Simon Fonteyne

EVALUATION OF CHILLING AND FROST STRESS TOLERANCE IN MISCANTHUS: FROM WINTER SURVIVAL AND EARLY-SEASON GROWTH TO FINAL BIOMASS YIELD

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences: Agriculture

Promotors: Dr. ir. Peter Lootens
Plant Sciences Unit, Research Institute for Agricultural, Fisheries and Food Research, Caritasstraat 39, 9090 Melle (Belgium)

Prof. dr. ir. Dirk Reheul
Department of Plant Production, Ghent University,
Coupure Links 653, 9000 Ghent (Belgium)

Members of the jury: Prof. dr. ir. Kris Verheyen
Department of Forest and Water Management, Ghent University, Geraardsbergsesteenweg 267, 9090 Gontrode (Belgium)

Prof. dr. ir. Christian Stevens
Department of Sustainable Organic Chemistry and Technology, Ghent University, Coupure Links 653, 9000 Ghent (Belgium)

Prof. dr. Isabel Roldán-Ruiz
Plant Sciences Unit, Research Institute for Agricultural, Fisheries and Food Research, Caritasstraat 39, 9090 Melle (Belgium) and
Department of Plant Biotechnology and Bioinformatics, Ghent University, Technologiepark 927, 9052 Ghent (Belgium)

Prof. dr. ir. Marie-Christine Van Labeke
Department of Plant Production, Ghent University,
Coupure Links 653, 9000 Gent (Belgium)

Prof. dr. Ivan Nijs
Department Biology, Plant and Vegetation Ecology, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk (Belgium)

Dr. Paul Robson,
IBERS, Energy Crop Biology, Aberysthwyth University, Penglais, Aberystwyth, Ceredigion, SY23 3DA (United Kingdom)

Dean: Prof. dr. ir. Marc Van Meirvenne

Rector: Prof. Anne De Paepe

Dutch translation of the title:

Evaluatie van kilte- en vorststresstolerantie in miscanthus: Van winteroverleving en groei in het begin van het seizoen tot uiteindelijke biomassaopbrengst.

Illustration on the cover: UAV VIS image of flowering and senescent Miscanthus genotypes on the miniplots and cold tolerance trials at ILVO, Melle, Belgium. Photo taken on 12/10/2015 by Peter Lootens.

Citation: Fonteyne, S., 2017. *Evaluation of chilling and frost stress tolerance in miscanthus: from winter survival and early-season growth to final biomass yield*. PhD thesis, Ghent University, Ghent, Belgium.

The research preented in this thesis was funded by European Union's 7th framework project OPTIMISC (grant agreement no. 289159).

ISBN: 978-90-5989-973-5

Woord vooraf

Alhoewel slechts mijn naam op de kaft staat is deze thesis het resultaat van vele jaren werk door een hele groep. Daarom gaat mijn dank uit naar iedereen die aan deze thesis meegewerkt heeft, in het bijzonder aan:

Peter Lootens, Isabel Roldán-Ruiz, Tom De Swaef, Hilde Muylle en Dirk Reheul voor het uitdenken en schrijven van deze thesis.

Katleen Sucaet, Ariane Stalens, Carina Pardon, Thomas Vanderstocken, Luc Van Gyseghem, Nancy Mergan, en Katrien Liebaut voor hun onmisbare hulp in het uitvoeren van de experimenten. Mijn excuses als ik jullie in de kou heb laten staan, maar dat was nu eemaal niet te vermijden met zo`n onderwerp.

De studenten Robbrecht Cardon, Ellen De Sutter, Kim Maes, Evelien Mortaignie, Seppe Vantomme en Laurens Vergauwen, elk leverde een belangrijke bijdrage aan dit werk.

De mensen van OPTIMISC, Iris Lewandowski, John Clifton-Brown, Luisa M. Trindade, Gerard Van Der Linden, Kai-UweSchwarz, Karl Müller-Sämann, Alexander Anisimov, Charley Chen, Oene Dolstra, Kerrie Farrar, Graham Harding, Astley Hastings, Laurie Huxley, Yasir Iqbal, Nikolay Khokhlov, Andreas Kiesel, Heike Meyer, Michal Mos, Chris Nunn, Mensure Ozguven, HeinrichSchüle, Ivan Tarakanov, Tim Van Der Weijde, Qingguo Xi en Olena Kalinina, het was een enorm voordeel om resultaten en ervaringen met hen te kunnen delen.

Wim van den Ende en Rudy Vergauwen van KU Leuven wiens hulp en expertise op het vlak van suikers onmisbaar was en Pavel Kerchev van het VIB voor zijn hulp bij het analyseren van ROS.

Verder ook Michael, Jochen en Margot voor hun morele steun en vooral ook Carolina, zonder wie ik het niet had kunnen doen.

Contents

Summary

Miscanthus is a genus of perennial C4 grasses originating in East Asia. *M. x giganteus*, a sterile triploid hybrid between *M. sinensis* and *M. sacchariflorus*, is increasingly used as a biomass crop. In this thesis the species is referred to as `*Miscanthus*`, while the crop is referred to as `miscanthus`. The biomass produced by miscanthus can be used for numerous purposes, for example as burning fuel for heating, feedstock for second generation bio-ethanol, mulch, bedding in stables or in construction materials. *M. x giganteus* is able to produce high biomass yields in comparison to other biomass crops and this for only limited inputs of fertilizers, pesticides and labor. It is a robust crop, ideally suited for marginal lands. It can be easily harvested using widely available harvest machinery and generally does not need drying after harvest. However, due to the sterility of *M. x giganteus* it has to be propagated vegetatively, either using rhizome cuttings or *in vitro* propagated plantlets. Both methods are expensive and put a limit on the uptake of the crop by farmers. Currently, breeders are developing new, seed based varieties of miscanthus that will bring down establishment costs drastically and will allow farmers to choose varieties better suited to local conditions. Ideally, these new varieties will be as high or even higher yielding than *M. x giganteus*.

The main limits to miscanthus biomass yield are abiotic stresses such as low temperatures (frost and chilling), drought or salinity. This thesis focuses on the tolerance of miscanthus to frost and chilling stress. These stresses limit miscanthus yield in different ways. Frost damage, for example, has been reported to be the main cause of winter mortality in *M. x giganteus* in the first year after planting. Developing varieties that can withstand lower temperatures in winter would allow expanding the potential miscanthus growing area to colder regions, for example Eastern and Northern Europe, regions were more marginal land is available. Frost damage can also kill above ground plant parts in early spring, causing the death of young plants or decreased biomass yield in older plantations. Tolerance to chilling stress is essential in developing early emerging varieties with a strong early-season growth and an early canopy formation. Varieties that are able to grow faster at low temperatures and develop a canopy earlier in the growing season would be able to intercept more solar radiation throughout the year and could theoretically produce more biomass than *M. x giganteus*.

The general aims of the research in this thesis were to study the variation in frost tolerance, chilling tolerance and early-season growth in miscanthus, to determine the underlying physiological and biochemical mechanisms and to establish the relationship between early-season growth and final biomass yield.

Compared to related C4 crops, such as maize, sugarcane or sorghum, miscanthus tends to be relatively cold tolerant and has been subject of several studies, most of which compared *M. x giganteus* with maize. These studies have shown that, while maize has higher maximum

photosynthetic rate, *M. x giganteus* is able to photosynthesize at lower temperatures, and this, combined with a longer growing season results in higher yields of *M. x giganteus* compared to maize. While maize under chilling stress displays a reduction in the activity of photosynthetic enzymes, *M. x giganteus* has been reported to show an increase in the activity and RNA expression of photosynthetic enzymes when exposed to chilling stress. It is thought that this higher expression counteracts the lower enzyme kinetics at lower temperatures, allowing *M. x giganteus* to maintain its higher photosynthetic rates. Although a couple of studies have compared different miscanthus genotypes under chilling stress in growth chamber experiments, to date little is known about the underlying mechanisms that distinguish genotypes differing in chilling tolerance and about the variation in chilling tolerance and early-season growth under field conditions.

We obtained, through the OPTIMISC EUFP7 project, a collection of over 100 miscanthus genotypes, comprising of *M. sinensis, M. sacchariflorus, M. sinensis x sacchariflorus* and *M. x giganteus* accessions. These genotypes were evaluated and compared in growth chamber experiments and in field trials. Additional data was obtained from trials already established at ILVO before the start of the project and from other trials in the OPTIMISC project.

In order to screen the variation in rhizome frost tolerance available in the germplasm collection, we determined the temperature at which 50% of the rhizomes were killed (LT_{50}) in 95 miscanthus genotypes. The LT_{50} in the collection ranged between −0.4 and −5.9°C, while the average LT_{50} for *M. x giganteus* was −2.6 ± 0.3°C. On average LT_{50} was −3.5 ± 0.1°C in *M. sinensis*, −2.6 ± 0.3°C in *M. sacchariflorus* and −3.9 ± 0.2°C in the *M. sinensis x sacchariflorus* hybrids. Rhizome frost tolerance was correlated to the timing of flowering and senescence but not to rhizome moisture content. Wide variation in shoot damage was observed in field-grown plants after a cold spell in early spring. Determination of apex height indicated that the shoot apex was probably still below ground when the frost event occurred, explaining the rapid recovery of damaged shoots later on. This study was the largest screening of rhizome and shoot frost tolerance in miscanthus reported to date and demonstrated the availability of frost tolerant genotypes in the miscanthus breeding material, potentially supporting the development of new frost tolerant varieties.

To investigate the variation available for chilling tolerance, we first investigated two highly productive miscanthus genotypes, *M. x giganteus* and the *M. sinensis* 'Goliath'. Measurements in the field as well as under controlled conditions were combined to create basic comparison tools in order to investigate chilling tolerance in miscanthus in relation to its field performance. Under field conditions, *M. x giganteus* was higher yielding and had a faster growth rate early in the growing season. Correspondingly, *M. x 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 non-photochemical quenching in *M.* x *giganteus* than that in *M. sinensis* 'Goliath' when exposed to chilling temperatures. Soluble sugar content markedly increased in both genotypes when grown at 12°C compared to 20°C. The results showed that while growth chamber screening might be useful to distinguish chilling tolerance, validation in field trials is necessary because of the variable conditions in the field.

Using the variation in early-season growth in the germplasm collection using a common garden experiment in Belgium during two seasons, and compared these results to those obtained under controlled conditions at low temperature and to observations of early-season growth in the OPTIMISC multi-location field trial in six locations across Europe and Turkey. A large variation in early-season growth was observed among the genotypes in both seasons, with strong between-year correlation for most parameters investigated. Several genotypes, both *M. sinensis, M. sacchariflorus* as well as *M. sinensis* x *sacchariflorus* hybrids displayed stronger early-season growth than *M. x giganteus*. The observations in the multi-location trial showed a strong genotype by environment interaction indicating that locally adapted genotypes are necessary in order to maximally take advantage of an extended growing season. The substantial variation in early-season growth parameters indicates that selecting for early emergence and chilling tolerance should be possible. Shoot length based traits evaluated in a field trial were most consistent between years and appear well suited to screen large germplasm collections for early-season growth.

The chilling tolerance of *M. x giganteus* has been predominantly studied under controlled conditions and our understanding of the underlying mechanisms contributing to chilling tolerance in the field and their variation in different miscanthus genotypes remains largely unexplored. To address these questions, we selected five miscanthus genotypes which varied chilling sensitivity and scored a comprehensive set of physiological traits throughout the spring season. Chlorophyll fluorescence was measured as indication of photosynthesis and leaf samples were analyzed for biochemical traits related to photosynthetic activity (chlorophyll content and PPDK activity), redox homeostasis (malondialdehyde, glutathione and ascorbate contents, and catalase activity) and water soluble carbohydrates content. The overall physiological response of chilling tolerant genotypes was distinguishable from that of chilling sensitive genotypes, while *M. x giganteus* was intermediate between both groups. Chilling tolerant genotypes were characterized by higher levels of malondialdehyde, raffinose, sucrose and higher catalase activity while the chilling sensitive genotypes were characterized by higher concentrations of glucose, fructose and higher pyruvate-Pi-dikinase activity later in the growing season. *M. x giganteus* responses were similar to the tolerant genotypes early in the growing season, but more similar to the chilling sensitive genotypes, which also combined a high biomass yield, later on.

Early emergence and early canopy formation theoretically allow plants to intercept more radiation in the long days in spring which would in turn allow them to produce more biomass. Reports in literature are contrasting however and therefore we also studied the relationship between early-season growth and biomass production. We combined the early-season growth measurements with further growth measurements performed throughout the rest of the growing season in order to find the growing season traits that are most strongly related to biomass production. We found that all growing season traits were highly correlated between years. Overall, early-season growth was indeed correlated with higher biomass yield.

Breeding new varieties requires a screening of the germplasm for yield potential and other agronomic traits. We therefore investigated the yield potential of our germplasm collection on a miniplots level and report the variation in emergence, flowering and senescence among the genotypes in the trial. We did not observe a relationship between early-season growth and biomass yield in this trial due to the later harvest, which canceled out most differences in early-season growth among the genotypes. We observed a large variation in flowering and senescence in our field trial but no relation between flowering and biomass production was found. Senescence also varied widely among the genotypes. only in the *M. sacchariflorus* genotypes a relation between later senescence and biomass yield was found.

In conclusion we found a large variation in chilling and frost tolerance in the miscanthus germplasm. Genotypes with higher chilling and frost tolerance than *M. x giganteus* were identified in both *M. sinensis* and *M. sacchariflorus*. This large variation and the high heritability of these traits may allow successful breeding for more cold tolerant varieties that will allow the production of miscanthus in areas that are currently too cold for the crop.

Samenvatting

Miscanthus is een geslacht van meerjarige C4 grassen dat oorspronkelijk uit Oost-Azië stamt. *M. x giganteus*, een steriele triploïde hybride tussen *M. sinensis* en *M. sacchariflorus*, wordt in toenemende mate gebruikt als biomassagewas. In deze thesis wordt `*Miscanthus*` gebruikt wanneer verwezen wordt naar het genus en `miscanthus` wanneer verwezen wordt naar het gewas. De biomassa van miscanthus kan worden gebruikt voor verschillende doeleinden, bijvoorbeeld als brandstof voor verwarming, grondstof voor tweede generatie bio-ethanol, mulch, strooisel in stallen of voor de productie van bouwmaterialen. *M. x giganteus* heeft een hoge biomassaopbrengst in vergelijking met andere biomassagewassen en dit voor slechts beperkte inputs van meststoffen, pesticiden en arbeid. Het is een robuust gewas, ideaal geschikt voor teelt op marginale gronden. Het kan gemakkelijk worden geoogst met algemeen beschikbare oogstmachines en drogen van de biomassa na de oogst is over het algemeen niet nodig. Vanwege de steriliteit van *M. x giganteus* moet deze vegetatief worden vermeerderd, door wortelstokstekken of in vitro vermeerderde plantjes. Beide methoden zijn duur en beperken de aanplant van het nieuwe gewas door landbouwers. Momenteel zijn nieuwe, zaaibare rassen van miscanthus onder ontwikkeling bij veredelaars die kosten om nieuwe velden aan te leggen drastisch zouden kunnen verlagen en de telers zullen toestaan rassen te kiezen die beter geschikt zijn voor de lokale omstandigheden. Idealiter zullen deze nieuwe rassen een hoger opbrengst dan *M. x giganteus* hebben.

De belangrijkste limieten voor de biomassaopbrengst van miscanthus zijn abiotische stressfactoren zoals lage temperaturen (vorst en kilte), droogte of zout. Het onderzoek in deze thesis richt zich op de tolerantie van miscanthus voor vorst en kiltestress. Deze stressfactoren beperken de opbrengst van miscanthus op verschillende manieren. Vorstschade bijvoorbeeld, wordt beschouwd als de belangrijkste oorzaak van wintersterfte van *M. x giganteus* in het eerste jaar na aanplant. Het ontwikkelen van rassen die lagere temperaturen in de winter kunnen overleven zou het potentieel teeltgebied van miscanthus naar koudere gebieden kunnen uitbreiden, bijvoorbeeld Oost- en Noord-Europa, regio's waren meer marginale gronden beschikbaar zijn. Vorstschade kan ook de bovengrondse delen van de plant afdoden in het vroege voorjaar, hetgeen jonge planten kan doden of de biomassaopbrengst kan verlagen in oudere plantages. Kiltestresstolerantie is van essentieel belang bij de ontwikkeling van vroeg opkomende rassen met een sterke groei in het begin van het groeiseizoen en een vroege ontwikkeling van het bladerdek. Rassen die sneller kunnen groeien bij lage temperaturen en eerder een bladerdek vormen in het groeiseizoen kunnen meer zonnestraling onderscheppen doorheen het jaar en zouden in theorie meer biomassa dan *M. x giganteus* kunnen produceren.

De algemene doelstellingen van het onderzoek in deze thesis waren om de variatie in vorsttolerantie, kiltetolerantie en vroege groei in miscanthus te bestuderen, om de onderliggende fysiologische en biochemische mechanismen vast te stellen en om de relatie tussen vroege groei en de uiteindelijke biomassaopbrengst te bepalen.

Vergeleken met verwante C4 gewassen, zoals maïs, suikerriet of sorghum, is miscanthus relatief koudetolerant. Deze koudetolerantie is daarom is al onderwerp geweest van een aantal studies, waarvan de meeste *M. x giganteus* met maïs hebben vergeleken. Deze studies hebben aangetoond dat, terwijl maïs een hogere maximale fotosynthese heeft, *M. x giganteus* kan fotosynthetiseren bij lagere temperaturen en dat dit in combinatie met een langer groeiseizoen essentieel is voor de potentieel hogere biomassaopbrengsten van *M. x giganteus* vergelijking met maïs. Terwijl bij maïs onder kiltestress een vermindering van de activiteit van fotosynthetische enzymen werd gerapporteerd vertoont *M. x giganteus* een toename van de activiteit en expressie van RNA dat codeert voor fotosynthetische enzymen bij blootstelling aan kiltestress. Men denkt dat deze verhoogde expressie de verminderde enzymekinetiek bij lagere temperaturen tegengaat, waardoor *M. x giganteus* een hogere fotosynthese kan behouden. Hoewel een aantal studies verschillende miscanthusgenotypen onder kiltestress hebben vergeleken in groeikamerexperimenten is er tot dusver weinig bekend over de moleculaire mechanismen achter de verschillen in kiltetolerantie tussen genotypen en over de variatie in kiltetolerantie en groei in het vroege groeiseizoen in het veld.

We verkregen via het EUFP7 project OPTIMISC, een collectie van meer dan 100 miscanthusgenotypen, van de soorten *M. sinensis*, *M. sacchariflorus*, *M. sinensis x sacchariflorus* hybriden en *M. x giganteus*. Deze genotypen werden geëvalueerd en vergeleken in groeikamerexperimenten en in veldproeven. Aanvullende gegevens werden bekomen van reeds op ILVO aangelegde experimenten en van de andere proeven in het OPTIMISC-project.

Om de variatie in vorsttolerantie in de collectie te screenen bepaalden wij de temperatuur waarbij 50% van de wortelstokken niet meer overleefde (LT_{50}) in 95 miscanthusgenotypen. De LT_{50} in de verzameling varieerde tussen -0,4 en -5,9°C, terwijl de gemiddelde LT_{50} voor *M. x giganteus* -2,6 ± 0,3°C was. Gemiddeld was de LT_{50} -3,5 ± 0,1°C in *M. sinensis*, -2,6 ± 0,3°C in *M. sacchariflorus* en -3,9 ± 0,2°C in de *M. sinensis x sacchariflorus* hybriden. De vorsttolerantie van de wortelstokken was gecorreleerd met de timing van de bloei en afrijping van de planten in het veld, maar niet met het vochtgehalte van de wortelstokken. Grote variatie in schade aan de jonge scheuten werd waargenomen in een veldproef na een koudegolf in het vroege voorjaar. Bepaling van de hoogte van de apexen gaf aan dat deze waarschijnlijk nog

steeds onder de grond zaten bij de meeste genotypen toen de vorst optrad, wat het snelle herstel van beschadigde scheuten na de vorst verklaarde. Deze studie was de grootste screening van vorsttolerantie van wortelstokken en scheuten in miscanthus tot nog toe en toonde de beschikbaarheid van vorsttolerante genotypen in het veredelingsmateriaal van miscanthus aan.

Om de variatie in kiltetolerantie in de collectie in te schatten werden eerst twee zeer productieve miscanthusgenotypen, *M. x giganteus* en *M. sinensis* 'Goliath' uitvoerig bestudeerd. Metingen in het veld en onder gecontroleerde omstandigheden werden gecombineerd met het doel een fundamentele vergelijkingstools te ontwikkelen om kiltetolerantie ten opzichte van prestaties in het veld te onderzoeken. Onder veldomstandigheden had *M. x giganteus* een hogere opbrengst en een snellere groei in het begin van het groeiseizoen. *M. x giganteus* vertoonde ook een minder drastische vermindering van bladgroei en van de netto-fotosynthese onder continue kiltestress in de groeikamer. Dit ging gepaard met hogere fotochemische ‘quenching’ en lagere niet-fotochemische ‘quenching’ van lichtenergie in *M. x giganteus* dan in *M. sinensis* 'Goliath' bij blootstelling aan lage temperaturen. Het gehalte aan oplosbare suikers verhoogde aanzienlijk in beide genotypen bij groei bij 12°C vergeleken met 20°C. Dit onderzoek werd gebruikt om verdere experimenten te plannen, het toonde aan dat groeikamers gebruikt kunnen worden om kiltetolerante genotypes te identificieren, maar dat validatie in veldproeven noodzakelijk is omwille van de variabele condities in het veld.

