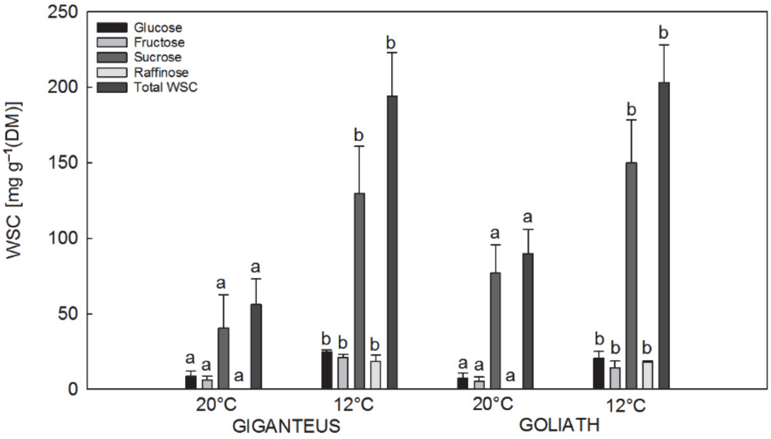

Fig. 5. Water soluble carbohydrate concentrations (WSC) in mature leaves of Giganteus and Goliath grown at 12 and 20°C. Error bars show standard errors (n = 4). *Letters* indicate significant differences between genotypes and temperatures for a specific WSC. DM – dry mass.

a more efficient use of light energy. The lower NPQ and higher q_P in Giganteus showed that this genotype dissipated less light energy as heat and was able to utilize more light energy for photochemistry.

Changes in sugar content associated to chilling treatment: The concentration of soluble sugars in leaves was measured at the end of the leaf growth measurements at both 20 and 12°C (Fig. 5). At both temperatures, sucrose was the most abundant sugar in leaves, with a concentration of 61 ± 14 mg g^{-1}(DM) at 20°C and 140 ± 20 mg g^{-1}(DM) at 12°C. The plants grown at 12°C had significantly higher contents of all measured water soluble sugars than those grown at 20°C. This agrees with previous reports in different grasses and other species under chilling stress (Koster and Lynch 1992, Equiza *et al.* 1997, Morsy *et al.* 2007, Tarkowski and Van den Ende 2015). As the variation among replicates was large, differences between the genotypes were not significant at both temperatures. Within both genotypes, raffinose concentration and total sugar concentration were significantly higher at 12°C. Although the relation between the accumulation of soluble sugars and chilling tolerance is not straightforward, there is often a correlation between compatible solute pools and chilling tolerance (Tarkowski and Van den Ende 2015). In sugarcane, chilling-tolerant varieties accumulate sucrose in the leaves after a chilling shock, but chilling-sensitive varieties do not (Du and Nose 2002). In contrast, maize genotypes tolerant to chilling have been found to accumulate lower sugar concentrations in the leaves than sensitive ones (Hodges and Andrews 1997). In *Miscanthus*, glucose, fructose, and sucrose have been shown to increase rapidly in the first 12 h after a sudden chilling shock; but the accumulation in Goliath happens faster than that in Giganteus (Purdy *et al.* 2013). To date, an increase in soluble sugars after prolonged chilling stress has not yet been reported in *Miscanthus*. Raffinose concentration displayed the strongest response to temperature. At 20°C, raffinose concentrations were very low with 0.21 ± 0.07 and 0.22 ± 0.04 mg g^{-1}(DM), while the concentration at 12°C was significantly elevated to 18.5 ± 4.4 and 18.0 ± 1.0 mg g^{-1}(DM) in Giganteus and Goliath, respectively. There are no reports of the accumulation of raffinose in *Miscanthus* yet, but Spence *et al.* (2014) found that several enzymes of the raffinose synthesis pathway are upregulated in Giganteus under chilling stress. The accumulation of soluble sugars can be a result of a reduced sink demand by reduced growth and respiration. Sugars may function as stress signals (Van den Ende and El-Esawe 2014), protect membranes or proteins (Keunen *et al.* 2013), or could be involved in direct scavenging of reactive oxygen species (ROS), such as hydroxyl radicals (Matros *et al.* 2015). In *Arabidopsis*, cold tolerance studies demonstrated that a high capacity for sucrose synthesis (Nägele *et al.* 2012) and high sucrose/hexose balances during early stress stages are associated with a tolerance (Nägele and Heyer 2013), indicating that a certain sucrose threshold value should be passed to initiate sugar-mediated signalling as well as for raffinose biosynthesis. In our experiments, growth slowed at 12°C, thus, the accumulation of glucose, fructose, and sucrose could occur due to source sink imbalance. However, raffinose was not produced in absence of chilling stress, therefore the accumulation of this sugar was more than merely a result of a decline in the sink demand. Raffinose has been shown to protect cells against chilling stress. It stabilizes cell membranes (Valluru and Van den Ende 2008, Janská *et al.* 2010) and could play a role in the protection against oxidative stress (Nishizawa *et al.* 2008). It can be speculated that raffinose increases under cold stress may not necessarily lead to improved cold tolerance (Nägele and Heyer 2013). Perhaps the capacity to import raffinose into chloroplasts (Schneider and Keller 2009) may be a crucial factor in this respect. Raffinose may be specifically involved in the protection of photosystems and overall chloroplast stability under cold, through ROS scavenging and/or other mechanisms (Matros *et al.* 2015).

Conclusions: Although Giganteus produced higher biomass yields than Goliath, Giganteus did not start growing earlier than Goliath but rather had a higher growth rate early in the spring under field conditions. The higher growth rate of Giganteus in the field was reflected by the relatively faster leaf growth rate under chilling stress under controlled conditions in the growth chamber. The higher growth rate was supported by a higher photosynthesis at low temperatures under controlled conditions. If the results obtained in the growth chambers hold true under field conditions, this could allow Giganteus to form a canopy faster and assimilate more carbon early in the growing season. However, both genotypes showed remarkable chilling tolerance for plants with C_4 photosynthesis. Both genotypes could form new, photosynthetically active leaves at a constant temperature of 12°C. Chlorophyll fluorescence indicated that Giganteus was relatively more photoinhibited when growing at low temperatures, but could use more light energy than Goliath. Under field conditions in the spring, Giganteus can have a higher photosynthetic capacity than that of Goliath. Screening a larger collection of *Miscanthus* genotypes for higher photosynthesis and growth at low temperatures might thus reveal useful variation that would allow breeders to produce more chilling-tolerant varieties.

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