We onderzochten de variatie van de groei in het vroege groeiseizoen in de collectie genotypen in een veldproef gedurende twee seizoenen, en vergeleken deze resultaten met groei bij lage temperatuur onder gecontroleerde omstandigheden en met de groei in het vroege groeiseizoen in de OPTIMISC-veldproeven op zes verschillende locaties. Een grote variatie in groei werd waargenomen tussen de genotypen in beide seizoenen, met een sterke correlatie tussen beide jaren voor de meeste onderzochte parameters. Verschillende genotypen, zowel van *M. sinensis*, *M. sacchariflorus* als *M. sinensis x sacchariflorus* hybriden vertoonden een sterkere groei in het begin van het groeiseizoen dan *M. x giganteus*. De waarnemingen in de OPTIMISC-veldproeven op meerdere locaties toonden een sterke interactie tussen genotype en omgeving aan en die wees erop dat lokaal aangepaste genotypen nodig zijn om maximaal te profiteren van een langer groeiseizoen. De aanzienlijke variatie in de gemeten parameters geeft aan dat het veredelen van verbeterde rassen door selectie voor vroege opkomst en kiltetolerantie mogelijk moet zijn. Parameters gebaseerd op de lengte van de scheuten waren het meest consistent tussen de jaren en lijken meest geschikt voor het screenen van grote genotypencollecties.

De kiltetolerantie van *M. x giganteus* is voornamelijk bestudeerd onder gecontroleerde omstandigheden en de onderliggende mechanismen die bijdragen aan het kiltetolerantie in het veld en de variatie hierin tussen verschillende miscanthusgenotypen is grotendeels onbekend. Om dit te bestuderen werden vijf miscanthusgenotypen met verschillende kiltegevoeligheid geselecteerd in de veldproef en onderzocht gedurende het vroege groeiseizoen op een uitgebreide set van fysiologische eigenschappen. Chlorofylfluorescentie werd gemeten als indicatie van de fotosynthese en bladstalen werden geanalyseerd op biochemische parameters die verband houden met fotosynthetische activiteit (chlorofylgehalte en pyruvaat-Pi-dikinase-activiteit), redox homeostase (malondialdehyde, glutathion en ascorbaatinhoud, en catalase-activiteit) en de gehaltes aan wateroplosbare koolhydraten. De fysiologische reactie van kiltetolerante genotypen was te onderscheiden van die van kiltegevoelige genotypen, terwijl *M. x giganteus* intermediair was tussen beide groepen. Kiltetolerante genotypen werden gekenmerkt door hogere niveaus van malondialdehyde, raffinose, sucrose en hogere katalase-activiteit terwijl kiltegevoelige genotypen werden gekenmerkt door hogere concentraties glucose, fructose en hogere pyruvaat-Pi-dikinase activiteit later in het groeiseizoen. *M. x giganteus* was vergelijkbaar met de tolerante genotypen vroeg in het groeiseizoen, maar werd later meer vergelijkbaar met de kiltegevoelige genotypen, die ook een hogere biomassaopbrengst hadden.

Vroege opkomst en een vroege ontwikkeling van het bladerdek maken het theoretisch gezien mogelijk om meer straling te onderscheppen tijdens de lange dagen in de lente, wat op zijn beurt planten in staat zou stellen om meer biomassa te produceren. De rapporten in de literatuur zijn echter tegenstrijdig en daarom hebben we de relatie tussen groei in het vroege groeiseizoen en de productie van biomassa onderzocht. Wij combineerden de metingen van voorjaarsgroei met groeimetingen in de rest van het groeiseizoen teneinde de eigenschappen te vinden die de sterkste relatie met biomassaproductie hebben. We vonden dat alle groeiseizoenparameters sterk gecorreleerd waren tussen de jaren en dus bruikbare kenmerken zijn voor selectie. Over het algemeen vonden we dat de groei in begin van het seizoen inderdaad gecorreleerd was met een hogere biomassaopbrengst, alhoewel de correlaties niet bijzonder sterk waren. Planten met een vroege ontwikkeling produceerden meer biomassa.

De veredeling van nieuwe rassen vereist een screening van het veredelingsmateriaal voor opbrengstpotentieel en andere agronomische eigenschappen. Daarom onderzochten we het opbrengstpotentieel van onze collectie op een miniplots-niveau en bespreken we de variatie in opkomst, bloei en afrijping van de genotypen in de veldproef. In deze veldproef kon de relatie tussen voorjaarsgroei en biomassaopbrengst niet waargenomen worden als gevolg van de

latere oogst (in het voorjaar), die de meeste verschillen tussen de genotypen in het begin van het groeiseizoen uitvlakte. We observeerden een grote variatie in bloeitijdstip en afrijping in de veldproef in beide jaren. Terwijl sommige genotypen al in juni bloeiden, bloeide geen enkele van de *M. sacchariflorus* genotypen. Tussen het bloeitijdstip en biomassaproductie werd geen relatie gevonden. Afrijping was ook sterk variabel tussen de genotypen, sommige genotypen rijpten reeds af in september, terwijl andere groen bleven tot aan de oogst. Alleen in de *M. sacchariflorus*-genotypen werd een relatie tussen afrijpen en biomassaopbrengst gevonden, latere afrijpende genotypen van deze soort produceerden meer biomassa.

Over het algemeen vonden we een grote variatie in vorst- en kiltetolerantie in de miscanthuscollectie. Genotypen met een betere vorst- en kiltetolerantie dan *M. x giganteus* werden gevonden in zowel *M. sinensis* als *M. sacchariflorus*. Deze grote variatie en de hoge erfelijkheid van deze eigenschappen kan succesvolle verdeling voor meer koudetolerante rassen mogelijk maken wat de productie van miscanthus in gebieden die op dit moment te koud zijn voor het gewas zal toestaan.

List of abbreviations and symbols

50%Sen: Day of the year less than 50% of the plant was green

90%Sen: Day of the year less than 90% of the plant was green

AGR: Absolute shoot growth rate

ANT: Anthesis

Asat: Light-saturated rates of CO2 assimilation

Asc: Ascorbate

CanDur: Canopy duration

Car: Carotenoids

Cat: Catalase

CC: DOY canopy closure

Chl: chlorophyll

CL: Competition index based on length of surrounding plants

CN: Competition index based on number of surrounding plants

CS: Competition index based on number of shoots of surrounding plants

CY: Competition index based on yield of surrounding plants

DM%: Dry matter content at harvest

DOY: Day of the year

DTT: Dithiothreitol

EDTA: Ethylenediaminetetraacetic acid

FL: Flag leaf appearance

Fru: Fructose

FT1: Field trial 1

FT2: Field trial 2

FT3: Field trial 3

FT4: Field trial 4

F_v/F_m: maximal quantum yield of PSII photochemistry

F_v'/F_m': quantum yield of open PSII reaction centers

GD: Duration of shoot growth

GDD: growing degree day

Glc: Glucose

g_s: stomatal conductance

GSH: Gluthathione

GxE: genotype by environment

H^2: broad sense heritability

HD: Heading date

L30: TT at which the longest shoot reached a length of 30 cm

L50: TT at which the longest shoot reached a length of 50 cm

LAI: Leaf area index

Leaf4: TT at which the fourth leaf emerged

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

LFR: Leaf formation rate

L_m: maximum leaf/shoot length

LT_{50}: Temperature at which 50% of the rhizomes are killed

Mal: Maltose

MDA: Malondialdehyde

MGR: Maximum shoot growth rate

NDVI: Normalized difference vegetation index

NDVI0.5: DOY NDVI value of 0.5 reached

NIR: Near infra-red light

N_{max}: Maximum shoot number

NPQ: non-photochemical quenching

PAR: Photosynthetically active radiation

PCA: principal component analysis

PEPc: Phosphoenolpyruvate carboxylase

P_N: net photosynthetic rate

PPDK: pyruvate-Pi-dikinase

PVP: Polyvinylpyrrolidone

q_P: photochemical quenching Raf: Raffinose

ROS: Reactive oxygen species

RQ: Research question

RuBisCo: Ribulose-1,5-bisphosphate carboxylase/oxygenase

S50: TT at which 50% of maximum shoot number was formed

Stem height: Length longest stem

Stem%: Stem content biomass

Suc: Sucrose

$t_{10\%:}$ Time until 10% of shoot length reached

$t_{50\%}$: Time until 50% of shoot length reached

$t_{90\%}$: Time until 90% of shoot length reached

T_a: air temperature

T_b: Base temperature for growth

TBA: Thiobarbituric acid

t_e: Time until end of shoot growth

t_m: Time until maximum shoot growth rate achieved

T_{max}: Maximum temperature for growth

T_o: Optimal temperature for growth

TT: thermal time

VegDur: Duration of vegetative growth

VIS: Visible light

WSC: water soluble carbohydrates

Yield: Final biomass yield

Φ_{CO2}: quantum yield of photosynthesis

Φ_{PSII}: effective quantum yield of PSII photochemistry

Chapter 1: Introduction and literature review

This chapter is based on: Fonteyne, S., Roldán-Ruiz, I., Muylle, H., De Swaef, T., Reheul, D., Lootens, P. (2016). A review of frost and chilling stress in *Miscanthus* and its importance to biomass yield. In: Barth, S., Murphy-Bokern, D., Kalinina, O., Taylor, G. and Jones, M. (eds): Perennial Biomass Crops for a Resource Constrained World. Springer, New York.

Introduction

Climate change and finiteness of fossil fuel reserves create the need for a shift away from a fossil fuel-based economy to a renewable economy. One strategy to increase renewability is the shift to a more biobased economy. This growing biobased economy creates a need for biomass feedstocks that can be produced in large quantities. Miscanthus (*Miscanthus* spp., in this thesis '*Miscanthus'* will be used to refer to the genus, while 'miscanthus' will be used to refer to the crop), a perennial rhizomatous C4 grass originating in East Asia, has become increasingly important as a biomass crop in the last few decades. Currently the crop has already been planted on 20 000 ha in temperate Europe (Lewandoski, 2015), where it can achieve high biomass yields of 10-25 ton ha^{-1} $year^{-1}$. It is an undemanding crop, requiring only limited inputs of fertilizers or pesticides and is suitable for growth on marginal land (Lewandowski et al., 2000; Heaton et al., 2010; Jones et al., 2015). In a long term field trial at ILVO in Flanders including miscanthus, switchgrass (*Panicum virgatum*), short rotation coppice willow (*Salix* spp.), reed canary grass (*Phalaris arundinacea)* and common reed (*Phragmites australis*), miscanthus proved to be the highest yielding perennial biomass crop (Van Hulle et al. 2012; Muylle et al. 2015). Miscanthus biomass is currently used for direct combustion at farm level, co-firing with coal in power plants, as animal bedding and plant mulch. Higher added value applications such as the use of miscanthus fiber in paper and cardboard (Cappelletto et al. 2000), fiber boards (Ferrando and Salvado 2002), bioplastics (Nanda et al. 2013) and concrete (Pude et al. 2005) are also possible. Miscanthus is also a leading candidate for second generation ethanol production in temperate climates (van der Weijde et al. 2013).

The crop miscanthus

Currently, most miscanthus production fields use *Miscanthus x giganteus Greef & Deuter ex Hodkinson & Renvoize*, a sterile hybrid of *M. sinensis* Anderss. and *M. sacchariflorus* (Maxim.) Franch.. Although several *M. x giganteus* clones are available, most commercial plantings descend from a single Japanese clone first introduced to Denmark (Greef et al., 1997; Głowacka et al., 2014b). *M. x giganteus* is generally planted using rhizome cuttings which can be planted using adapted potato planters. In the first year growth is limited and not enough biomass is

produced to justify harvesting. In the first year after planting herbicide application is necessary due to the slow development of the crop but in later years the rapid canopy formation and leaf litter make herbicide application unnecessary. To date no diseases or pests have been observed in commercial *M. x giganteus* fields that cause economical damage. In the first winter after planting, mortality of young plants has often been observed but older plants normally do not suffer winter mortality (Clifton-Brown and Lewandowski 2000; Heaton et al. 2010). Fields of *M. x giganteus* reach their full productivity after two to four years, depending on local conditions and stay productive for at least 20 years, although in old fields yield does decline somewhat (Lewandowski et al. 2000; Arundale et al. 2014).

Starting from the second growing season the crop is harvested yearly. Miscanthus is grown for its aboveground biomass, mainly stems. It is harvested either chipping it directly on the field using a maize forage harvester (Fig. 1.1) or by mowing and baling. It can be harvested either in autumn or in winter. Winter harvests are about 33% lower on a dry matter basis due to leaf loss (Clifton-Brown et al. 2007), but are of higher quality due to lower moisture and ash contents. During autumn nutrients and carbohydrates are stored in the rhizomes. During winter nutrients are further leached out of the standing biomass and are dropped with the leaves. This allows nutrients to be reused in the next growing season and significantly reduces fertilization requirements. In general, miscanthus requires little to no fertilization (Heaton et al. 2004). Although fertilization can increase yields, the marginal increases in yield are generally not enough to justify fertilization costs (Miguez et al. 2008).

While very high yielding, *M. x giganteus* has some disadvantages. Since it is sterile, it has to be propagated vegetatively, either by rhizome cuttings or through in vitro produced plantlets. This increases the cost of field establishment and is one of the reasons why miscanthus breeding currently focusses on developing seed based varieties, which could be established at a fraction of the cost of rhizome propagation (Muylle et al. 2015; Xue et al. 2015; Clifton-Brown et al. 2016). Furthermore, using only one single clone over broad geographical ranges implies a risk of the rapid spreading of diseases and pests and does not allow farmers to plant varieties adapted to local environmental conditions. There is thus a need to breed new miscanthus varieties. These genotypes should ideally combine a high yield potential with high resource use efficiency, the traits that have made of *M. x giganteus* a successful biomass crop.

Figure 1.1: *M. x giganteus* at harvest. Picture by Hilde Muylle 19/04/2011.

The high yield potential of *M. x giganteus* in temperate regions has been, at least partly, ascribed to its remarkable cold tolerance: it has higher CO_2 assimilation rates at cool temperatures than other C4 crops (e.g. maize, sugarcane and sorghum) of the temperate zone (Long and Spence, 2013; Sage et al., 2015). This is certainly true for the *M. x giganteus* clones currently used in commercial plantations, but research has shown the possibility of identifying genotypes in the miscanthus germplasm pool with an even higher level of cold tolerance (Clifton-Brown and Jones, 1997; Głowacka et al., 2015b). The availability of varieties with improved cold tolerance would theoretically allow expansion of the potential miscanthus growing area and would reduce the risk of winter mortality in the first year after planting (Clifton-Brown and Lewandowski, 2000; Hastings et al., 2009b; Peixoto et al., 2015). Increased growth at low temperatures could also prolong the growing season, allowing plants to capture more light energy, thereby potentially increasing yield (Dohleman and Long, 2009; Robson et al., 2013a). Available knowledge on cold tolerance in miscanthus is fragmentary, however, with limited understanding of its physiological basis. In particular, variation available in the miscanthus germplasm pool has not been sufficiently explored. Consensus on the relationship between cold tolerance and biomass yield in miscanthus is also lacking. In this chapter we systematically review the state of knowledge of cold tolerance in miscanthus, the effects of low temperature on miscanthus survival and growth, and discuss the expected and proven relationships between cold tolerance and biomass yield. Knowledge gaps are identified and directions for future research are suggested.

Sources of cold tolerance in the miscanthus germplasm pool

The genus *Miscanthus* belongs to the *andropogoneae* tribe, which contains mainly tropical grasses, amongst which important crop species such as maize (*Zea mays* L.), sugar cane (*Saccharum officinarum* L.) and sorghum (*Sorghum bicolor* (L.) Moench). The genus, contains

about 12 species, but taxonomy is confused and the exact number of species is debated and complicated by frequent interspecific hybridization (Hodkinson et al. 2002; Clifton-Brown et al. 2008). *Miscanthus* species have a broad geographic distribution ranging between 50°N to 22°S (Hodkinson et al., 2002). The most studied miscanthus species are *M. sinensis* and *M. sacchariflorus,* which are the parent species of *M. x giganteus*.

M. sinensis Anderss. is found mainly in China, Korea and Japan but occurs as far north as Sakhalin in East-Russia. In its natural range it is commonly found on mountain slopes, in open grasslands, on roadsides and in open coastal areas. It thrives in areas that are infrequently disturbed by mowing, grazing or burning (Stewart et al. 2009). It generally has a clumped growth and does not form large rhizomes (Fig. 1.2). It is generally diploid (2n=38). Traditionally it is used for thatching, grazing and as a fodder crop. In Europe it is frequently planted as an ornamental and numerous cultivars have been released (Darke 1994).

M. sacchariflorus (Maxim.) Hack. is found in southern Siberia, China, Korea and Japan. It is mainly diploid on the mainland (2n=38) but often tetraploid in Japan (4n=76). *M. sacchariflorus* genotypes have a spreading rhizomatous growth (Fig. 1.2). It is commonly found along river banks and lakes. *M. sacchariflorus* var. *lutarioriparius* is extensively cultivated in China for paper production and for its edible young shoots.

Figure 1.2. Typical *M. sinensis* and *M. sacchariflorus* genotypes. On the left the flowering *M. sinensis* OPM95 with a clumped growth and on the right *M. sacchariflorus* OPM18, which does not flower in Belgium and which spreads out through its rhizomes. Picture taken on 25/9/2014 on the FT1 field trial in Merelbeke, Belgium.

M. x giganteus Greef & Deuter ex Hodkinson & Renvoize is a sterile triploid (3n=57) hybrid of a tetraploid *M. sacchariflorus* and a diploid *M. sinensis*. It was found in the wild in the 1930s on the island of Honshu in Japan. Many different names for *M. x giganteus* genotypes have been used in literature, but genetic studies indicate little to no genetic variation among the various clones

used in literature (Greef et al. 1997; Głowacka et al. 2014b). *M. x giganteus* has large but not creeping rhizomes (Fig. 1.3) and thick, tall stems. *M. x giganteus* clones are the main miscanthus variety planted for biomass production. It is also widespread as an ornamental plant in gardens in temperate Europe.

Figure 1.3: A clump of *M. x giganteus* rhizomes.

The natural geographic distribution of these species ranges between eastern Russia in the north to Papua New Guinea in the south (Fig. 1.4). They can grow in a wide range of climatic conditions, so it is expected that considerable genetic variation exists for climatic adaptation within the genus. This view is supported by results of different studies. For example, seeds of Japanese *M. sinensis* accessions from higher latitudes germinate earlier than those from southern accessions, under both high and low temperature (Dwiyanti et al., 2014), indicating adaption to local climatic conditions. Yan et al. (2011) evaluated seedlings of *M. sinensis, M. sacchariflorus* and *M. sacchariflorus* var. *lutarioriparius* from populations originating across China at three locations representing the northern plains, the loess plateau and the warmer regions of central China. They found that accessions from northern locations showed greater winter survival. Clear interspecific variability was detected, as the majority of the *M. sacchariflorus* genotypes was able to survive the first winter in the northern plains, while most of the *M. sinensis* and *M. sacchariflorus* var. *lutarioriparius* genotypes did not. In contrast, Anzoua et al. (2015) detected no significant differences regarding winter survival among 43 *M. sinensis* accessions collected across Japan, where the species is found in climate conditions ranging from subarctic to subtropical (An et al., 2008; Stewart et al., 2009). When grown in Hokkaido, a location characterized by long winters and cool summers, the survival rate of accessions from subarctic and subtropical regions did not differ

significantly. These apparently contradictory conclusions are probably due to the particular set of accessions compared and the location chosen for field evaluation.

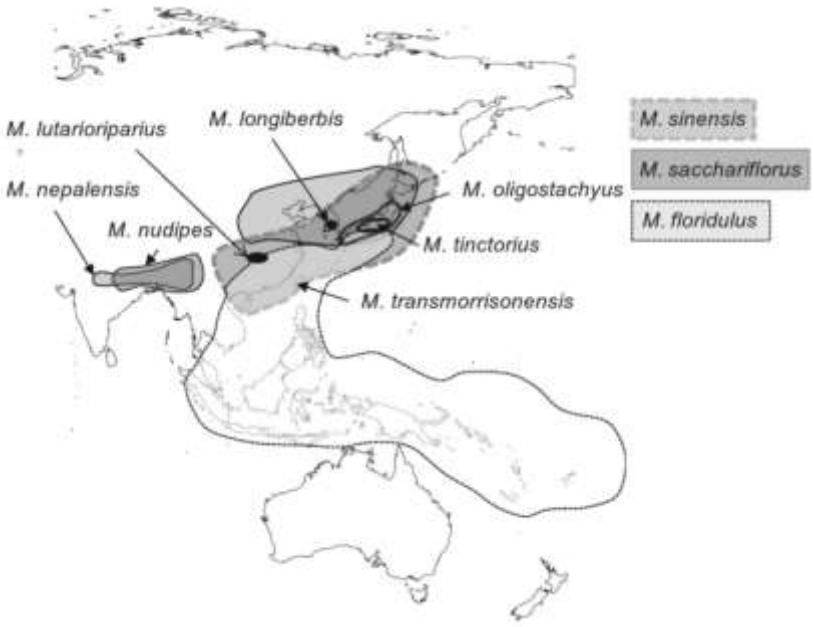


Figure 1.4: Distribution of *Miscanthus* species in Asia (Clifton-Brown et al. 2011).

Adaptation to local conditions has also been demonstrated along altitudinal gradients. For example, in Taiwan miscanthus grows from the coastal lowlands up to altitudes of 3200 m. Below an elevation of 2200 m, *M. floridulus* is the most common species, while above 2200 m. *transmorrisonensis* is the most common (Chou and Chang, 1988). *M. floridulus* genotypes collected at 1200 m above sea level do not survive the colder winter when transplanted to a location at 2600 m (Chou et al., 1991). Similarly, *M. transmorrisonensis* has a higher photosynthesis and biomass accumulation at 10 and 15°C, while at 25 and 30°C it is higher for *M. floridulus* (Kao et al., 1998). Miscanthus genotypes from coastal Taiwan reach maximum photosynthetic rates at higher temperatures than genotypes from higher altitudes; the latter reach higher values of photosynthesis at lower temperatures (Weng and Ueng, 1997; Weng and Hsu, 2001). This adaptation to different climatological conditions is also reflected in specific adaptations such as thicker leaves, fewer stomata and a thicker wax layer in accessions from higher altitudes (Kao and Chang, 2001; Weng and Hsu, 2001).

All this evidence demonstrates the existence of climatic adaptation within the genus *Miscanthus* which could be exploited in breeding. However, genotypes adapted to colder conditions seem to be characterized by lower yields when grown in milder climates. For example, in a collection of 23 genotypes collected across China and grown in Wuhan (30°33' N, 114°25' E), biomass yield was negatively correlated with the latitude of the locality where the accession had been collected (Yu et al., 2013). A study of 459 *M. sinensis* accessions collected across China yielded similar results

(Zhao et al., 2013). It is therefore important to consider possible trade-offs between cold tolerance and yield (see below).

Cold stress

Ruelland et al. (2009) divide cold stress into chilling stress and frost stress (Fig. 1.5). Chilling stress occurs when plants are exposed to temperatures that are too low for growth but still are above 0°C. At chilling temperatures biochemical processes are disrupted: the speed of metabolic reactions decreases, enzymes become less active and less stable, and the rigidity of the cell membranes increases (Ruelland et al., 2009; Yadav, 2010). This all disrupts the metabolic equilibria in the cell. For example, the capture of light energy during the light reactions of photosynthesis is less temperature-dependent than the use of this energy in the dark reactions. As a consequence, the chloroplast electron transfer chain can become overreduced under high light intensities at low temperature. This leads to the production of reactive oxygen species (ROS), which can damage cells and cause photoinhibition, especially if the activity of ROS scavenging enzymes and the synthesis of antioxidants is also reduced. Frost stress occurs at below zero temperatures and is mainly related to cell dehydration. At below zero temperatures, extracellular ice crystals are formed and attract water from the cells. When these crystals grow larger they can cause mechanical damage by penetrating the symplast (Ruelland et al., 2009).

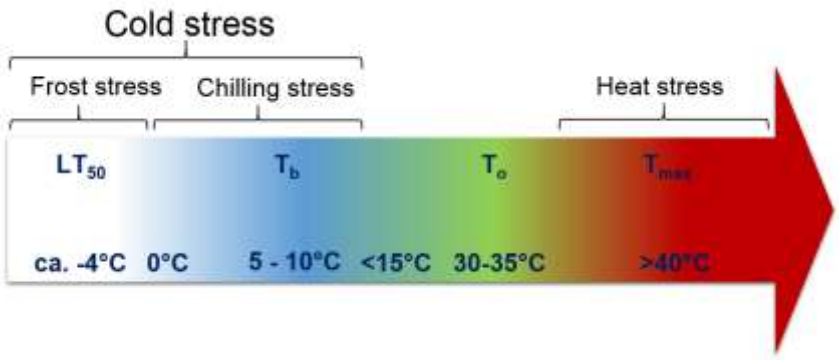


Figure 1.5: Graphical representation of the temperature effect on plant growth. At the optimal temperature (T_o) plant growth is maximal. When temperature increases above T_o, heat stress occurs. Above the maximum temperature (T_{max}) growth ceases. If temperature is lower than T_o, first chilling stress will occur. Below the base temperature (T_b) growth ceases. Below 0°C frost stress starts occurring. Below the LT_{50} 50% of the plants dies. Approximate temperature stress ranges in miscanthus are given.

The temperatures at which a plant or plant organ experiences these different kinds of stresses depend on the species, and even on the genotype. For example, chilling stress severely reduces growth in sugarcane below 20°C, while some miscanthus genotypes can still grow at 5°C (Clifton-Brown and Jones, 1997) and *M. x giganteus* rhizomes die around -3.4°C while some other perennial grasses can survive temperatures below -20°C (Clifton-Brown and Lewandowski, 2000; Belintani et al., 2012; Friesen et al., 2015). In general, miscanthus is more tolerant to cold stress

than other C4 grasses such as maize, sorghum or sugarcane (Long and Spence, 2013; Sage et al., 2015), but variability for this trait has also been described in the miscanthus germplasm pool (Clifton-Brown and Jones, 1997; Clifton-Brown and Lewandowski, 2000; Farrell et al., 2006; Friesen et al., 2014; Głowacka et al., 2014a; Głowacka et al., 2015b), as discussed below.

Frost tolerance in miscanthus

In temperate and continental climates, frost can affect miscanthus plants in different ways (Fig. 1.6). Shoots can be damaged by frost at the beginning or at the end of the growing season, while rhizomes can be killed by severe frost during winter. Frost damage to rhizomes is thought to be the main cause of winter mortality (Clifton-Brown and Lewandowski, 2000; Peixoto et al., 2015) and it is one of the biggest problems in miscanthus production in northern, colder areas, especially in the first year after planting (Lewandowski et al., 2000).

Frost tolerance at the rhizome level and winter mortality

Winter mortality has been investigated in several field trials established in multiple locations with different levels of winter severity. Within the 'European Miscanthus Improvement' (EMI) project (1997-1999), field trials including four *M.* x giganteus, one *M. sacchariflorus*, five *M. x sinensis x M. sacchariflorus* hybrids and five *M. sinensis* genotypes were established in Portugal, England, Germany, Denmark and Sweden. In Portugal, England and Germany, winter losses of the *M. x giganteus* genotypes did not surpass 1%, while in Sweden and Denmark mortalities up to 100% were observed. Furthermore, in the 15 field trials of the European Miscanthus Network (1993), *M. x giganteus* showed good winter survival in southern Europe, but unreliable survival in the trials in northern Europe (Christian and Haase, 2001; Clifton-Brown et al., 2001a). In the USA, Maughan et al. (2012) reported winter survival rates of 99 and 100% for *M. x giganteus* in the warmer locations in New Jersey and Kentucky and 79% and 25% in the colder Nebraska and Illinois locations. Some studies have demonstrated a higher winter survival for *M. sinensis* (Clifton-Brown et al., 2001b), but this was not supported by Rosser (2012), who reported higher winter mortality for the genotypes of this species in a trial in Ontario, Canada. In this trial twenty miscanthus genotypes were planted at three locations of varying winter severity. The diploid *M. sinensis* x *M. sacchariflorus* hybrids investigated displayed the highest survival rates, followed by *M. x giganteus* and *M. sinensis* (Rosser, 2012). Generalizations at the species level are therefore not possible, and large intra-species variability seems to exist for this trait. It is possible to breed genotypes with higher winter survival rates than *M. x giganteus*, however, as demonstrated by Fritz et al. (2009).

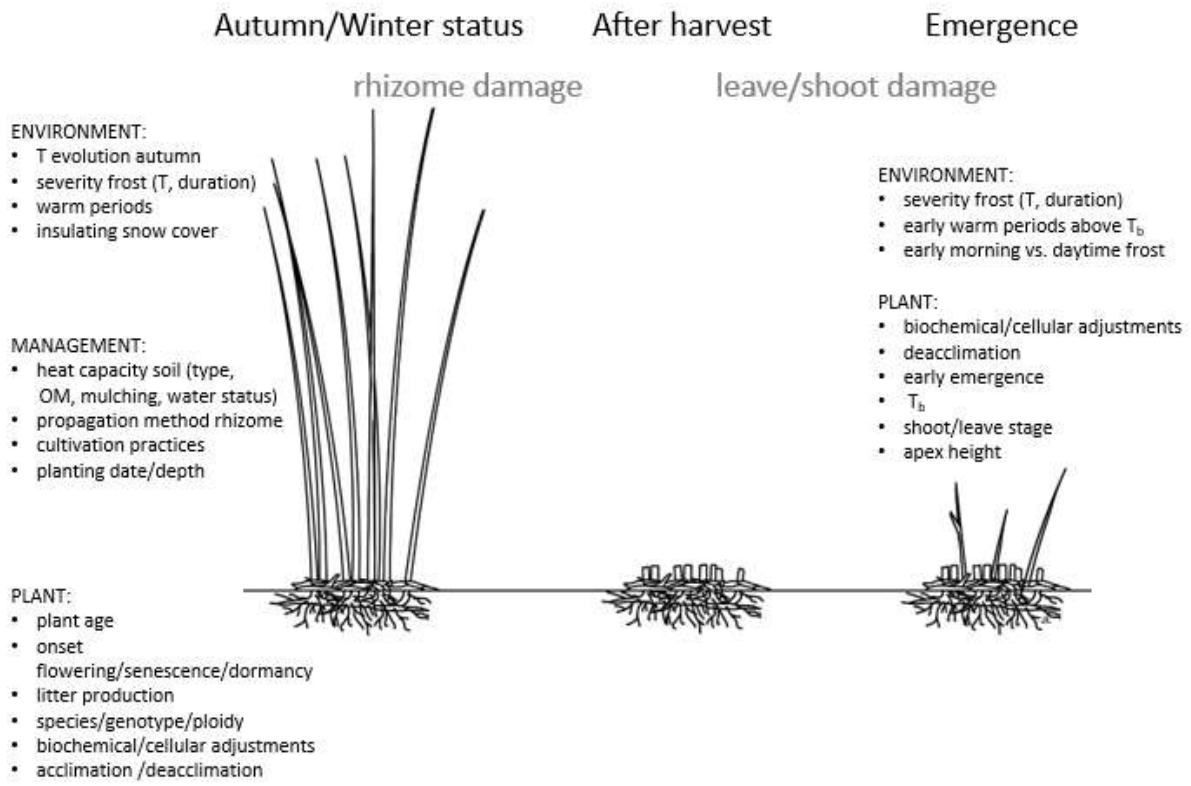


Figure 1.6: Schematic overview of the factors contributing to frost stress and frost tolerance in miscanthus. T: temperature, T_b: base temperature.

Winter mortality seems to be particularly relevant in young miscanthus fields. Since miscanthus plants often do not senesce normally the first year after planting (Jørgensen and Muhs, 2001; Clifton-Brown and Lewandowski, 2002), they do not achieve a sufficient level cold acclimation before winter. Instead, the aboveground parts are killed by the first frosts, thus eliminating the possibility to store reserves that should otherwise be transported to the rhizomes. Consequently, the plant's ability to form new shoots and grow vigorously may be impaired during the following spring (Jørgensen and Muhs, 2001). The relationship between phenological and developmental aspects and winter mortality is unclear (Jørgensen and Muhs, 2001). Most literature reports support no relationship between flowering date (senescence occurs after flowering) and winter mortality, but larger plants might be better prepared to survive the winter (Jørgensen and Schwarz, 2000; Rosser 2012). The use of ethephon to induce senescence and reduce winter mortality has been proposed, but correct application is difficult due to temperature requirements for effectiveness (Fritz et al., 2009). Comparison of trials is complicated by the use of in vitro propagated plants in some of the field trials because the effect of the phytohormones used in in vitro culture can be long lasting. In some trials plants propagated through rhizomes have shown greater winter survival rates than in vitro propagated plants (Fritz et al., 2009), but in other trials rhizome-propagated plants had lower winter survival (Christian and Haase, 2001). Hormonal

treatments during in vitro propagation and hormonal status at planting can also affect the survival capacity of the plants (Christian and Haase, 2001).

Miscanthus rhizomes are able to survive sub-zero temperatures to a certain extent, but frost tolerance of miscanthus seems to be low compared to other rhizomatous C4 grasses, such as switchgrass or prairie cordgrass (*Spartina pectinata*) (Hope and McElroy, 1990; Peixoto et al. 2015). Within the genus, LT_{50}, the temperature at which 50% of the plants are killed, ranged from -3.4°C for *M. x giganteus* to -6.3°C for certain *M. sinensis* genotypes (Clifton-Brown and Lewandowski, 2000). The frost tolerance of the rhizomes as investigated in the laboratory was correlated with winter survival in the EMI trial described above, with the two *M. sinensis* hybrids surviving winter at all sites whereas *M. x giganteus* and *M. sacchariflorus* only survived on sites where soil temperatures at 5 cm depth did not fall below -2.8°C (Clifton-Brown et al., 2001a). This suggests that predicting the frost stress tolerance of miscanthus rhizomes under field conditions using controlled freezing experiments might be an option for large scale screening.

Peixoto et al. (2015) tested five hybrid miscanthus genotypes and reported LT_{50} values between -1.5 and -6.7°C depending on genotype and harvesting date. For some genotypes no difference in LT_{50} between harvesting dates was observed, but for some others, LT_{50} values were 2-3°C higher when rhizomes were harvested in summer, suggesting that at least in some genotypes deacclimatization happens. In addition, it has been shown that the speed at which temperature decreases influences mortality rates. Indeed, when Peixoto et al. (2015) applied a staged cooling protocol, in which the temperature was decreased by 2.5°C every 24 h, they observed LT_{50} values between -6.3 and -14.4°C. Furthermore, hardening *M. x giganteus* plants at 12 or 5°C increases the tolerance of rhizomes to freezing stress up to -3°C (Płażek et al., 2011) and rhizomes of plants that have survived one winter can survive severer frost events than rhizomes that have not yet overwintered.

Several physiological mechanisms have been suggested to mediate frost tolerance in miscanthus. For example, it has been shown that hardening increases abscisic acid contents in the leaves and the rhizomes and decreases moisture content in the rhizomes. Hardening also increases the amount of low molecular weight antioxidants and phenolic compounds in leaves and rhizomes, and decreases catalase activity (Płażek et al., 2011). Withers (2015) has demonstrated the accumulation of raffinose, linoleic acid (C18:2n6) and alpha-linolenic acid (C18:3n3) during cold acclimation. Linoleic acid and alpha-linolenic acid are known to stabilize cell membranes at low temperatures and increase their fluidity.

Frost tolerance of new shoots

Late frosts in spring affect shoot growth. When shoots emerge during early warm periods, they can be killed by subsequent frost events. This is particularly relevant if resprouting is compromised

by a shortage of reserves in the rhizome, as discussed above during the first year after planting. If shoots are killed completely by a late frost, any yield advantage of early emergence will be lost. It is therefore important to understand how plants react to freezing temperatures at the start of the growing season. Farrell et al. (2006) exposed leaves of young, hardened plants of one *M. x giganteus*, one *M. sacchariflorus* and two *M. sinensis* hybrids to controlled freezing temperatures up to -10°C. Considerable variation was found with LT_{50} values ranging from -6°C to -9°C. Frost tolerance was different for plants at different developmental stages, with plants in the third or fifth leaf stage being more tolerant than those with six or seven leaves. In plants with three to five leaves, the shoot apex is most probably still underground, where it is protected from frost damage (Zub et al., 2012a). These four genotypes were part of the 15 genotypes planted at five locations by the EMI project. The *M. x giganteus* (LT_{50} -8°C) and the *M. sacchariflorus* (LT_{50} -7°C) did not survive the first winter in Sweden and Denmark, while the two *M. sinensis* hybrids (LT_{50} of -6°C and -9°C) did survive (Clifton-Brown et al., 2001a). Interestingly, leaf frost tolerance was not associated with greater winter survival, which was rather more related to rhizome frost tolerance as discussed above.

Chilling stress in miscanthus

C4 photosynthesis evolved from C3 photosynthesis to reduce photorespiration, the process in which RuBisCo uses O_2 as a substrate instead of CO_2, leading to a loss of assimilated CO_2. To avoid photorespiration C4 plants elevate CO_2 concentration at the site of RuBisCo, which is confined to the bundle sheet cells of the leaves in C4 plants. CO_2 is first bound to oxaloacetate (OAA) in the mesophyll cells by phoenolpyruvate carboxylase (PEPC) (Matsuoka et al. 2001; Sage and Kubien 2007). Oxaloacetate is then reduced to malate or transaminated to arginine and transported to the bundle sheet cells, where it is decarboxylated to release CO_2. The affinity of RuBisCo for O_2 increases with temperature and decreases with increasing intracellular CO_2 concentration. As C4 species are common in warmer climates than in temperate and cold climates, it has long been assumed C4 photosynthesis is inherently chilling sensitive. However, the chilling sensitivity of C4 species such as maize or sorghum seems to be a consequence of their origins in warm climates rather than an inherent characteristic of C4 photosynthesis (Long and Spence 2013; Sage et al. 2015). It is the growing season temperature, rather than the winter temperature, that determines survival and production of C4 species. In this section, an overview is provided of the effects of chilling temperature on the photosynthetic apparatus, metabolism and plant development, and yield in miscanthus.

Effect of chilling stress on plant growth and development

Plant growth is strongly dependent on the temperature of the environment (T_e) (Fig. 1.4). If this temperature is below the base temperature (T_b) or above the maximum temperature (T_{max}), growth will cease. Above T_b growth increases up to an optimum temperature (T_o), and when temperature

gets higher than T_o growth decreases. The effect of temperature on the growth and the development of crops is generally expressed in thermal time, or the summation of the number of degrees the mean daily temperature is above T_b over a certain period. Shoot production of four miscanthus genotypes was reduced strongly at low temperatures, with only one genotype producing shoots on more than 50% of its rhizomes at 7°C (Farrell et al., 2006). The degree days needed for emergence was different for different genotypes and varied between 60 and 180 degree days, with T_b between 6 and 8.6°C. This corresponds with the Tb of 6.8°C that Zub et al. (2012b) calculated using the formula developed by Yan and Hunt (1999). Clifton-Brown et al. (2000) calculated 10°C as a base temperature for *M. x giganteus,* as this temperature gave the highest correlation between the increase in leaf area index and accumulated degree days. As T_b seems to vary according to genotype, it is necessary to use the appropriate T_b when determining growing season duration of a particular genotype, expressed in thermal time. However, this is not common practice and a general T_b value of 10°C (Clifton-brown et al., 2004; Angelini et al., 2009; Jensen et al., 2013; Arundale et al., 2014) or 0°C (Miguez et al., 2008; Hastings et al., 2009a; Maughan et al., 2012) is used in most studies. Inter-genotype differences have also been reported with regards to plant elongation rates at chilling temperatures (Clifton-Brown and Jones, 1997; Głowacka et al., 2014a), with 43-fold differences at 10/5°C between the most chilling tolerant genotype and the least chilling tolerant genotype among a set of 50 (Głowacka et al., 2014a). In this latter study *M. x giganteus* was amongst the genotypes showing the least reduction in growth rate when transferred from 25°C to 10/5°C.

Effects of low temperature on the photosynthetic apparatus

M. x giganteus can achieve high rates of photosynthesis, with CO_2 assimilation rates up to 35 μmol $m^{-2}s^{-1}$ under field conditions (Beale et al., 1996). There are however large differences among miscanthus species and genotypes in chilling tolerance of photosynthesis. Several studies have shown that CO_2 assimilation rate declines relatively little in *M. x giganteus* after chilling shock compared to *M. sinensis and M. sacchariflorus* (Purdy et al., 2013; Friesen et al., 2014; Głowacka et al., 2014a; Fonteyne et al., 2015). Large differences in chilling tolerance of photosynthesis are found among miscanthus species and genotypes. Several studies have shown that CO_2 assimilation rate declines relatively little in *M. x giganteus* after chilling shock compared to *M. sinensis and M. sacchariflorus* (Purdy et al., 2013; Friesen et al., 2014; Głowacka et al., 2014a, Fonteyne et al., 2015).

The temperature optimum for photosynthesis lies around 30-35°C for both *M. x giganteus* and maize (Naidu et al., 2003), but compared to other C4 crops such as maize or sugarcane, *M. x giganteus* can still achieve high photosynthetic rates at low temperatures (Beale et al., 1996; Naidu and Long, 2004; Friesen et al., 2014). While maize displays an 80% reduction in assimilation rate when grown at 14/11°C with respect to growth at 25°C, Naidu et al. (2003) and Wang et al. (2008a)

report nearly the same light-saturated rates of CO_2 assimilation (A_{sat}) for *M. x giganteus* grown at 14°C or at 25°C. *M. x giganteus* grown at 10°C however, does show a marked decrease in the quantum efficiency of CO_2 fixation (Φ_{CO2}) and assimilation rate (Farage et al., 2006). The ratio between the quantum efficiency of electron transport in photosystem II (Φ_{PSII}) and Φ_{CO2}(Φ_{PSII}/ Φ_{CO2}) is similar in *M. x giganteus* grown at 25°C, 14°C or 12°C but it increases when grown at 10°C. This indicates an increase in linear electron transport at lower temperature, i.e. more electrons are transported through PSII than are used for the assimilation of CO_2. These electrons can be directed to alternative electron sinks, for instance O_2 via the Mehler reaction, which generates superoxide and hydrogen peroxide (Naidu and Long, 2004; Farage et al., 2006; Fonteyne et al., 2015). So *M. x giganteus* suffers chilling stress and has a risk of oxidative damage at temperatures below 12°C.

Stomatal conductance limitations do not appear to lie at the basis of the decrease in assimilation rate at low temperatures in miscanthus (Naidu et al., 2003). This is supported by the conclusions of Głowacka et al. (2015b), in a study of 11 miscanthus genotypes and of Głowacka et al. (2014a) in a comparison of 13 miscanthus genotypes. Stomatal conductance decreased in all genotypes, but this was mainly a consequence of lower assimilation rates, rather than a cause. The main reason for low assimilation rates was light induced chilling damage of photosystem II. Głowacka et al. (2015b) and Friesen et al. (2014) used chlorophyll fluorescence measurements in the field and found that genotypes that show a higher chilling tolerance measured by chlorophyll fluorescence under controlled conditions also tend to show less cold stress under field conditions. Measurements of photosynthesis under controlled conditions might thus be representative of chilling tolerance under field conditions.

Biochemical adaptations to chilling temperatures

The chilling tolerance of *M. x giganteus* is a result of its ability to maintain high rates of photosynthesis at low temperatures, while at the same time effectively dissipating excess light energy. At low temperatures, the activity and stability of enzymes such as ribulose-1,5-bifosfaat carboxylase oxygenase (RuBisCo), phosphoenolpyruvate carboxylase (PEPc) and pyruvate phosphate dikinase (PPDK) becomes limiting for C4 photosynthesis (Matsuoka et al., 2001; Sage and Kubien, 2007). At low CO_2 concentration PEPc is the rate limiting enzyme, but in miscanthus under chilling stress the intracellular CO_2 concentration is generally not limiting (Głowacka et al., 2015a). This implies that enzymatic activity and stability of RuBisCo and PPDK are probably more important.

PPDK contents in leaves of both maize and *M. x giganteus* decline when transferred from 25°C to 14°C. While PPDK in maize leaves remains at a low level, PPDK contents in *M. x giganteus* leaves increase again after the initial decline, reaching significantly higher levels compared to 25°C after seven days of cold treatment (Wang et al., 2008b). This is accompanied by higher PPDK mRNA

abundances and higher photosynthetic rate. Higher concentrations of PPDK increase the stability of the protein and thus its activity at low temperatures (Wang et al., 2008b). This agrees with the observation that the amount of RuBisCo, PEPc and PPDK is significantly lower in maize grown at 14°C compared to 25°C, while in *M. x giganteus* no difference in protein concentration between these growing temperatures could be observed. Similarly, in chilling tolerant sugarcane genotypes (Naidu et al., 2003) PPDK mRNA expression and enzyme activity increase under chilling stress, while they decline in sensitive genotypes (Du et al., 1999; Nogueira et al., 2003).

Although two PPDK genes have been described in *M. x giganteus*, their protein products are highly similar, and they even display a high level of sequence similarity when compared to orthologous genes of miscanthus, maize and sugarcane (Du et al., 1999; Naidu et al., 2003; Wang et al., 2008b). Furthermore, there seems to be no functional difference between recombinant PPDK from both miscanthus and maize expressed in *E. coli* (Wang et al., 2008b). Likewise, no differences were found with regards to catalytic properties, activity and leaf concentrations of RuBisCo from maize and *M. x giganteus* grown at 14°C or 25°C (Wang et al., 2008a). However, higher RuBisCo contents were detected under chilling stress in *M. sinensis* (Spence, 2012) and *M. x giganteus* (Spence et al., 2014). When exposed to 14°C, *M. sinensis* accessions from colder climates displayed higher PPDK and RuBisCo contents (38 and 50% higher, respectively), while PPDK content declined by 28% and RuBisCo content did not change in comparison to a genotype from a warmer climate. Furthermore, in a microarray experiment, Spence et al. (2014) showed that in *M. x giganteus* the expression of all genes coding for photosynthetic proteins or proteins protecting PSII tested was higher under chilling stress. *M. x giganteus* thus counteracts the lower activity and stability of these proteins at low temperature by increasing the mRNA levels for their synthesis (Spence et al., 2014). In conclusion, increasing enzyme content under chilling stress is probably a general strategy of miscanthus in response to cold, but this should be confirmed in other species and genotypes.

Tolerance to chilling stress not only involves maintaining high levels of photosynthesis at low temperatures, but it is also necessary to avoid chilling-induced damage of the photosynthetic apparatus. High light intensities at low temperature can cause photoinhibition of photosystem II. Correspondingly, dissipation of excess light energy to heat through reversible photoprotective processes such as conversion of violaxanthin to zeaxanthin is increased in *M. x giganteus* under chilling stress (Farage et al., 2006). Compared to more chilling sensitive miscanthus genotypes, *M. x giganteus* shows a more pronounced response under chilling stress (Friesen et al., 2014). The role of reactive oxygen species (ROS) in chilling stress in miscanthus has not received much attention in the past, but ROS are also likely to play a role in miscanthus. This is supported by the relatively high tolerance of *M. sinensis* genotypes to oxidative stress caused by high heavy metal concentrations (Scebba et al., 2006; Ezaki et al., 2008).

Chilling stress has a marked influence on the carbohydrate concentrations in miscanthus leaves. Purdy et al. (2013) report an increase of soluble sugars and starch after a chilling shock in four miscanthus genotypes. These changes were ascribed to a decrease in growth, resulting in a decreased demand for carbohydrates (Purdy et al., 2013). The soluble sugar concentration remains high as long as the plants are under chilling stress (Mortaignie, 2014), supporting the view that sugars might play a protective role. Indeed, in *M. x giganteus* and *M. sinensis* Goliath grown at 12°C, raffinose is present in the leaf, while it is absent at 20°C (Fonteyne et al., 2015). Raffinose is known to protect cells against the effects of chilling stress by stabilizing cell membranes (Valluru and Van den Ende, 2008; Janská et al., 2010) and against ROS damage (Nishizawa et al., 2008). In our view, the role of soluble sugar changes in chilling tolerance of miscanthus deserves further investigation. The role of raffinose as protective agent is of particular interest.

Cold stress tolerance and biomass yield

Investigating the cold tolerance of miscanthus is of great interest because of the close phylogenetic relationship of miscanthus with cold sensitive crops such as maize or sugarcane (Friesen et al., 2014; Głowacka et al., 2015a). However, from the application point of view the main question remains whether increasing cold tolerance may lead to higher biomass yields in miscanthus. From a theoretical point of view, the capacity to form shoots and grow at low temperature, results in an increase of the canopy duration, allowing to intercept a higher amount of radiation and thus a potentially higher biomass yield. For example, while miscanthus has a lower photosynthetic capacity than maize, its larger leaf area, combined with a much longer growing season, allows it to accumulate more biomass (Dohleman and Long, 2009). The positive effect of low temperature tolerance can however be counteracted if it results in a yield penalty that reflects the costs of improved low temperature tolerance (Trudgill et al., 2005). Although there is evidence that improved abiotic resistance does not always have a yield penalty, at least in *Arabidopsis* (Raineri et al., 2015; Yu et al., 2013), currently available literature does not allow to determine unequivocally whether this is the case for miscanthus. The miscanthus growing season is essentially delimited by the last spring frost and the first autumn/winter frost (Jørgensen and Muhs, 2001). Within these limits, genotypes that emerge early and grow fast at low temperature in early spring have an advantage because early canopy closure leads to more interception of solar radiation (Fig. 1.7) (Clifton-Brown and Jones, 1997; Sage et al., 2015). Farrell et al. (2006) modelled miscanthus biomass production and concluded that breeding new cultivars with an improved growth rate at low temperature could potentially increase yields. They showed that a hypothetical genotype with a similar growth rate as *M. x giganteus* at optimal temperature but lower base temperature for growth, a lower thermal time requirement for emergence, and a better leaf frost tolerance could yield up to 25% more than *M. x giganteus* in southern Germany. Similarly,

in a simulation by Davey et al. (2015), a lower base temperature or an earlier leaf emergence predicted a significantly higher yield. Nevertheless, these simulation studies did not take into account a possible trade-off between growth rate and base temperature, as reported by Clifton-Brown and Jones (1997), in a study of the growth rate of 32 miscanthus genotypes at different temperatures, and of Farrell et al. (2006) in four genotypes. According to the conclusions of these two studies, genotypes with higher growth at low temperatures were unlikely to be higher yielding compared to the other genotypes due to relatively lower growth rates at higher temperatures. Whether this relationship is of general application in miscanthus, and whether there is a genetic linkage between these two aspects or just a correlation specifically for the set of genotypes investigated should be the topic of future research. Furthermore, it is currently unknown if breeding for increased cold tolerance will have impact, either positive or negative, on tolerance to other abiotic stresses, such as drought or nutrient deficiency. To our knowledge there are currently no reports available about possible interactions between different abiotic stress tolerances in miscanthus. Some information on this topic is expected soon, as one of the objectives of the EU-FP7 research project OPTIMISC (Lewandowski et al., 2015) is to identify miscanthus genotypes that are tolerant to multiple types of stress.

A study of the results of field experiments reveals strong genotype x environment effects. Indeed, Zub et al. (2011) and Zub et al. (2012b) found the highest yields among the 21 genotypes they examined in late emerging genotypes with high maximum growth rates in summer and negatively related to growth duration (Zub et al., 2012b). This is contradicted by Robson et al. (2013a), who reported that canopy duration has a positive effect on yield and concluded that both early emergence and late senescence lead to higher yields, after examining 244 genotypes. These different conclusions might be related to differences in the locations at which these two experiments were carried out. In the French location where Zub et al. (2012b) carried out the experiment, it is possible that early emergence and growth are less relevant than in Wales where Robson et al. (2013a) established their field trials. Weather data was not reported for neither study, however. The differences in the methodologies and genotypes used in the trials might also explain the different conclusions (Robson et al., 2013a). Furthermore, in the EMI trial described above (Clifton-Brown et al., 2001a), the two genotypes with the highest T_b among a set of 15 did not survive the first winter in the trials at the two coldest locations but yielded almost 10 tons per hectare more than the two genotypes with lower T_b in the trial at the warmest location (Farrell et al., 2006). The trials thus demonstrated the need for varieties adapted to local environmental conditions to maximize yield potential.

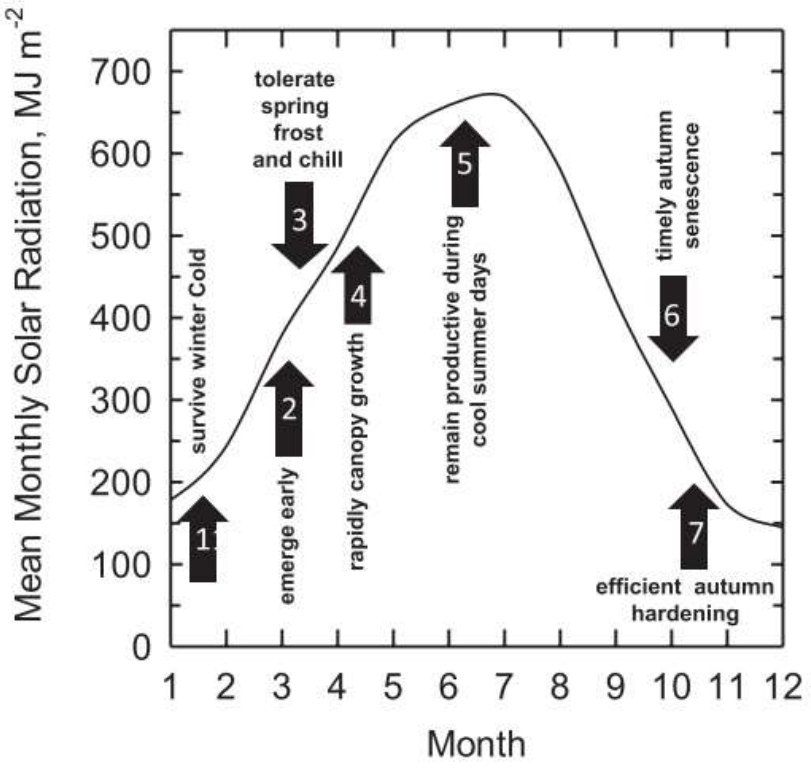


Figure 1.7: Seven criteria necessary for a high yielding perennial biomass crop during the annual solar radiation cycle. The curve shows the average radiation in southern Canada (Sage et al. 2015).

Rhizome frost tolerance will generally not increase yield on a plant basis, but improved winter survival can increase yield on a field basis in regions where frost kill is of concern. The real relevance of frost tolerance at the rhizome level for miscanthus plantings is difficult to estimate, because in the investigated areas, temperatures in the soil (at the level of the rhizomes) seldom reach the lethal temperatures given by Clifton Brown et al. (2000). For example, Etzinger et al. (2012) reported that while air temperatures dropped to -10°C in a miscanthus field in Austria, soil temperatures never dropped below zero (Eitzinger and Kössler, 2002). It is possible that in most field trials investigated to date, lethal soil temperatures were not reached, which explains at least partially the large variation in winter mortality observed among locations and years. Hastings et al. (2009a) identified a once-in-ten-year occurrence of lethal frost as the upper limit for commercial exploitation of miscanthus. Using the MISCANFOR miscanthus yield model, they showed that a hypothetical improved variety with a LT_{50} of -6°C would have a substantially larger growing area compared to *M. x giganteus* with at LT_{50} of -3.4°C (Hastings et al., 2009b). Kucharik et al. (2013) simulated minimum soil temperatures in the Midwest region of the USA and showed that in the northern parts of this region, these minimum soil temperatures are often reached. Indeed, soil cover by miscanthus straw or snow can have an insulating effect of 2 to 6°C, protecting the rhizomes from lethal frost. A thick straw cover is not present the first winter after planting, however, which may explain higher mortalities. Furthermore, in older plantings, the large density of rhizomes may have an insulating effect, protecting the lower rhizomes from frost damage (Peixoto et al.,

2015). Earlier emergence increases the risk of exposure of young leaves to frost, and the risk that the shoot apices are already above ground when a late frost strikes. To date it has not been quantified what the effect hereof could be on final yield. It appears however necessary to take shoot frost tolerance into account when breeding for earlier emergence.

Chapter 2: Thesis objectives and outline

M. x giganteus is a high yielding biomass crop, but the lack of availability of other varieties limits the adoption of miscanthus by farmers. The absence of other high yielding miscanthus varieties does not allow farmers to choose varieties adapted to local climatic and soil conditions, restricts the potential growing area and might allow for the rapid spread of pests and diseases. This doctoral thesis is part of the OPTIMISC project which aims to resolve these issues by optimizing the miscanthus biomass production chain. In this project research is conducted on all aspects of miscanthus production, from screening of germplasm, trialing of advanced lines and biomass quality improvement to life cycle analysis of biomass value chains. One of the traits under interest for the development of improved new varieties is tolerance to cold stress, as cold stress is one of the main constraints to miscanthus productivity as discussed in chapter one. Frost stress can lead to winter mortality and can kill shoots early in the growing season, while chilling temperatures limit emergence, growth and photosynthesis in spring. Improved cold stress tolerance can potentially lead to higher biomass yields, through earlier emergence and canopy formation and would allow expanding the miscanthus growing area eastwards and northwards in Europe. Considerable variation for frost tolerance and for growth and photosynthesis under chilling stress has been reported in the genus *Miscanthus*, including genotypes more tolerant to cold stress than *M. x giganteus*. It should thus be possible to develop varieties with higher cold stress tolerance. In order to incorporate improving cold stress tolerance in a breeding program, it is important to screen the germplasm for cold tolerance, to better understand the physiological and biochemical mechanisms that lead to higher cold tolerance and to determine the relationship between cold tolerance and final biomass yield.

Therefore, the general aims of this PhD thesis were:

1. To screen a broad miscanthus germplasm collection for rhizome and leaf frost tolerance.
2. To screen a broad miscanthus germplasm collection for chilling tolerance and early-season growth.
3. To determine the biochemical and physiological parameters underlying chilling tolerance.
4. To analyze the relationship between early-season growth and final biomass yield.

For each of these aspects we postulated a number of hypotheses and posed several research questions to guide the research. These research questions have been addressed in the chapters of this thesis (Table 2.1).

Hypothesis 1: There exists a useful variation in frost tolerance in the genus *Miscanthus*, with genotypes with a lower LT_{50} than *M. x giganteus.*

- RQ1: Does the variation in rhizome frost tolerance in miscanthus exceed −5°C?
- RQ2: How large is the variation in shoot frost tolerance in miscanthus?

- RQ3: Which phenological characteristics relate to frost tolerance in miscanthus?

Hypothesis 2: There exists a useful variation in chilling tolerance and early-season growth in the genus *Miscanthus*, with genotypes allowing an earlier growing season than *M. x giganteus*.

- RQ4: How large is the variation in chilling tolerance and early-season growth in miscanthus?
- RQ5: What is the most efficient method to measure chilling tolerance and early-season growth?
- RQ6: Can chilling tolerance and early-season growth be screened in growth chamber experiments?
- RQ7: How large is the genotype x environment effect on early-season growth?

Hypothesis 3: Variation in chilling tolerance in *Miscanthus* is linked to variation in biochemical and physiological traits.

- RQ8: Which biochemical traits, such as ROS, PPDK or soluble sugars relate to chilling stress tolerance in miscanthus?

Hypothesis 4: Increased cold tolerance and early season growth are linked with increased biomass yield.

- RQ9: Is there a relationship between growing season duration and final yield?
- RQ10: Is there a relationship between early-season growth and final yield?

Table 2.1: Overview of hypotheses, research questions and the chapters in this thesis in which these are addressed.

Hypothesis	Research question	Addressed in chapter:
H1	RQ1	4
	RQ2	4
	RQ3	4
H2	RQ4	5-6-7
	RQ5	5-6-7
	RQ6	7
	RQ7	7
H3	RQ8	8
H4	RQ9	9-10
	RQ10	9-10

Chapter 3: General Materials and Methods: Description of genotypes and trials

In this chapter a general overview of the plant material and field trials used to generate the results presented in this thesis is given. More detailed descriptions of measurements and trials are provided in the respective chapters.

Plant materials

In total 121 miscanthus genotypes were used for the experiments described in this PhD thesis (Table 3.1). Of these, 80 were *M. sinensis*, 17 *M. sacchariflorus*, 11 *M. x giganteus* and 13 were the result of interspecific crosses between *M. sinensis* and *M. sacchariflorus* (referred to as '*M. sinensis x sacchariflorus*' or 'hybrids' in this thesis). Species classification was based on information supplied by the providers of the germplasm and on visual observations of morphology in the field. For some genotypes genome size was determined using flow cytometry (Vergauwen, 2016). Ploidy data from other genotypes, as shown in Table 3.1 was obtained from literature (Clifton-Brown et al., 2001a). Most of the genotypes (all OPTIMISC genotypes except for OPM12-OPM15) were in vitro propagated, while the genotypes coming from the ILVO collection where rhizome propagated. The hormonal treatments during the in vitro propagation might have influenced results and should be taken into account when comparing genotypes starting with OPM to genotypes starting with EMI or IL.

Table 3.1: Main characteristics of the genotypes used in this PhD thesis, with indication of the chapters in which results are shown. Ploidy: 2: diploid (2x=2n=38), 3: triploid (2x=3n=57), 4 tetraploid (2x=4n=76), an.: aneuploid. Ploidy values in italics were obtained from literature. Propagation indicates whether plants where propagated in vitro or through rhizomes prior to planting. 'Seed' in this column indicates the four seed based populations used in a multilocation field trial (see below). Source indicates the supplier of the genotype (AU: Aarhus university; OPM: genotypes supplied by the OPTIMISC project; BR: Bruckeveld, Belgium; IBERS: IBERS, Aberystwyth University, UK; ILVO: Institute for Agriculture and Fisheries Research, Belgium; JD: J. Deplanque, Belgium; KU: Krakow University, Poland; TE: Testelmans, Belgium; AG: Agrimiscanthus, The Netherlands; WU: Department of plant breeding, Wageningen University, The Netherlands).

Genotype	Species	Ploidy	Propagation	Chapter 4	Chapter 5	Chapter 6	Chapter 7	Chapter 8	Chapter 9	Chapter 10	Source
OPM01	*M. sacchariflorus*	4	In vitro	x			x			x	OP (IB)
OPM02	*M. sacchariflorus*	4	In vitro	x			x			x	OP (IB)
OPM03	*M. sacchariflorus*	4	In vitro	x			x			x	OP (IB)
OPM04	*M. sacchariflorus*	2	In vitro	x			x			x	OP (IB)
OPM05	Hybrid	2	In vitro	x			x		x	x	OP (IB)
OPM06	Hybrid	2	In vitro	x			x	x	x	x	OP (IB)
OPM07	Hybrid	2	In vitro	x			x		x	x	OP (IB)
OPM08	Hybrid	2	In vitro	x			x		x	x	OP (IB)
OPM09	*M. x giganteus*	2	In vitro	x			x	x	x	x	OP (IB)
OPM10	Hybrid	an.	In vitro	x			x		x	x	OP (IB)
OPM11	*M. sinensis* Goliath	3	In vitro	x			x		x	x	OP (IB)

Genotype	Species	Ploidy	Propagation	Chapter 4	Chapter 5	Chapter 6	Chapter 7	Chapter 8	Chapter 9	Chapter 10	Source
OPM12	*M. sinensis*	2	seed				x				OP (IB)
OPM13	*M. sinensis*	2	seed				x				OP (WU)
OPM14	*M. sinensis*	2	seed				x				OP (WU)
OPM15	*M. sinensis*	2	seed				x				OP (IB)
OPM16	Hybrid	2	In vitro	x			x		x	x	OP (IB)
OPM17	Hybrid	2	In vitro	x			x		x	x	OP (IB)
OPM18	*M. sacchariflorus*	2	In vitro	x			x			x	OP (IB)
OPM19	*M. sacchariflorus*	2	In vitro	x			x			x	OP (IB)
OPM20	Hybrid	2	In vitro	x			x		x	x	OP (IB)
OPM21	*M. sacchariflorus*	2	In vitro	x			x			x	OP (IB)
OPM22	*M. sacchariflorus*	2	In vitro	x			x			x	OP (IB)
OPM23	*M. sacchariflorus*	4	In vitro	x			x			x	OP (IB)
OPM24	*M. sacchariflorus*	4	In vitro	x			x			x	OP (IB)
OPM25	*M. sacchariflorus*	4	In vitro	x			x			x	OP (IB)
OPM26	*M. sacchariflorus*	4	In vitro	x			x			x	OP (IB)
OPM27	*M. sacchariflorus*	4	In vitro	x			x			x	OP (IB)
OPM28	*M. sacchariflorus*	4	In vitro	x			x			x	OP (IB)
OPM29	*M. sinensis*	2	In vitro	x			x		x	x	OP (IB)
OPM30	*M. sinensis*	2	In vitro	x			x		x	x	OP(IB)
OPM31	*M. sinensis*	2	In vitro	x			x		x	x	OP (IB)
OPM32	*M. x giganteus*	3	In vitro	x			x		x	x	OP (IB)
OPM33	Hybrid	2	In vitro	x			x		x	x	OP (IB)
OPM34	*M. sacchariflorus*	4	In vitro	x			x			x	OP (IB)
OPM35	Hybrid	2	In vitro	x		x	x	x	x	x	OP (IB)
OPM36	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM37	*M. sinensis*	2	In vitro	x		x	x		x	x	OP (WU)
OPM38	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM39	*M. sinensis*	2	In vitro	x			x			x	OP (WU)
OPM40	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM41	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM42	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM43	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM44	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM45	*M. sacchariflorus*	4	In vitro	x			x		x	x	OP (WU)
OPM46	*M. sinensis*	2	In vitro				x		x		OP (WU)
OPM47	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM48	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM49	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM50	*M. sinensis*	2	In vitro	x		x	x		x	x	OP (WU)
OPM51	*M. sinensis*	2	In vitro	x		x	x	x	x	x	OP (WU)
OPM55	*M. sinensis*	2	In vitro				x		x		OP (WU)
OPM56	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM57	*M. sinensis*	2	In vitro				x		x	x	OP (WU)
OPM59	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM60	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM62	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM63	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM64	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM65	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM66	*M. sinensis*	2	In vitro	x			x	x	x	x	OP (WU)
OPM67	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM68	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM69	*M. x giganteus*	3	In vitro	x			x		x	x	OP (WU)
OPM71	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM72	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM73	*M. sinensis*	2	In vitro	x						x	OP (WU)
OPM74	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)

Genotype	Species	Ploidy	Propagation	Chapter 4	Chapter 5	Chapter 6	Chapter 7	Chapter 8	Chapter 9	Chapter 10	Source
OPM75	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM76	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM77	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM78	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM79	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM80	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM81	*M. sinensis*	2	In vitro	x			x			x	OP (IB)
OPM82	*M. sinensis*	2	In vitro	x							OP (WU)
OPM83	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM84	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM85	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM86	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM87	*M. sinensis*	2	In vitro	x						x	OP (WU)
OPM88	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM89	*M. sinensis*	2	In vitro	x					x	x	OP (WU)
OPM90	*M. sinensis*	2	In vitro	x			x			x	OP (WU)
OPM91	*M. sinensis*	2	In vitro	x			x			x	OP (WU)
OPM92	*M. sinensis*	2	In vitro	x						x	OP (WU)
OPM93	*M. x giganteus*	3	In vitro	x			x		x	x	OP (WU)
OPM94	*M. sinensis*	2	In vitro	x							OP (WU)
OPM95	*M. sinensis*	2	In vitro	x						x	OP (IB)
OPM96	*M. sinensis*	2	In vitro	x			x		x	x	OP (IB)
OPM98	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM99	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM100	*M. sin.'* Silberfeder'	2	In vitro	x			x		x	x	OP (ILVO)
OPM101	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM102	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM103	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM104	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM105	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM106	*M. sinensis*	2	In vitro	x						x	OP (WU)
OPM107	*M. sinensis*	2	In vitro	x			x		x	x	OP (WU)
OPM108	*M. sinensis*	2	In vitro	x						x	OP (WU)
OPM109	Hybrid	2	In vitro	x			x		x	x	OP (IB)
OPM110	*M. sinensis*	2	In vitro	x						x	OP (IB)
EMI-1	*M. x giganteus*	*3*	rhizome	x			x		x		AU
EMI-5	*M. sacchariflorus*	*4*	rhizome	x							AU
EMI-8	Hybrid	*an.*	rhizome	x			x		x		AU
EMI-9	*M. sinensis*	*2*	rhizome	x			x		x		AU
EMI-10	Hybrid	*2*	rhizome	x			x		x		AU
EMI-11	*M. sinensis*	*2*	rhizome	x			x		x		AU
EMI-12	*M. sinensis*	*2*	rhizome	x			x		x		AU
EMI-13	*M. sinensis*	*2*	rhizome	x			x		x		AU
EMI-14	*M. sinensis*	*2*	rhizome	x			x		x		AU
EMI-15	*M. sinensis*	*2*	rhizome	x			x		x		AU
IL2	*M. sin.* 'Gracillimus'	2	rhizome				x		x		BR
IL4	*M. x giganteus*	3	rhizome	x			x		x		KU
IL6	*M. x giganteus*	3	rhizome	x			x		x		JD
IL7	*M. x giganteus*	3	rhizome	x			x		x		TE
IL8	*M. x giganteus*	3	rhizome	x			x		x		TE
IL9	*M. x giganteus*	3	rhizome	x			x		x		TE
IL10	*M. x giganteus*	3	rhizome	x	x	x				x	AG
IL11	*M. sin.* 'Goliath'	3	rhizome	x	x						BR

Field trials and growth chamber experiments

Six field trials were used for the experiments presented in this thesis. Four of the trials were established at ILVO facilities (Melle - Merelbeke, Belgium) as part of the EU project OPTIMISC (Field trial 1-4), one was already present at this location when this thesis was initiated (Bioenergy trial, Muylle et al., 2015; Van Hulle et al., 2012) and one was established at six different locations across Europe and Turkey by other OPTIMSC project partners (Multilocation field trial).

The five OPTIMISC trials were set up mainly with genotypes from the collections of breeders at Wageningen University and Aberystwyth University, while the bio-energy trial was set up with commercially available genotypes. All trials, except for the multilocation trial, were planted in the area Melle - Merelbeke (51°0'N, 3°48'E), on light sandy loam soil and a temperate maritime climate with mean rainfall of 800 mm per year and annual mean temperature of 10.5°C over the past 10 years. The maximum distance between these trials was 2 km.

A number of other experiments were performed under controlled conditions in growth chambers. Table 3.2 provides an overview of the chapters in which the different field trials were used or for which growth chamber experiments were run.

Table 3.2: Overview of the use of field trials and growth chamber experiments in this PhD thesis.

		Chapter						
Trial	Code	4	5	6	7	8	9	10
Field trial 1	FT1	x						x
Field trial 2	FT2	x			x	x	x	
Field trial 3	FT3			x				
Field trial 4	FT4	x		x				
Bioenergy field trial			x					
Multilocation field trial					x			
Growth chambers		x	x		x			

Weather data (temperature, precipitation, radiation, wind speed and relative humidity) were collected on a daily basis in a weather station in Merelbeke at approximately 1 km from the field trials (Fig. 3.1). The 2014 growing season was markedly wetter than the 2015 growing season, with 494 mm precipitation between March and September 2014 while only 371 mm of rain fell during the same period in 2015. Both years were warmer than average. The total accumulated thermal time with a base temperature of 0°C at the end of the year was 4333 growing degree days (GDD) in 2014 and 4078 GDD in 2015, compared to an average of 3918 GDD in the last 25 years. Especially the spring of 2014 was warmer than usual (Fig. 3.2). By 30/6/2014 the accumulated thermal time was 1885 GDD, compared to 1567 GDD in 2015 and the average of 1636 GDD. All

calculations growing degree days units in this thesis were calculated with a base temperature of 0°C. Growing degree days were calculated as the sum of the daily temperatures starting from January 1st.

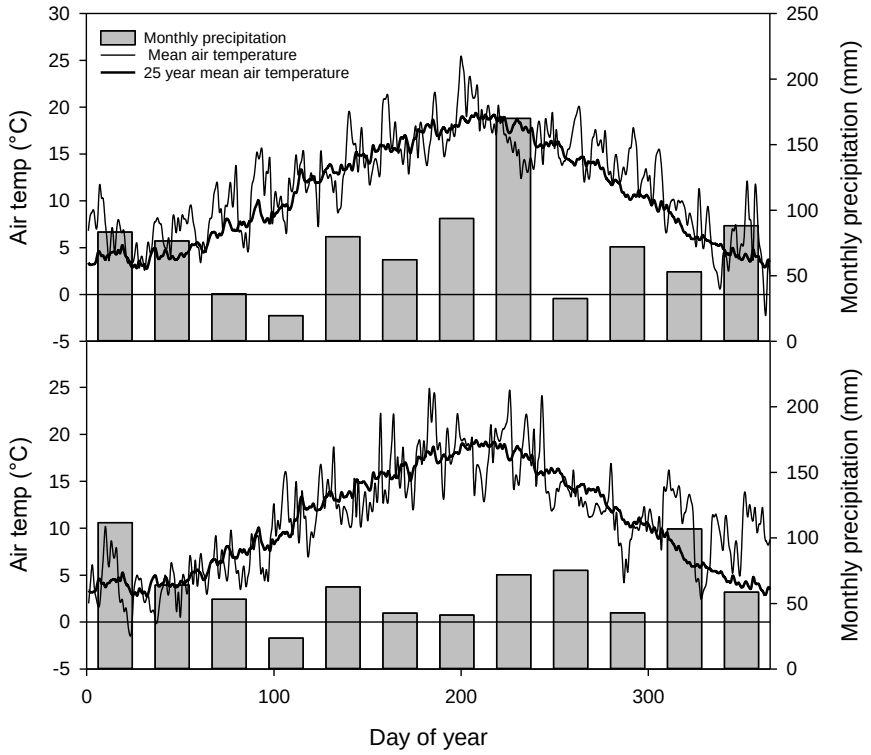


Figure 3.1: Daily temperature and monthly precipitation data for 2014 (upper figure) and 2015 (lower figure) in Melle, Belgium. Data were collected on a daily basis in a weather station in Merelbeke, approximately 1 km from the field trials.

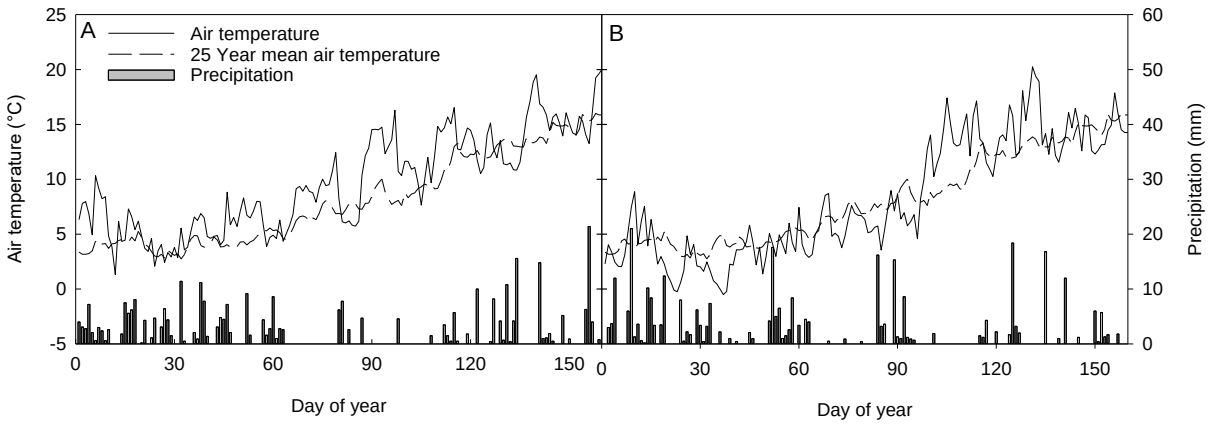


Figure 3.2: Daily mean air temperature and daily precipitation between January 1st and May 31st in 2014 (A) and 2015 (B) in Merelbeke, Belgium.

Field trial 1

The main goal of FT1 was to evaluate the biomass yield and quality of 100 genotypes. Additionally, a number of phenology and physiology related characteristics were determined, such as emergence, canopy temperature, flowering time and senescence.

The trial was a randomized complete block design with two blocks of 100 miniplots (Fig. 3.3 and 3.4). Each miniplot was planted with 12 (3x4) plants of one genotype at a between-plant distance of 0.7 m. The middle two plants of each miniplot were used for measurements, while the outer ten served as a border.

The trial was established on 6-8/05/2013, additional plants were planted on 5/08/2013, 23/09/2013 and 13/05/2014. In total the trial contained 97 genotypes. The trial was harvested in 2014 and 2015 by the end of March when the moisture content of the *M. x giganteus* genotypes was approximately 20%.

28	89	67	10	96	92	83	108	47	IL10	49	84	5	60	57	79	100	3	4	18
110	71	31	39	86	17	7	68	38	88	50	35	16	44	65	90	27	24	6	95

81	102	78	48	106	33	63	105	66	62	64	101	22	93	73	74	85	25	29	2
45	26	1	34	107	20	99	32	19	109	75	23	9	76	59	77	69	21	30	80

51	8	56	43	31	42	6	72	37	41	58	40	11	91	103	36	98	18	87	104
36	74	IL10	84	58	73	91	104	57	85	105	72	10	92	108	33	49	63	78	37

80	59	21	30	95	16	65	77	50	75	17	26	109	88	32	31	110	7	86	38
87	60	4	5	3	100	40	101	98	22	28	47	8	102	81	62	42	41	6	43

23	76	24	9	69	90	6	27	35	44	34	39	1	20	45	107	71	68	19	99
11	18	2	18	79	29	64	103	25	93	56	51	83	89	96	66	43	48	67	106

Figure 3.3: Map of Field trial 1: White: block 1, Grey: block 2. Numbers without letter code indicate OPM genotypes as listed in Table 2.1. Plots are 2.1 by 2.8 m in size.

Figure 3.4: Field trial 1 (left) and field trial 2 (right). Aerial picture taken on 12/10/2015.

Field trial 2

FT2 was set up to intensively monitor cold stress symptoms and early-season growth in a large collection of miscanthus genotypes during winter and spring. This field was harvested at the end of December or the beginning of January, in order to enable the counting and measuring of new emerging shoots (impossible to be done on Field trial 1 due to standing biomass until harvest in March). On this trial the length of the longest shoot per plant was measured, the number of leaves on this shoot was recorded and the number of shoots per plant was counted twice weekly from February until the end of May in 2014 and 2015. Thereafter the length of the longest shoot per plant was measured monthly until the end of the growing season.

The trial was laid out as a complete randomized block design with six blocks, in each of which a single plant of 120 genotypes was planted randomly at a distance of 0.7 m (Fig. 3.4 and 3.5). The whole trial was surrounded by one row of plants of genotype *M. sinensis* OPM50. Of the planned 120 genotypes, 114 genotypes were successfully established, but the 16 *M. sacchariflorus* genotypes spread out too widely and were destroyed in May 2014.

The trial was established on 6/5/2013, but at that time not all genotypes had been propagated in vitro. Therefore, additional plantings were performed on 5/8/2013 and 25/9/2013. In September 2013, the remaining empty spaces were filled with random plants of genotypes OPM06, OPM07, OPM08, OPM50 and OPM60, which were not used for the analyses, these genotypes are indicated in italics.

8	*8*	97	*8*	37	*8*	41	10	29	*8*	28	*6*	10	100	56
7	71	99	4	45	65	EMI10	*8*	60	81	*6*	6	37	*6*	*6*
25	EMI14	101	38	28	38	78	35	46	45	1	6	34	25	93
65	1	43	95	9	*8*	23	IL4	40	71	49	71	35	40	EMI11
64	85	*8*	86	92	EMI13	*8*	34	105	*8*	107	11	41	2	19
107	EMI1	102	106	31	61	22	50	*8*	*8*	*6*	51	*6*	IL2	*6*
8	57	IL2	36	47	18	67	*50*	3	IL6	98	29	45	22	78
50	17	56	*8*	3	EMI7	EMI1	*50*	79	89	88	*6*	*6*	EMI8	IL9
34	87	*8*	11	49	IL7	48	36	85	1	*6*	*6*	*6*	43	*6*
8	44	76	27	*8*	28	76	49	74	90	59	95	83	*6*	33
72	74	110	78	96	107	108	2	7	43	30	IL8	EMI12	92	74
84	*8*	55	98	105	9	EMI14	64	26	6	104	23	20	18	EMI14
39	66	100	EMI9	8	21	16	42	4	68	109	6	*6*	*6*	27
18	8	89	5	93	106	31	110	80	*50*	26	105	*6*	64	36
26	48	40	*8*	*8*	98	IL2	32	47	109	106	9	21	38	8
8	68	EMI8	75	IL8	EMI15	EMI8	93	19	*50*	72	110	IL7	42	*6*
EMI10	20	79	90	10	*50*	24	*50*	44	IL9	*6*	*6*	80	*6*	*6*
30	80	33	23	IL7	*50*	100	66	72	59	60	50	7	84	EMI15
24	35	77	108	21	55	25	102	84	30	66	*6*	*6*	62	16
29	22	81	59	*8*	IL8	96	63	EMI12	92	99	*6*	47	102	101
8	6	67	EMI12	32	8	37	75	17	27	31	67	3	69	*7*
104	*8*	42	EMI5	*8*	86	*50*	87	95	104	4	17	24	77	75
41	62	103	2	*8*	*50*	20	69	5	51	*6*	63	32	48	44
8	109	60	51	19	*50*	EMI9	EMI11	99	11	86	68	76	5	65
8	71	11	87	*8*	67	5	46	IL4	*50*	EMI11	IL8	87	*7*	56
EMI8	27	*8*	EMI12	IL8	105	37	25	*50*	100	IL7	80	86	*7*	*7*
8	46	77	41	60	69	EMI8	9	76	IL7	51	100	22	*7*	*7*
92	79	*8*	23	75	*50*	1	17	59	60	40	*7*	42	*7*	66
36	110	86	8	EMI9	32	101	85	*50*	74	*7*	78	*7*	*7*	71
74	43	EMI10	EMI11	44	68	71	99	EMI12	EMI1	7	92	1	89	59
103	*8*	7	37	96	96	83	*50*	48	28	*7*	76	18	9	*7*
101	84	21	93	55	26	45	19	21	*50*	85	*7*	IL2	23	36
8	69	IL9	17	25	78	*6*	89	40	87	*7*	41	101	57	88
72	20	40	*8*	9	49	77	92	22	34	109	6	*7*	*7*	8
28	26	*8*	*8*	IL6	EMI9	33	110	107	65	81	50	4	60	93
76	EMI1	47	*8*	45	10	55	50	106	4	77	*7*	*7*	46	95
8	99	31	67	*8*	36	*6*	62	104	43	*7*	25	44	27	19
78	*8*	IL2	*8*	102	24	*6*	EMI13	3	72	105	98	10	20	28
1	3	6	56	38	*6*	31	EMI15	*6*	27	102	IL9	72	3	99
19	49	10	80	*8*	86	18	*6*	102	*6*	37	62	*7*	26	84
8	109	48	EMI13	34	75	7	8	56	42	29	48	17	75	45
8	*8*	98	100	4	IL8	64	*6*	109	30	EMI9	*60*	11	2	16
22	29	50	64	68	47	57	EMI10	6	*6*	47	EMI8	38	EMI10	EMI15
83	*8*	42	90	107	35	*6*	41	88	95	63	EMI14	49	*60*	21
33	62	*8*	88	35	84	*6*	90	93	EMI5	106	*60*	43	32	68
105	66	2	57	106	20	*6*	98	79	66	103	24	64	*60*	31
IL7	5	30	51	18	51	IL2	38	11	2	67	*60*	34	35	79
104	39	24	32	65	44	23	80	6	29	*60*	74	*60*	5	69

Figure 3.5: Map of Field trial 2. Numbers without letter code indicate OPTMISC OPM genotypes. The complete trial was surrounded by a border of OPM50 plants. Genotype codes as in Table 2.1.

Field trial 3

On FT3 detailed plant measurements were combined with destructive biomass harvests to determine the correlation between early-season growth and biomass production. Early-season growth measurements performed in FT1 and FT2 were combined with sequential destructive harvests of aboveground biomass in FT3.

FT3 was established in September 2013 (Fig. 3.6) using in vitro propagated plants of three genotypes (hybrid OPM35, *M. sinensis* OPM37 and *M. sinensis* OPM50). FT3 consisted of nine miniplots of three plants per genotype. All 'miniplots' were surrounded by rows of other miscanthus genotypes (OPM6, 8, 20, 31, 37, 38, 43, 45, 48, 50) as shown in figure 3.7. The distance between all plants in this trial was 0.7 m. The field trial was harvested at the same time as FT1.

Figure 3.6: Field trial 3 on 12/05/2015

OPM 37	OPM50	OPM08	OPM 37	OPM06	OPM 48	OPM 48
OPM 37	OPM50	OPM08	OPM 37	OPM06	OPM 35	OPM 48
OPM 37	OPM50	OPM08	OPM 37	OPM06	OPM 35	OPM 48
OPM 37	OPM50	OPM08	OPM 37	OPM06	OPM 35	OPM 48
OPM 37	OPM50	OPM08	OPM 37	OPM06	OPM 35	OPM 48
OPM 37	OPM50	OPM08	OPM 37	OPM06	OPM 35	OPM 48
OPM 37	OPM50	OPM08	OPM 37	OPM06	OPM 35	OPM 48
OPM 20	OPM50	OPM50	OPM 37	OPM50	OPM 35	OPM 43
OPM 20	OPM50	OPM50	OPM 37	OPM50	OPM 35	OPM 43
OPM 20	OPM50	OPM50	OPM 37	OPM50	OPM 35	OPM 43
OPM 20	OPM50	OPM50	OPM 37	OPM50	OPM 35	OPM 43
OPM 20	OPM50	OPM50	OPM 37	OPM50	OPM 35	OPM 43
OPM 20	OPM50	OPM50	OPM 37	OPM50	OPM 35	OPM 43
OPM 20	OPM50	OPM50	OPM 37	OPM50	OPM 35	OPM 43
OPM 20	OPM50	OPM50	OPM 37	OPM50	OPM 35	OPM 43
OPM 20	OPM50	OPM50	OPM 37	OPM50	OPM 35	OPM 43
OPM 20	OPM50	OPM50	OPM 37	OPM50	OPM 35	OPM 43
OPM 20	OPM50	OPM50	OPM 37	OPM50	OPM 35	OPM 43
OPM 20	OPM50	OPM50	OPM 37	OPM50	OPM 35	OPM 43

OPM 20	OPM50	OPM50	OPM 37	OPM50	OPM 35	OPM 43
OPM 20	OPM50	OPM50	OPM 37	OPM50	OPM 35	OPM 43
OPM 20	OPM50	OPM50	OPM 37	OPM50	OPM 35	OPM 43
OPM 31	OPM50	OPM08	OPM 37	OPM 38	OPM 35	OPM50
OPM 31	OPM50	OPM08	OPM 37	OPM 38	OPM 35	OPM50
OPM 31	OPM50	OPM08	OPM 37	OPM 38	OPM 35	OPM50
OPM 31	OPM50	OPM08	OPM 37	OPM 38	OPM 35	OPM50
OPM 31	OPM50	OPM08	OPM 37	OPM 38	OPM 35	OPM50
OPM 31	OPM50	OPM08	OPM 37	OPM 38	OPM 35	OPM50
OPM 31	OPM50	OPM08	OPM 37	OPM 38	OPM 35	OPM50
OPM 31	OPM50	OPM08	OPM 37	OPM 38	OPM 35	OPM50
OPM 31	OPM50	OPM08	OPM 37	OPM 38	OPM 35	OPM50
OPM 31	OPM50	OPM08	OPM 37	OPM 38	OPM 35	OPM50
OPM 31	OPM50	OPM08	OPM 37	OPM 38	OPM 35	OPM50
OPM 31	OPM50	OPM08	OPM 37	OPM 38	OPM 35	OPM50
OPM 31	OPM50	OPM08	OPM 37	OPM 38	OPM 35	OPM50
OPM 31	OPM50	OPM08	OPM 37	OPM 38	OPM 35	OPM50
OPM 31	OPM50	OPM08	OPM 37	OPM 38	OPM 35	OPM50

Figure 3.7: Map of FT3. The miniplots consist of the plants marked in grey, which were the plants used for measurements and biomass sampling and the surrounding border plants.

Field trial 4

FT4 was set up to propagate plant materials for the screening of rhizome frost tolerance and for the production of plant materials for other field trials and growth chamber experiments. No special design was used. The trial was planted in June 2013.

In 2014 four genotypes were selected in the field trial and used for the same measurements as performed on FT3 in 2015. On three plants each of these genotypes the early-season growth measurements performed in FT1 and FT2 were performed combined with sequential destructive harvests of aboveground biomass in FT4 on a weekly basis between from 2/04/14 till 23/05/14.

FT4 consisted of rows of up to 35 plants (depending on the success of in vitro propagation) per genotype, planted at a distance 0.7 m between-rows and 0.5 m within-the row (Fig. 3.8).

IL11	
IL10	
OPM-01	OPM-59
OPM-02	OPM-60
OPM-03	OPM-61
OPM-04	OPM-62
OPM-05	OPM-63
OPM-06	OPM-64
OPM-07	OPM-65
OPM-08	OPM-66
OPM-09	OPM-67
OPM-10	OPM-68
OPM-11	OPM-69
OPM-16	OPM-71
OPM-17	OPM-72
OPM-18	OPM-73

OPM-19	OPM-74
OPM-20	OPM-75
OPM-21	OPM-76
OPM-22	OPM-77
OPM-23	OPM-78
OPM-24	OPM-79
OPM-25	OPM-80
OPM-26	OPM-81
OPM-27	OPM-82
OPM-28	OPM-83
OPM-29	OPM-84
OPM-30	OPM-85
OPM-31	OPM-86
OPM-32	OPM-87
OPM-33	OPM-88
OPM-34	OPM-89
OPM-35	OPM-90
OPM-36	OPM-91
OPM-37	OPM-92
OPM-38	OPM-93
OPM-39	OPM-94
OPM-40	OPM-95
OPM-41	OPM-96
OPM-42	OPM-98
OPM-43	OPM-99
OPM-44	OPM-100
OPM-45	OPM-101
OPM-51	OPM-102
OPM-47	OPM-103
OPM-48	OPM-104
OPM-49	OPM-105
OPM-50	OPM-106
OPM-04	OPM-107
OPM-56	OPM-108
OPM-57	OPM-109
OPM-24	OPM-110

Figure 3.8: Field map of FT4. Genotypes indicated in grey were used for biomass sampling in 2014. Each row contains up to 35 plants of a genotype.

Bio-energy trial

The bio-energy trial was set up in May 2007 to compare the biomass yield of different crops, including miscanthus, over several seasons in Flanders. Before the initiation of the experiment this field had been used since 2004 to grow maize, with rye sown as winter catch crop. Before planting, compost was added at a rate of 25 t ha^{-1} (137N, $6P_2O_5$, and $14K_2O$ kg ha^{-1}). Three groups of cropping systems were considered: (i) annual crops, (ii) perennial grassland, and (iii) lignocellulosic crops. As annual crops maize, sorghum, and Italian ryegrass were evaluated, as grassland crops perennial ryegrass, cocksfoot, timothy and tall fescue and as lignocellulosic crops miscanthus, switchgrass, common reed, reed canary grass, and willow. The experimental layout

of the field trial was a randomized split-plot block design with three replications. Each replicate (block) was divided in three sub-blocks, corresponding to the three cropping systems. The position of the sub-blocks within each replicate was randomized, and the crops were randomized within the sub-blocks. The plots within the sub-blocks of cropping systems 'annuals crops' and 'perennial grassland' were divided into two subplots, referring to two fertilizer treatments (low F and medium F). This trial was described extensively in Muylle et al. (2015) and Van Hulle et al. (2012). In this thesis, this trial was used for the yield and early-season growth data of *M. x giganteus* IL10 and *M. sinensis* 'Goliath' IL11 presented in chapter three. The IL11 plants were obtained from *Bruckeveld* (Belgium) and the *IL10* plants 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. The second part of the field trial contains another complete randomized block design containing 26 *Miscanthus* genotypes with three repetitions per genotype. Each repetition consists of one row of ten plants (distance between rows was 1 m, distance between plants within the row was 0.6 m).

Multilocation field trial

The multi-location field trial consisted of six identical fields in Adana (Turkey), Aberystwyth (Wales), Hohenheim (Germany), Potash (Ukraine), Moscow (Russia) and Wageningen (The Netherlands) (Table 3.3, Fig. 3.9). The main aim was to compare biomass yield of fifteen genotypes and seed based populations in different locations. The six fields were planted in spring 2012, and the exact lay-out of the trials depended on local conditions. Each of the trials consisted of a complete randomized block design with three blocks of 15 plots. Each plot contained seven by seven plants of one single genotype planted at 0.7 m between and within rows. The eleven clonal genotypes included in the trial (Table 3.1) were multiplied using *in vitro* propagation, while for the four seed-based genotypes seedlings were generated in a greenhouse. The trials were harvested in winter after the plants had senesced and dried down. Several measurements were performed in these trials, in this thesis only the emergence measurements were used. In the first weeks before and after emergence of the plants in each site the length of the longest shoot of five plants per plot in each of the three plots was measured from soil level to shoot tip. The shoot length of five plants per plot was determined at 6 time points during the growing season in 2014 and 2015. These 6 time points were distributed from the day of first emergence until the plants had reached a height of approximately 50 cm.

Table 3.3: Description of the six sites used for the multi-location field trial and the site of our field trials.

Country	Location	Latitude (°N)	Longitude (°E)	Altitude (m a.s.l.)	$T_{\bar{a}}$, (°C) Annual mean	$T_{\bar{a}}$(°C) Mean April to September
Turkey	Adana	37.0	35.0	27	19.0	26.1
Germany	Stuttgart	48.7	8.9	463	9.8	16.4
Ukraine	Potash	48.9	30.4	237	8.9	18.5
Netherlands	Wageningen	51.6	5.4	10	10.3	15.8
Wales	Aberystwyth	52.4	-4.0	39	9.7	13.8
Russia	Moscow	55.0	37.0	140	4.1	14.8
Belgium	Merelbeke	51.0	3.8	17	10.7	15.1

Figure 3.9: Location of the multilocation field trials. Black dots represent the location of the multilocation field trial, the red square indicates the location of ILVO in Belgium.

Topic I

Frost tolerance

Chapter 4: How low can you go? – Rhizome and shoot frost tolerance in miscanthus germplasm

This chapter is based on: Fonteyne, S., Muylle, H., De Swaef, T., Reheul, D., Roldán-Ruiz, I., Lootens, P., 2016. How low can you go? - Rhizome and shoot frost tolerance in miscanthus germplasm. Ind. Crops Prod. 89, 323–331.

Introduction

Frost can kill miscanthus rhizomes during winter and damage newly emerged shoots in spring (Clifton-Brown & Lewandowski, 2000; Jørgensen & Schwarz, 2000). It is currently unknown whether genotypes with frost-tolerant rhizomes also have more frost-tolerant young shoots. Therefore, both responses must be differentiated when the frost tolerance of a given genotype is screened. The frost tolerance of miscanthus rhizomes can be determined in artificial freezing tests. Clifton-Brown & Lewandowski (2000) tested the rhizome frost tolerance of five genotypes. The temperature at which 50% of the rhizomes were killed (LT_{50}) varied from -3.4°C for *M. x giganteus* to -6.5°C for a *M. sinensis* hybrid. Furthermore, they demonstrated a correlation between these LT_{50} values and winter survival in the abovementioned EMI trial (Clifton-Brown & Lewandowski, 2000). Recently, Peixoto et al. (2015) tested five genotypes using a similar protocol and found LT_{50} values ranging between -1.5°C and -6.7°C, depending on genotype and harvesting date of the rhizomes. The range of rhizome freezing tolerance in a large collection of diverse genotypes is currently unknown. The LT_{50} of only 14 genotypes, five of which were *M. x giganteus*, have been reported in literature (Clifton-Brown & Lewandowski, 2000; Friesen et al., 2015; Peixoto et al., 2015; Płażek et al., 2011). A large scale screening would make it possible to estimate the potential for improvement, to determine breeding goals for rhizome frost tolerance and to select tolerant genotypes that could either be planted in colder locations or serve as parents for adapted varieties.

The frost tolerance of young miscanthus shoots has been screened in a small number of studies. Genotypic variation has been reported on a limited number of genotypes by Farrell et al. (2006) and Zub et al. (2012), both of whom report considerable leaf damage in all tested genotypes below a temperature of -8°C in a controlled environment. However, in field trials Friesen et al. (2014) and Głowacka et al. (2015b) observed leaf damage and severe reduction of photosynthesis after cold spells with minimum air temperatures of -1.8°C and -3.6°C, respectively. Frost sensitivity increases with leaf developmental stage, making frost spells later in the growing season generally more damaging (Zub et al., 2012). This effect is most likely due to the location of the shoot apex: in young shoots, the apex is located underground, protecting it from frost damage, whereas in

older shoots the apex is aboveground and thus more exposed to freezing temperatures. In *M. x giganteus* at least, the shoot apex remains underground until the shoot has developed 6-7 leaves (Zub et al., 2012). The range of shoot frost tolerance in the miscanthus germplasm is largely unknown and an extensive screening for this trait is necessary to assess the available variation and to select improved genotypes.

Our study was set up to determine the extent of the variation in frost tolerance of rhizomes and shoots of a large collection of miscanthus genotypes from diverse origins, representing the germplasm currently available to (and generated by) European miscanthus breeders. Our purpose was to identify genotypes that are better suited than *M. x giganteus* for cultivation in areas where frost damage during the winter and/or early spring is a real risk, or genotypes that might be used in breeding programs for improved frost tolerance. The specific aims of our study were therefore: (i) to test the rhizome and shoot frost tolerance of a large germplasm collection, and (ii) to determine the relationship between flowering, senescence and rhizome frost tolerance.

Materials and methods

The study consisted of two separate parts. In the first part the frost tolerance of miscanthus rhizomes was tested, while in the second part the frost tolerance of newly emerged shoots was evaluated. For rhizome frost tolerance, first the LT_{50} of the rhizomes was determined in artificial freezing tests. This data was then used to assess the biological reproducibility of the results (experiment RFT1) and to determine the variation in LT_{50} in the germplasm (RFT2). Thereafter the relation between LT_{50} and rhizome moisture content (RFT3) and phenological data (RFT4) were determined. In the second part of the experiments the frost tolerance of newly emerged shoots was scored in a field trial (SFT1) and the height of the shoot apex was measured in a growth chamber experiment (SFT2). An overview of the experiments performed for this study, the kind of plant materials and the number of miscanthus genotypes used is provided in Table 4.1.

Plant material

A large, diverse miscanthus germplasm collection representative of the current European breeding material was used. The collection consists of 117 genotypes including 76 *M. sinensis,* 17 *M. sacchariflorus,* 11 *M. x giganteus* and 13 interspecific (*M. sinensis* x *M. sacchariflorus*). This classification was based on information supplied by the providers of the germplasm and confirmed by phenotypic observations in the field and by determination of genome size through flow cytometry. The large majority of genotypes (99) was obtained through the European project OPTIMISC (Lewandowski et al., 2015) (*www.optimisc-project.eu*), of these 61 were supplied by Wageningen University, 37 by Aberysthwyth University and one by ILVO. Eighteen additional genotypes were obtained from various sources. Most genotypes are the results of crosses with

genotypes of unknown origin. As a result, no data about the climatic conditions at the location of origin was available for most genotypes.

In our study, 95 genotypes were screened for rhizome frost tolerance (RFT2). Of these, 91 were also screened for senescence and flowering time in field trial FT1 (RFT4). In field trial FT2, 104 genotypes were screened for shoot frost tolerance. Of these 104, 89 were OPTIMISC genotypes, 10 were genotypes used in the European Miscanthus Improvement (EMI) projects' trials (Clifton-Brown et al., 2001a) and five were commercially available *M. x giganteus* clones. All OPTIMISC plant material was propagated *in vitro*. Some OPTIMISC genotypes could not be propagated efficiently *in vitro* and were therefore either not screened for frost or not included in the field trials. Non-OPTIMISC genotypes were propagated using rhizome cuttings from field-established plants.

Table 4.1: Overview of the different experiments performed in this study and their purposes. The numbers in the 'Genotypes' column indicate the number of distinct genotypes used in each experiment, the numbers between brackets list the number of *M. sinensis, M. sacchariflorus, M. x giganteus* and hybrid genotypes, respectively, in each group.

Rhizomes	*Aim*	*Method*	*Plant material*	*Genotypes*
RFT1	Technical reproducibility of freezing test	Artificial freezing test	Rhizomes	12 (4/0/2/6)
RFT2	Determination of LT_{50}	Artificial freezing test	Rhizomes	95 (66/16/5/9)
RFT3	Relationship between LT_{50} and rhizome characteristics	Determination of moisture content and size	Rhizomes	95 (66/16/5/9)
RFT4	Relationship between LT_{50} and phenology	Scoring of flowering and senescence in a field trial	Field trial FT1	91 (62/16/4/9)
Shoots				
SFT1	Determination of shoot frost tolerance	Scoring of shoot frost stress in field trial	Field trial FT2	104 (64/17/10/13)
SFT2	Determination apex height	Destructive measurements	Pot plants	5 (1/2/2/0)

Determination of rhizome frost tolerance (RFT1-2)

All rhizome material screened for frost tolerance was produced in field trial FT4. Plants were dug up from the propagation field during winter on January 17th and February 18th in 2014 and on January 13th and 14th and February 10th and 24th in 2015. Most genotypes were only tested once (RFT2), but twelve genotypes were harvested multiple times, either both in 2014 and 2015 or twice in 2015, to test the reproducibility of the frost screening results (RFT1). After digging up the plants, all shoots were cut and the remaining rhizome clumps were covered with moist potting soil and stored at 3°C in the dark until needed. All rhizomes were used within maximum 55 days after harvest. For the freezing test, the protocol of Clifton-Brown & Lewandowski (2000) was followed. The plant material was washed to remove soil and separated into single rhizome pieces. The morphology of the rhizomes differed strongly among species. Most genotypes developed

rhizomes only in the top 10 cm top of the soil, but *M. x giganteus* formed a larger clump of rhizomes deeper in the ground, up to 30 cm below soil level. The *M. sinensis* genotypes formed rather small and thin rhizomes in a clump under the plant, while the *M. sacchariflorus* genotypes formed much thicker and longer rhizomes, leading to considerable variation between the genotypes in the rhizome material used for the freezing tests. For testing *M. sinensis,* rhizomes were pulled apart into pieces containing at least one bud; for *M. sacchariflorus,* rhizomes were cut into 10-cm pieces. *M. x giganteus* and hybrid rhizomes were intermediate in size between *M. sinensis* and *M. sacchariflorus.* The rhizome pieces were then wrapped in wet tissue paper, vacuum sealed in plastic and stored at 3°C until needed (max. 4 d). For each genotype, 16 rhizome pieces were evaluated per temperature, in two repetitions of eight rhizomes. Four temperatures (-2, -3, -4 and -6°C) were tested, and an additional 20 rhizome pieces were planted without treatment to test rhizome viability. The refrigerated bath (PC200-A24B, Thermo Scientific Haake, Waltham, MA, USA) was cooled at a rate of 3°C h^{-1}, followed by a constant temperature for 3 h, after which the temperature was raised by 3°C h^{-1} to 4°C (Clifton-Brown & Lewandowski, 2000). The rhizomes were left to thaw at 4°C and planted in wet sand. Afterwards they were placed in a dark growth chamber at a constant temperature of 20°C and a relative humidity of 70%. After four weeks the rhizomes were dug up and the number of rhizomes that had formed new shoots and/or roots was counted. Rhizomes producing only roots after four weeks were considered as dead for the determination of LT_{50}. The genotypes were screened sequentially, at a maximum rate of 12 genotypes per week.

Determination of rhizome characteristics (RFT3)

At the beginning of each test 10 rhizomes per genotype were used to determine the average rhizome mass and moisture content. Roots and soil were first removed from the rhizomes. Thereafter the rhizomes were weighed, dried at 70°C for 48 h in a ventilated oven (Binder Gmbh. Tuttlingen, Germany) and then weighed again.

Phenotyping flowering time and senescence in the field (RFT4)

In field trial 1, flowering and senescence were scored for 91 of the genotypes that had been screened for rhizome frost tolerance. This trial, established in Melle (Belgium) in May 2013, was set up as a complete randomized block design with two blocks. Each plot consisted of 12 plants of a single genotype, planted in four rows of three plants. The distance between plants was 0.7 m; plot size was 5.9 m². The two central plants were used for measurements, while the other 10 plants served as border. Flowering was scored visually from June 25th 2014 to September 21st 2014 with a score of 0 to 4 (0: no flowering; 1: flag leaf formed; 2: panicle emergence; 3: anthesis; 4: end of anthesis). The day of the year (DOY) when a certain score was reached was calculated using linear interpolation. Senescence was scored visually from September 25th 2014 to March 11th 2015 by estimating the proportion of the plant that was still green, as described in Robson et al. (2011).

The DOY when 50% of the plant had already senesced was then calculated through linear interpolation.

Determination of shoot cold stress tolerance (SFT1)

In field trial 2 shoot damage was scored after a frost event. This trial included 114 genotypes and was planted in Melle, Belgium in May 2013 as a complete randomized block design, planted in six blocks of 5 by 24 single plants, with 0.7 m spacing between plants. The trial was harvested in December 2013 to facilitate early-season emergence observations. All genotypes in field trial 2 were scored for cold stress symptoms on March 28th, 2014, two days after the end of a cold spell with three consecutive days of night frost during which the minimum temperature was -1.6°C. A score ranging from 1 to 9 was used to score shoot frost tolerance. The scores ranged from 1 (sensitive) to 9 (very tolerant) and were defined as follows: 1: all leaves killed by frost; 2: >50% leaves irreversibly damaged; 3: <50% leaves irreversibly damaged; 4: Leaves purple with white parts due to photo bleaching; 5: most of the leaves purple; 6: some purple coloring of the leaves; 7: yellowish green, light green plant, no other stress symptoms; 8: green plant; 9: dark green plant.

Determination of apex height (SFT2)

In order to estimate the position of the apex at the moment frost stress occurred in field trial 2, the apex height was determined for five genotypes (*M. sinensis* OPM30, *M. sacchariflorus* OPM04 and OPM24 and *M. x giganteus* OPM09 and OPM32). Per genotype, 15 rhizomes were planted in potting soil and placed in a growth chamber (20°C, 170 µmol PAR m^{-2} s^{-1}, 0.62 kPa vapor pressure deficit, 16 h day length). The rhizomes were allowed to grow for two weeks, thereafter three plants per genotype were analyzed weekly for a period of five weeks. On each plant the length of the longest shoot measured from the rhizome to the tip of the longest leaf and the number of leaves on this shoot were determined. The plants were then dissected and the distance between the top of the apex of the longest shoot and the rhizome was measured.

Statistical analyses

The lethal temperature was determined as the temperature at which 50% of the rhizomes failed to produce new shoots (LT_{50}). LT_{50} was calculated using a logistic regression with a probit link function (Finney, 1952). The analysis was performed in R 3.1.0 (R core team, Vienna, Austria) using the *glm* function of the built-in *stats* package. The effects of parameters such as species, moisture content, rhizome weight, harvest date on LT_{50} and differences in shoot frost tolerance between genotypes and species were evaluated using generalized linear models. The analysis was performed in R 3.1.0 (R core team, Vienna, Austria) using the *glm* function of the built-in *stats* package. The difference between the LT_{50} of the genotypes tested both in 2014 and 2015 or twice in 2015 was tested using paired Students' t-tests in the *stats* package. Correlations were calculated using Pearson's product moment correlation using the *cor.test* function from the *stats* package.

Results

Rhizome frost tolerance

Biological reproducibility of the screening protocol (RFT1)

The large set of genotypes included in this study made it logistically impossible to test all plant material in one evaluation round. To determine how this might have affected the results, we tested 12 genotypes twice: eight genotypes were harvested and tested both in 2014 and 2015, while four genotypes were harvested and tested twice in 2015. There was no significant difference in LT_{50} between years nor between both harvest dates in 2015 (Table 4.2), with an average difference between the different tests of one genotype of 0.7°C. Only for the hybrid OPM07 and the *M. sinensis* IL11 was a difference of more than 1°C observed between repeated tests. We can thus conclude that the procedure followed to estimate LT_{50} values for the larger collection of miscanthus genotypes was appropriate.

Table 4.2: Genotypes tested more than once for rhizome frost tolerance (RFT1). LT_{50} (°C), standard error and the difference in °C between the two runs are shown.

	2014	2015	Difference
OPM06	-3.9 ± 0.6	-3.1 ± 0.3	-0.8
OPM07	-2.2 ± 0.3	-4.3 ± 0.4	2.1
OPM08	-4.7 ± 0.5	-5.1 ± 0.8	0.4
OPM09	-2.2 ± 0.3	-3.1 ± 0.3	0.9
OPM10	-3.9 ± 0.4	-4.4 ± 0.4	0.5
OPM17	-3.9 ± 0.4	-3.2 ± 0.5	-0.7
OPM20	-4.7 ± 0.3	-3.9 ± 0.3	-0.8
OPM29	-3.7 ± 0.3	-3.5 ± 0.5	-0.2
	2015 - test 1	2015 - test 2	
OPM66	-3.7 ± 0.3	-3.7 ± 0.3	- 0.0
OPM79	-4.8 ± 0.3	-4.1 ± 0.3	-0.7
IL11	-4.5 ± 0.3	-2.9 ± 0.3	-1.6
IL10	-2.3 ± 0.3	-1.9 ± 0.2	-0.4

Range of rhizome frost tolerance in the germplasm (RFT2)

A large range of LT_{50} values was obtained by the freezing tests of the whole collection, with LT_{50} ranging from -5.9°C for OPM64 to -0.4°C for OPM44 (Fig. 4.1). Overall, the hybrid genotypes were more frost tolerant than the *M. sacchariflorus* and *M. x giganteu*s genotypes. However, the *M. sacchariflorus* genotype OPM28 had a LT_{50} of -5.1°C, similar to that of the most frost tolerant hybrid, OPM08. The *M. sinensis* genotypes were not different from either group (Table 4.3). The observed LT_{50} values of the *M. x giganteus* genotypes was on average -2.6°C. Thus in these tests *M. x giganteus* was less tolerant to freezing than most other miscanthus genotypes. The variability within *M. x giganteus* was larger than would be expected if these genotypes were all genetically identical. Whether the difference between the *M. x giganteus* genotypes was due to different hormonal treatments during propagation, due to genuine genetic differences between genotypes or due to variability in the results of the screening method could not be determined in the experiment.

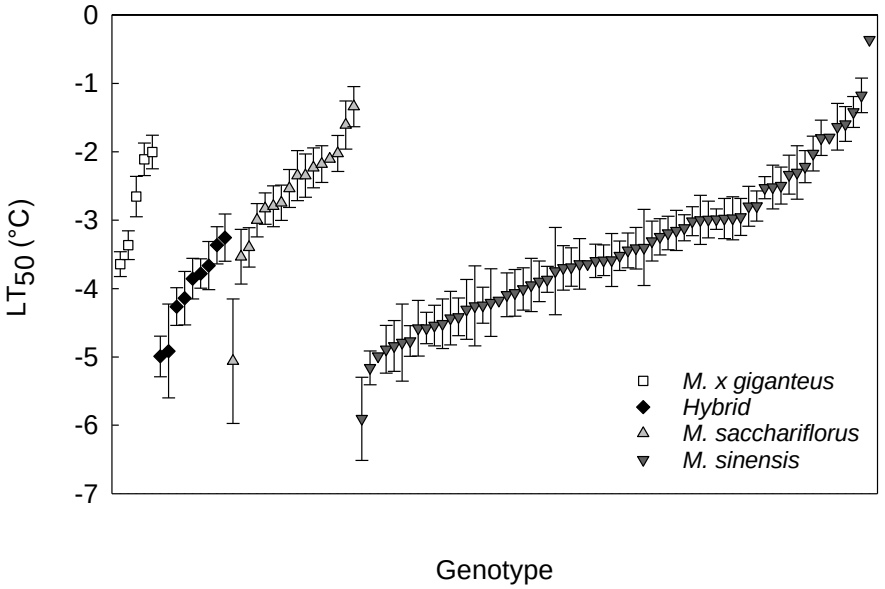


Figure 4.1: Average rhizome frost tolerance of the tested genotypes (RFT2). Symbols represent the average LT_{50} per genotype. Error bars show the standard error. Different symbols indicate different species groups. 'Hybrid' are M. sinensis x sacchariflorus genotypes.

Almost all rhizomes in the control treatment formed new shoots within two weeks after planting, indicating little or no dormancy requirement. Some genotypes, especially of *M. x giganteus*, formed a large amount of roots before producing new shoots, regardless of the treatment. Some rhizomes produced only new roots and no new shoots within four weeks after the frost treatment. When these rhizomes were put in soil for another four weeks, they rarely produced shoots. The production of roots but not shoots might indicate that although the buds had been killed by frost, the rhizome was still alive.

Table 4.3: Average LT50 and moisture content with standard error per species (RFT3) (*M. sinensis*: n = 70, *M. sacchariflorus*: n = 16, *M. x giganteus*: n = 7, Hybrid: n = 15. Within a column, different letters show significant differences as determined by Tukey HSD test ($p < 0.05$).

Species	Mean LT_{50} (°C)	Moisture content (% H_2O)
M. x giganteus	-2.6 ± 0.3^{a}	64.5 ± 3.3^{ab}
M. sacchariflorus	-2.6 ± 0.2^{a}	69.8 ± 2.3^{a}
M. sinensis	-3.5 ± 0.1^{ab}	51.1 ± 1.2^{c}
Hybrid	-3.9 ± 0.2^{b}	53.9 ± 1.7^{bc}

For 12 of the *M. sacchariflorus* genotypes tested, reliable information about the collection site was available and was used to calculate correlations between LT_{50} and characteristics of the original location. There was a significant negative correlation between LT_{50} and latitude, and a significant positive correlation between LT_{50} and the number of degree days above 10°C at the original location (Table 4.4). This indicates that genotypes from more northern locations are more frost tolerant, having a LT_{50} of approximately 0.1°C lower per °N, than genotypes from warmer locations. There were no significant correlations with other parameters describing the site of origin.

Table 4.4. Correlation between rhizome LT_{50} and characteristics of the location of origin of 12 *M. sacchariflorus* genotypes (RFT2). Correlations marked with * are significant ($p < 0.05$).

Parameter	Correlation	*p*
Altitude (m.a.s)	0.110	0.735
Latitude (°N)	-0.633*	0.027
Mean maximum monthly temperature (°C)	0.570	0.053
Mean minimum monthly temperature (°C)	0.429	0.164
Summer rain (mm)	0.169	0.600
Degree days above 10°C (DD)	0.630*	0.028

Relationship between rhizome characteristics and rhizome frost tolerance (RFT3)

The average moisture content of the rhizomes across all genotypes was 55.6%, with a range between 29.7% and 83.4%. Overall the moisture content was significantly correlated ($r = 0.322$) with LT_{50}, but moisture content was also significantly different between species (Table 4.3). The less frost tolerant *M. sacchariflorus* and *M. x giganteus* genotypes had rhizomes with a higher moisture content, whereas the *M. sinensis* and hybrid genotypes had on average lower moisture contents. Within species, there was no significant correlation between rhizome moisture content and LT_{50}, nor was a significant influence of other rhizome characteristics observed such as fresh or dry weight, rhizome diameter or harvest date on frost tolerance. Moisture content therefore is

not a good trait to distinguish frost tolerant genotypes, as the correlation detected between these two parameters was probably due to inter-species differences.

Relationship between phenology and rhizome frost tolerance (RFT4)

Flowering and senescence in the field were scored visually for 91 out of the 95 genotypes screened for frost tolerance (Fig. 4.2). Correlations were calculated separately per species group because of significant species by trait (anthesis or senescence) interactions. As the *M. x giganteus* and hybrid groups were too small, correlations were only calculated for *M. sinensis* and *M. sacchariflorus* (Table 4.5). None of the *M. sacchariflorus* genotypes flowered, but a significant although low correlation was found between LT_{50} and flowering date (r = 0.324) in *M. sinensis*. There was no difference in day of the year at which 50% of the plant had senesced between the species. Senescence was significantly correlated with LT_{50} values in both *M. sinensis* and *M. sacchariflorus*. The correlation was high in *M. sacchariflorus* (r = 0.723) but the correlation in *M. sinensis* was rather low (r = 0.330) (Fig. 4.2). Interestingly, the *M. sinensis* genotype OPM44 that did not senesce and remained green until harvest time in spring had very frost sensitive rhizomes (LT_{50} = -0.36°C).

Table 4.5: Correlation between the day a genotype reached first anthesis or was 50% senesced and the rhizome LT_{50} (RFT4). Significant correlations are marked with * (Pearson's product-moment, *M. sinensis*: n = 65, *M. sacchariflorus*: n = 16. Correlations marked with NA could not be calculated due to lack of data.

	M. sinensis		*M. sacchariflorus*	
Parameters	Correlation	p	Correlation	p
DOY first anthesis	0.324*	0.007	NA	NA
DOY 50% senescence	0.330*	0.007	0.723*	0.001

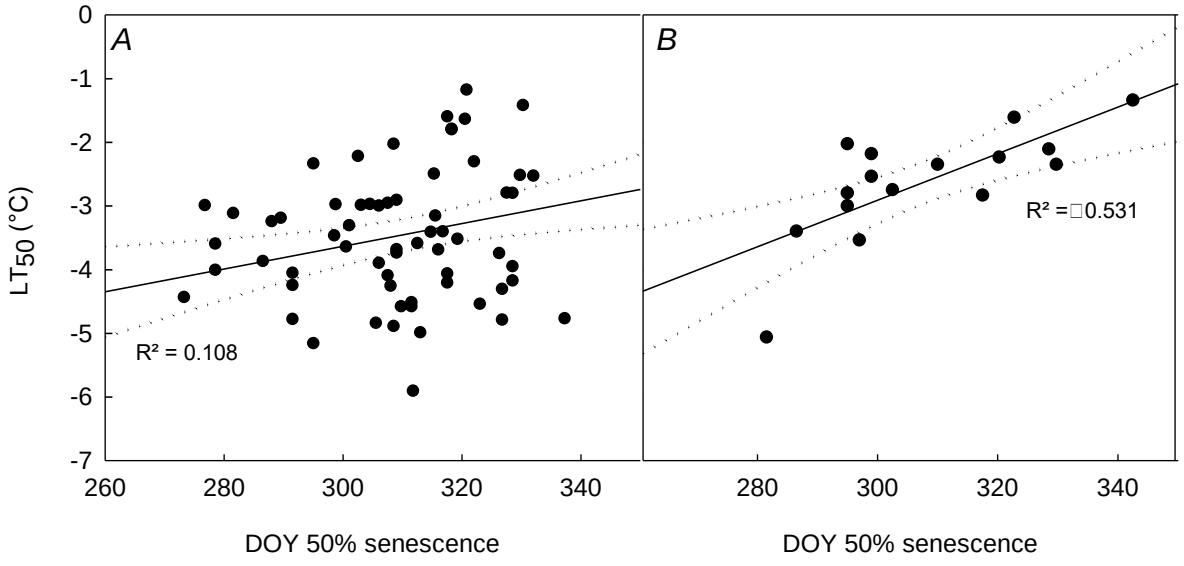


Figure 4.2: Relationship between rhizome LT_{50} and the day of the year (DOY) 50% of the plant was senesced (RFT4). A: M. sinensis, B: M. sacchariflorus. Dashed lines show 95% confidence intervals.

Shoot frost tolerance

Shoot frost stress scored in the field (SFT1)

In the 2014 growing season, emergence started very early (beginning of March) because of a warm winter and early spring. At the end of March, a colder period with night frost occurred, after which the extent of frost stress symptoms on the shoots was scored (Fig. 4.3). A large diversity of cold stress responses was observed among genotypes (Fig. 4.4). Plants of some genotypes remained green and displayed no stress symptoms, while others became yellowish and showed considerable anthocyanin formation. Other plants had purple leaves with white spots due to photobleaching. A relatively small number of the genotypes had leaves that were completely killed by frost (scores 1 to 3). Leaves suffering from photobleaching (score 4) did not recover; the bleached parts of the leaves were effectively killed and turned brown later on. Plants with a score higher than 4 did not show any permanent damage and all leaves turned green when temperatures increased in April. The shoots of *M. sacchariflorus* and *M. x giganteus* genotypes were on average more cold susceptible, with average scores of 4.5 ± 0.2 and 4.8 ± 0.2, respectively, than the *M. sinensis* and hybrid genotypes, which had average scores of 6.3 ± 0.1 and 5.3 ± 0.2, respectively. In 2015 no similar frost event occurred and no obvious stress symptoms were observed.

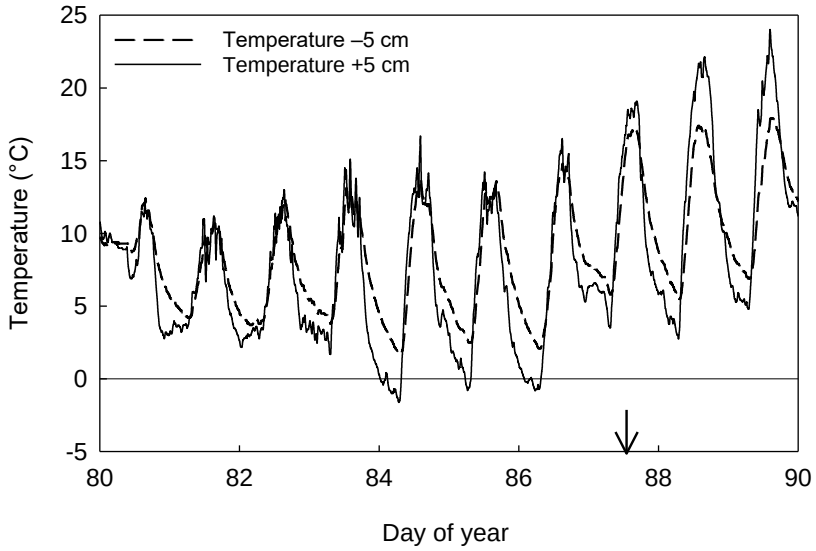


Figure 4.3: Temperature at 5 cm above soil level and at 5 cm below soil level from March 21, 2014 to March 30, 2014 in field trial 2 in Melle, Belgium (SFT1) The arrow marks the moment cold stress was scored.

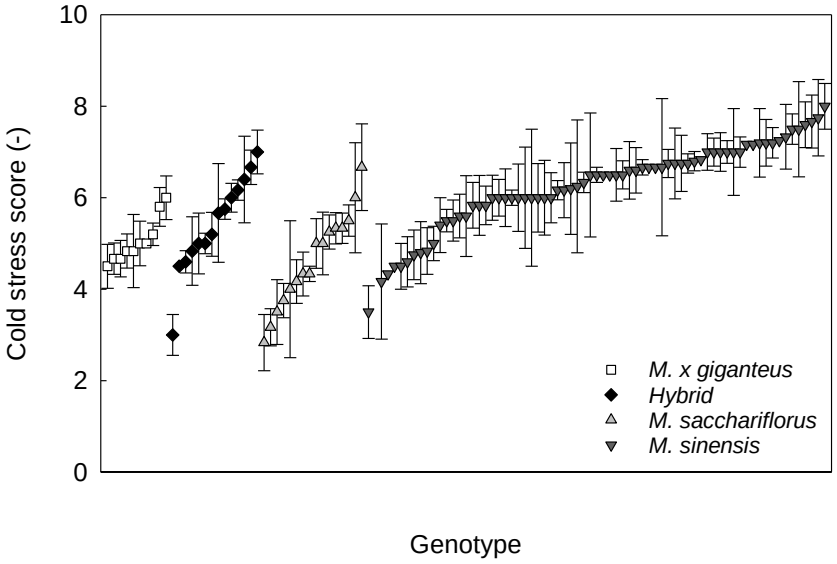


Figure 4.4: Average cold stress score of the shoots measured in the field trial in Melle (Belgium) (SFT1). Symbols represent average scores per genotype (n = 2-6). Different symbols indicate different species groups. Error bars show the standard error. Hybrid genotypes are *M. sinensis x sacchariflorus* genotypes. The scores were defined as follows: 1: all leaves killed by frost; 2: >50% leaves irreversibly damaged; 3: <50% leaves irreversibly damaged; 4: leaves purple with white parts due to photobleaching; 5: most of the leaves purple; 6: some purple colouring of the leaves; 7: yellowish green, light green plant, no other stress symptoms; 8: green plant; 9: no symptoms.

Apex height (SFT2)

The damaged leaves in the SFT1 experiment were quickly replaced by new ones, indicating that the shoot apex was probably not damaged. Soil temperature measurements showed that the temperature did not drop below 0°C at 5 cm (Fig. 4.3), which could explain why the shoot apex did not experience permanent damage due to low temperature. In order to test this hypothesis, the height of the apex of five genotypes as a function of shoot length and developmental stage (number of leaves) was determined in a growth chamber. In all genotypes tested, the top of the apex was less than 50 mm above the rhizome until the shoot was at least 60 cm long or had developed at least six leaves (Fig. 4.5). The rhizomes of field-grown plants are generally situated more than 50 mm below soil level (personal observation), and no shoot was longer than 50 cm when the cold spell occurred (data not shown). This is suggesting that in all the genotypes the shoot apex was still belowground when air temperature dropped below 0°C. These results should however be treated with some caution, as we did not determine the apex height in the field trial.

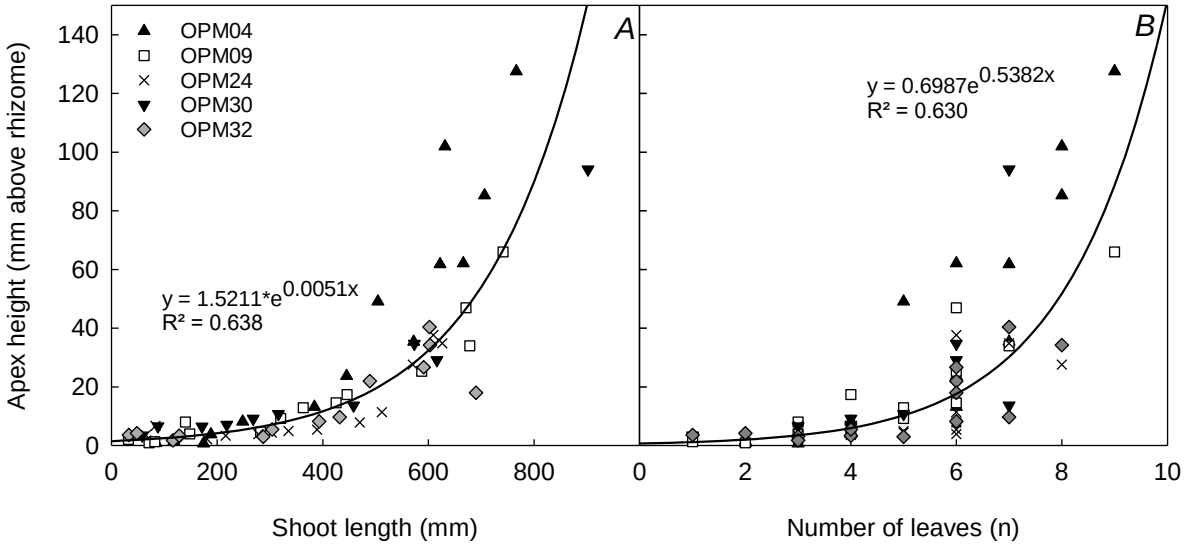


Figure 4.5: Shoot apex height above the rhizome as a function of shoot length (A) and number of leaves (B) determined on five genotypes grown in a growth chamber (SFT2). Symbols depict individual measurements.

Discussion

How large is the variation in rhizome frost tolerance in miscanthus?

To the best of our knowledge, this is the first large-scale study of rhizome frost tolerance in miscanthus. The most frost tolerant genotypes had LT_{50} values below -5°C, which was considerably lower than the LT_{50} values of *M. x giganteus*. Previous studies (Clifton-Brown & Lewandowski, 2000; Friesen et al., 2015; Peixoto et al., 2015; Płażek et al., 2011) reported results for a limited number of genotypes that did not allow generalization at the level of the genus or of the germplasm currently used in breeding programs. On average rhizomes of *M. sinensis* and *M. sinensis x sacchariflorus* hybrid genotypes had LT_{50} values of -3.5°C and -3.9°C respectively, about 1°C degree lower than the -2.6°C of *M. sacchariflorus* and *M. x giganteus* genotypes. This difference between species corresponds well with the observed differences for winter mortality in the EMI field trials (Clifton-Brown et al., 2001a) and the interspecies differences reported by Clifton-Brown & Lewandowski (2000) and Peixoto et al. (2015). It is however still not possible to generalize at the species level because the number of *M. sacchariflorus* genotypes screened here was much lower than the number of *M. sinensis* genotypes.

Furthermore, estimating possible consequences of different LT_{50} values for the different species investigated is not straightforward due to differences in rhizome morphology. *M. sacchariflorus* and *M. x giganteus* form new shoots each spring from belowground buds, while *M. sinensis* and *M. sinensis x sacchariflorus* hybrids have smaller, more superficial rhizomes and can form new shoots from within shoots from the previous year (personal observation) as well as from rhizome buds. The rhizomes and buds of *M. sacchariflorus* and *M. x giganteus* are thus likely better insulated by the soil than those of *M. sinensis* and *M. sinensis x sacchariflorus* hybrids. Because

of these differences in morphology, under field conditions *M. sacchariflorus* and *M. x giganteus* rhizomes are possibly less exposed to low temperatures during the same frost event than those of *M. sinensis* and hybrids.

The LT_{50} values obtained in our study were slightly higher than those reported by Clifton-Brown & Lewandowski (2000), Friesen et al. (2015) or Peixoto et al. (2015). Possible causes for this could be differences in the set of genotypes screened, in acclimation prior to the tests or in experimental protocols. In our study the same freezing prototcol as in Clifton-Brown & Lewandowski (2000) was followed, which differed from that of Friesen et al. (2015) and Peixoto et al. (2015) in that the cooling rate was faster i.e. 3°C h^{-1} instead of 1°C h^{-1}. The rate of cooling and length of exposure can strongly impact LT_{50} (Peixoto & Sage, 2016). For example, Peixoto et al. (2015) also applied a staged cooling protocol wherein the temperature was lowered by 2.5°C every 24 hours and obtained LT_{50} values of -6.3 to -14.4°C. When they used a continuous cooling protocol, more similar to the one used in our study, the LT_{50} values obtained were more comparable to our results. However, they used different genotypes for the different protocols, so it is unclear whether genotype ranking was affected. The winters of 2013-2014 and 2014-2015, when the rhizomes used for freezing tests in our study were harvested, were rather mild. As a consequence, the plants senesced slowly and possibly had not undergone a similar level of cold acclimation in the field as in the other studies. Peixoto et al. (2015) reported that genotypes harvested in the summer were about 2-3°C less frost tolerant than genotypes harvested in the winter but genotype ranking was not strongly affected.

What is the extent of shoot frost tolerance in miscanthus?

The variation in shoot frost tolerance observed in this study demonstrates that numerous miscanthus genotypes are more tolerant than the widely cultivated *M. x giganteus*. While some genotypes (mostly *M. sacchariflorus* and *M. x giganteus*) in our trial showed extensive leaf damage, many genotypes (mostly *M. sinensis*) did not display any sign of damage due to frost. Similarly, Kaiser & Sacks (2015) observed *M. x giganteus* to be relatively susceptible to frost in a field trial including 95 mostly ornamental miscanthus genotypes. However, Friesen et al. (2014) and Głowacka et al. (2015b) found the photosynthetic capacity of *M. x giganteus* to recover faster after minor frost events compared to most other genotypes in their trials.

Farrell et al. (2006) reported LT_{50} values between -6°C and -9°C for miscanthus shoots, and Kaiser & Sacks (2015) recently reported more than 50% survival at -10°C in two out of four seedling populations tested. The lowest air temperature measured at 2 m above the soil level in our trial was -1.6°C, however the temperature at plant level near the soil surface might have been lower. After the cold spell the damaged plants quickly formed new leaves and no strong delay in growth was observed. The apex heights obtained in our study were similar to those reported by Zub et al. (2012) for *M. x giganteus* and suggest that the shoot apex was probably located belowground in

all plants when the frost event occurred. While in the more frost susceptible genotypes leaves were killed, the below ground apex was probably not damaged and shoot growth was not markedly reduced compared to the frost tolerant genotypes. Similarly, it has been reported that in maize frost damage is less severe in younger plants, which still have below ground apices (Carter, 1995).

How does frost tolerance relate to geographical origin and phenology?

The *M. sacchariflorus* genotypes in our study originating from colder regions were more frost tolerant than genotypes originating from warmer areas. Similarly, Yan et al. (2011) observed that winter survival was significantly correlated with the latitude of origin in a collection of 93 *M. sacchariflorus, M. sinensis* and *M. lutarioriparius* seedling populations planted in a field trial in Northern China. In their study the *M. sacchariflorus* populations had significantly better winter survival than the *M. sinensis* and *M. lutarioriparius* populations. In contrast, Anzoua et al. (2015) reported no significant difference in winter survival in a field trial in northern Japan between *M. sinensis* accessions from northern and southern Japan. Genotypes that flowered and senesced earlier tended to have higher frost tolerance in our study. These genotypes likely had more time to reach a state of dormancy and to acclimate to lower temperatures. Phenotypic traits such as flowering or senescence also vary along latitudinal and altitudinal gradients in the natural range of miscanthus in East Asia (Jensen et al., 2013; Slavov et al., 2013). Unfortunately for most of the genotypes studied here the geographical origin is unknown and we could not carry out a more in depth analysis of these correlations.

Is rhizome moisture content a good predictor of frost tolerance?

The molecular mechanisms behind frost tolerance in miscanthus remain unexplained. In other crops several mechanisms have been reported to protect the cell against ice formation, cell dehydration or membrane damage (Ruelland et al., 2009; Sandve et al., 2011; Tarkowski & Van den Ende, 2015). Clifton-Brown & Lewandowski (2000) analyzed the relationship between rhizome frost LT_{50} values and rhizome characteristics such as moisture and carbohydrate content, osmotic potential of cell sap, and mineral composition. Of these traits, only moisture content was significantly correlated with LT_{50}. Likewise, in our study there appeared to be a relationship between LT_{50} and moisture content. However, the correlation observed in both studies was most likely caused by interspecific differences for both moisture content and rhizome frost tolerance. *M. sacchariflorus* genotypes tended to have higher moisture contents and LT_{50} values and *M. sinensis* genotypes tended to have low moisture contents and LT_{50} values. Nevertheless, within each species we did not detect a significant correlation between moisture content and LT_{50}. Moisture content is thus not an accurate predictor of LT_{50} in miscanthus. This agrees with findings in switchgrass, for which Hope & McElroy (1990) found that crown moisture content was not a good predictor of frost tolerance. Whether moisture content is related with frost tolerance on the genotype level could not be determined from our study since most genotypes were only sampled

once. Although Clifton-Brown & Lewandowski (2000) did not detect a relationship between biochemical traits and LT_{50}, other traits than the ones measured in their study might affect rhizome frost tolerance. It has been demonstrated that during autumn carbohydrates are translocated from the shoot to the rhizome (Purdy et al., 2014). Furthermore, during the same period the concentrations of raffinose, linoleic and alfalinoleic acid, which can protect the cell against the effects of frost stress, increase in the rhizomes (Withers, 2015). Correspondingly in miscanthus leaves cold stress has also been shown to induce the accumulation of non-structural carbohydrates, including raffinose (Fonteyne et al., 2016; Purdy et al., 2014).

What are the implications of this study for breeding?

Breeding for improved frost tolerance will require either screening new material in locations where frost damage and winter mortality are likely to occur, or an efficient, fast and cheap screening protocol under controlled conditions. Płażek et al. (2011) and Peixoto et al. (2015) used the ion leakage method to determine rhizome frost kill. That method has important drawbacks: not only is it labor-intensive, the relationship between ion leakage and LT_{50} is not always well determined (Peixoto & Sage, 2016). Frost primarily kills the rhizome buds, while ion leakage is generally measured on a whole rhizome level. Alternatively, studying regrowth provides more information because it is more representative of field conditions (Mortaignie, 2014). For example, Płażek et al. (2011) reported LT_{50} values of -4.2 to -12.1°C for a *M. x giganteus* clone by measuring electrolyte leakage, taking 50% electrolyte leakage as a threshold, while Peixoto et al. (2015) showed that 50% rhizome mortality occurs already around the level of 20% electrolyte leakage. This suggests that a screening test based on controlled freezing and regrowth is more appropriate. For breeding purposes, a less accurate procedure to estimate frost tolerance than determining the LT_{50} might suffice. It might be sufficient to test whether rhizomes survive a certain threshold value, for example -6°C (Hastings et al., 2009). Such an approach would greatly reduce the number of rhizomes needed, consequently reducing time and labor needed for the tests. It would also allow to apply slower cooling rates and longer exposure times, simulating more closely the field situation, in large scale screening experiments. Anyhow, results of screenings of rhizome frost tolerance done under controlled conditions, as in our study, require further validation under realistic field conditions, where factors, such as plant size, rhizome depth, soil type and moisture content, snow and leaf litter cover and the intensity of the frost event will likely affect survival (Clifton-Brown et al., 2015; Kucharik et al., 2013; Roy, 2016).

The rhizome LT_{50} observed in our study varied considerably, with a 5.5°C difference between the most and the least frost tolerant genotype. Such a difference may have a large impact on the potential growing area. For example, using the MISCANFOR miscanthus yield model, Hastings et al. (2009) calculated that improved hybrids with a LT_{50} of -6°C could be commercially grown in a significantly larger part of Europe compared to *M. x giganteus* (with a LT_{50} of -3°C). Likewise, an

LT_{50} of -6°C may markedly increase the potential growing area compared to *M. x giganteus* in the USA (Kucharik et al., 2013). These values mentioned in literature are similar to the extremes found in our study. According to Farrell et al. (2006) genotypes which combine improved frost shoot tolerance with earlier emergence could theoretically take advantage of a longer growing season compared to *M. x giganteus* in areas where late severe frost events are common. They simulated yield of a theoretical genotype with increased frost tolerance and concluded that it might produce up to 25% higher biomass yields. Both simulations should be interpreted with caution however, as the other model parameters that determine growth and biomass accumulation were assumed to be the same as for the high yielding *M. x giganteus.* Increased tolerance to frost stress may come with a yield penalty however. In the trial by Yan et al. (2011) the genotypes with the best winter survival yielded less biomass in the more southern locations. In *Arabidopsis lyrata* and among tree species a trade-off between frost tolerance and growth rate has been reported (Loehle, 1998; Wos & Willi, 2015) while in *Triticum durum* no such trade-off was found (Longin et al., 2013). Field trials will be needed to clarify the relationship between cold tolerance and final biomass yield and whether there is a yield penalty attached to increased stress tolerance (Fonteyne et al., 2016).

Compared to other perennial C4 grasses the rhizomes of miscanthus are relatively susceptible to frost damage. For example, the rhizomes of Johnsongrass (*Sorghum halapense* (L.) Pers.) and zoysiagrass (*Zoysia japonica* Steud.) can survive temperatures up to -10°C, and the rhizomes of switchgrass (*Panicum virgatum* L.) or prairie cordgrass (*Spartina pectinata* Bosc ex Link) can even survive temperatures below -20°C (Friesen et al., 2015; Hope & McElroy, 1990; Stoller, 1977; Warmund et al., 1998). This relatively higher frost susceptibility in miscanthus clearly shows the necessity of breeding for improved frost tolerance. Although rhizome and shoot frost tolerance were not significantly correlated on species level (results not shown), *M. sinensis* and *M. sacchariflorus* genotypes that combine both were found. The genotypic variation observed in our study indicates that it should be possible to develop new high yielding varieties with improved winter and spring frost survival, provided that the heritability for frost tolerance is sufficiently high. In a study by Kaiser & Sacks (2015) a seedling population generated in a cross with a northern adapted *M. sacchariflorus* genotype had significantly higher survival at -10°C than other seedling populations, which suggest some degree of heritability of cold tolerance. Furthermore, in crops such as wheat and barley, frost tolerance has been shown to be highly heritable (Doerffling et al., 1997; Longin et al., 2013). Heritability needs further study in miscanthus in order to determine if effective breeding for frost tolerance is indeed possible.

Conclusion

Developing new miscanthus varieties with improved tolerance to frost stress is necessary to reduce winter mortality and to expand the potential growing area. A large variation in frost tolerance was observed in the tested miscanthus germplasm, which represented current

European breeding programs. Many of the genotypes investigated are more tolerant to frost temperatures than the currently planted *M. x giganteus*. Provided that the heritability for frost tolerance is sufficiently high, the tolerant genotypes identified here can be used as parents to breed frost tolerant varieties.

Topic II

Chilling tolerance and early-season growth

Chapter 5: Chilling tolerance and early-season growth-related characteristics evaluated in two *Miscanthus* genotypes to identify useful tools and methods for large-scale screens

This chapter is based on: Fonteyne, S., Lootens, P., Muylle, H., Van den Ende, W., De Swaef, T., Reheul, D., Roldán-Ruiz, I., 2016. Chilling tolerance and early vigour related characteristics evaluated in two *Miscanthus* genotypes. Photosynthetica. 54, 295–306.

Introduction

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. 2014a), photosynthesis-related characteristics (Purdy et al. 2013, Friesen et al. 2014, Głowacka et al. 2014a), and/or soluble sugar contents (Purdy et al. 2013). With the exception of the study of Yan et al. (2011), who analyzed 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, ecophysiological studies have mainly investigated the effects of short term chilling stress. This might not be representative of the field situation, however, and 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.* x *giganteus* genotype might comprise clones from different sources with slightly different responses to chilling stress, making extrapolation of results among studies and the comparison of field and growth chamber results of different studies difficult. This ambiguity might 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. (2015b), 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.* x *giganteus*

clone (IL10) and one *M. sinensis* Goliath clone (IL11). *M.* x *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.* x *giganteus* was consistently higher yielding than *M. sinensis* Goliath in a field trial established in Melle, Belgium in 2007 (Muylle et al. 2015). *M.* x *giganteus* has been reported to display a relatively smaller decline in leaf elongation rate (LER) when transferred from 28 to 12°C than *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.* x *giganteus*, as also shown by Clifton-Brown and Jones (1997).

The main purpose of the research presented in this chapter was to create basic comparison tools to investigate chilling tolerance in miscanthus in relation to field performance. Additional parameters are investigated in chapter six. In a later stage, some of tools are used for the screening of a large collection of genotypes. The following specific questions are investigated in this chapter: (*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? A schematic overview of the research performed for this chapter is given in Figure 5.1.

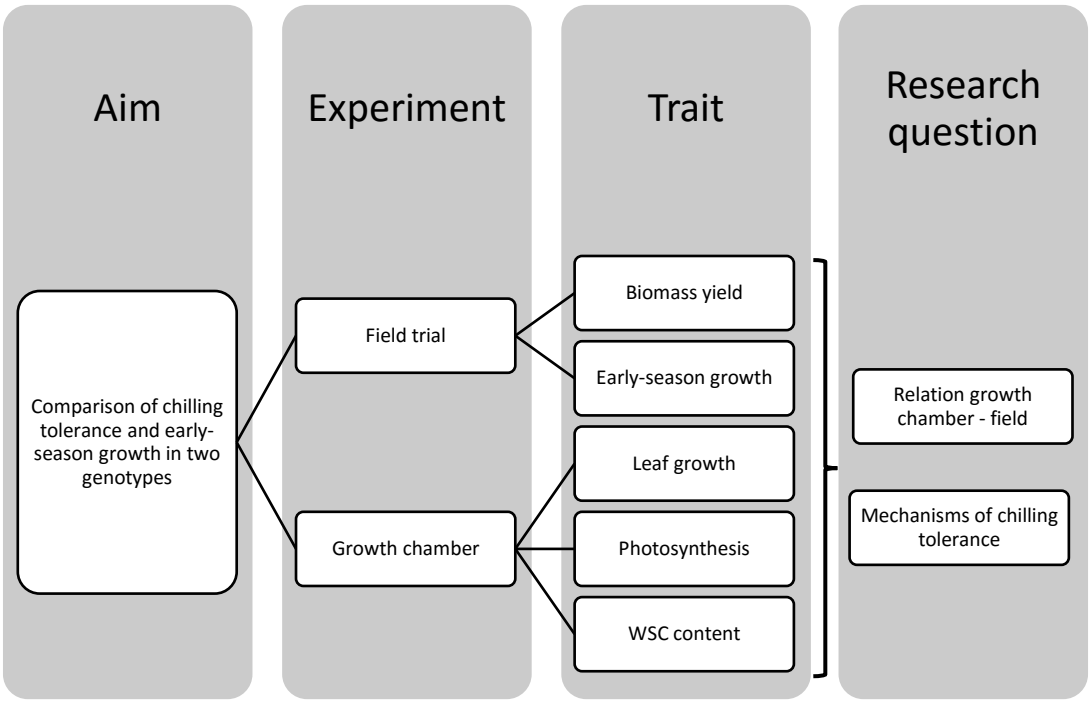


Figure 5.1: Schematic overview of the research conducted in this chapter.

Materials and methods

Experiment 1: field trial

Field measurements were performed on the bio-energy trial. The results have been partly reported before (Van Hulle et al. 2012, Muylle et al. 2015). The trial consists of two parts. In the first part yield was determined. 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. In each plot of IL10 and IL11, 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 length, 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 day length]. 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 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 day length. 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. (2016). The derivative of the sigmoid function, representing the leaf elongation rate, 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. (2016).

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. Samples were analysed with HPAEC-PAD on an ICS3000 system (Thermo Scientific Dionex). Analysis and detection were performed at 32 °C and the flow rate was 250 µL per minute. 15µL of

sample was injected on a Guard CarboPac PA100 (2 x 50 mm) in series with an analytical CarboPac PA100 (2 x 250mm) equilibrated for 9 minutes with 90 mM CO_2-free NaOH. Sugars were eluted in 90 mM NaOH, with an increasing NaAc-gradient: from 0 to 6 minutes, the NaAc-concentration increased linearly from 0 to 10 mM; from 6 to 16 minutes the concentration increased linearly from 10 to 100 mM; from 16 to 26 minutes, the concentration increased linearly from 100 to 175 mM, then the columns were regenerated with 500 mM NaAc for 1 minute and equilibrated with 90 mM NaOH for 9 minutes for the next run.

Statistical analyses

Differences in leaf growth parameters between treatments or between genotypes were analyzed using *t*-tests. Differences in photosynthesis, Chl fluorescence parameters, and sugar contents were analyzed independently for each light intensity using analysis of variance (*ANOVA*). The effect of genotype and temperature on Φ_{PSII}/Φ_{CO2} was analyzed 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

IL10 consistently yielded more biomass per hectare than IL11 over a course of seven years (19.1 ± 1.5 t ha^{-1} for IL10 and 10.1 ± 3.0 t ha^{-1} for IL11; Table 5.1). Both genotypes reached maturity after three years (Muylle et al. 2015), after which the yield was relatively stable. IL10 was thus higher yielding compared to IL11 at our location, which is consistent with the findings of Larsen et al. (2013) for Denmark. The higher chilling tolerance of IL10 is unlikely to be the only factor of its higher yield, as the genotypes also differ in their morphology: IL10 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 IL11's growing season occurred earlier because it flowered earlier than IL10, which had not even flowered every year under Flemish growth conditions.

Table 5.1: Average yield with standard deviation [t ha^{-1}] of IL10 and IL11 in the field trial in Melle, Belgium installed in 2007. Plots were harvested in February–March each year.

Genotype	2008	2009	2010	2011
IL10	3.3 ± 0.7	15.4 ± 0.6	25.7 ± 1.0	19.8 ± 1.3
IL11	0.5 ± 0.1	4.1 ± 0.5	14.0 ± 3.3	14.3 ± 3.3
	2012	2013	2014	
IL10	28.0 ± 4.6	17.9 ± 1.2	23.9 ± 1.2	
IL11	13.2 ± 4.7	12.8 ± 4.7	11.8 ± 4.4	

In the field, both genotypes started growing shortly when the mean weekly temperature rose above 8°C (Fig. 5.2*A*). IL10 resumed its growth from underground rhizome buds, while the growth of IL11 was partly the result of the elongation of shoots formed the year before and of newly formed shoots. As a consequence, emerging shoots appeared aboveground later in IL10 than those in IL11 (Fig. 5.2*B*). IL11 reached an average height of 5 cm at day of the year (DOY) 110 (20 April), while IL10 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 IL10 surpassed IL11. After DOY 120, when the average temperature was around 12°C, IL10 had an average growth rate of 2.7 ± 0.3 cm per day, while IL11 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, IL10 displayed the higher growth rate than that of IL11. Shoots of IL10 emerged later than those of IL11, but had a higher growth rate afterwards. This conferred IL10 an advantage over IL11 at the start of the growing season.

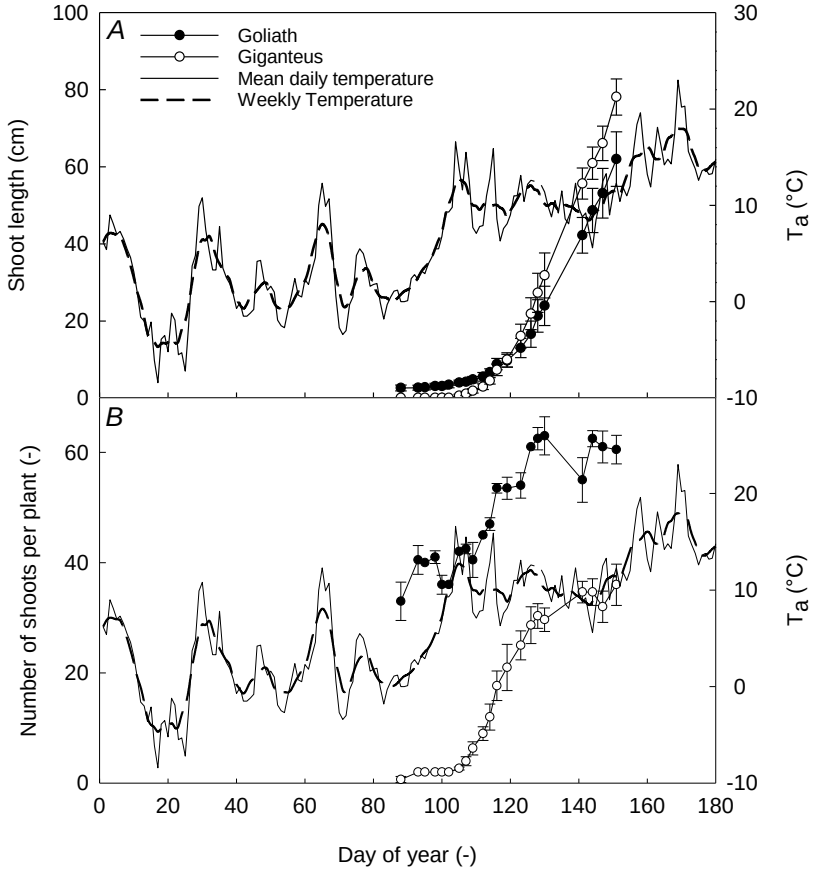


Figure 5.2: Average shoot length (A) and a number of shoots per plant (B) of IL10 and IL11 in the field trial and mean daily and weekly air temperature (T_a) in the spring of 2013. Error bars show standard errors (n = 15).

Under controlled conditions, IL11 produced significantly longer leaves at 20°C than IL10 did, but when grown at 12°C, the final leaf length (L_m) was reduced more in IL11 (24%) than that in IL10

(13%) (Table 5.2). Leaf length reductions caused by chilling stress have also been reported for maize (Rymen et al. 2007). If chilling stress also causes IL11 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 IL10 than for IL11 (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. 5.3). This is in agreement with the higher shoot elongation rate early in the season under the abovementioned field conditions. The moment at which LER_{max} was reached was not affected by chilling treatment in IL10, while in IL11 a delay of 37% was observed (data not shown). The duration of leaf elongation ($LED_{10–90\%}$) was about 20 d for both genotypes at 20°C, but at 12°C a lower value was obtained for IL10 (33.7 d) than that for IL11 (40.5 d). The growth curves were also fitted in 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 IL10 at 12 and 20°C overlapped, while those of IL11 did not (data not shown). This could either mean that IL11 is relatively more chilling stressed, or that it has a lower base temperature than IL10.

Overall, the higher growth rates of IL10 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, LER_{max} and LED at 12°C were less affected in IL10 than in IL11. Similar results have been reported by Głowacka et al. (2014a) in a comparison of a larger set of genotypes. They found that IL10 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 IL11 and one of the IL10 accessions investigated, but a relatively higher growth reduction at low temperature for the other IL10 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. (2014a) 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 IL10 to a decrease in temperature is stronger

than that of IL11, 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.

Table 5.2: Leaf growth parameters of IL10 and IL11 calculated by LEAF-E as a function of time. L_m – maximum leaf length, LER_{max} – maximum leaf elongation rate, $LED_{10–90\%}$ – 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	20°C	12°C	% change
IL10			
L_m [cm]	92.3 ± 2.6[a]	80.2 ± 5.9	–13
LER_{max} [cm d^{-1}]	4.5 ± 0.2[ab]	2.2 ± 0.1[ab]	–50
$LED_{10–90\%}$ [d]	20.0 ± 1.2[b]	33.7 ± 1.8[b]	68
IL11			
L_m [cm]	113.7 ± 4.1[ab]	86.2 ± 5.5[b]	–24
LER_{max} [cm d^{-1}]	5.4 ± 0.2[ab]	1.9 ± 0.1[ab]	–64
$LED_{10–90\%}$ [d]	19.4 ± 0.6[b]	40.6 ± 3.0[b]	110

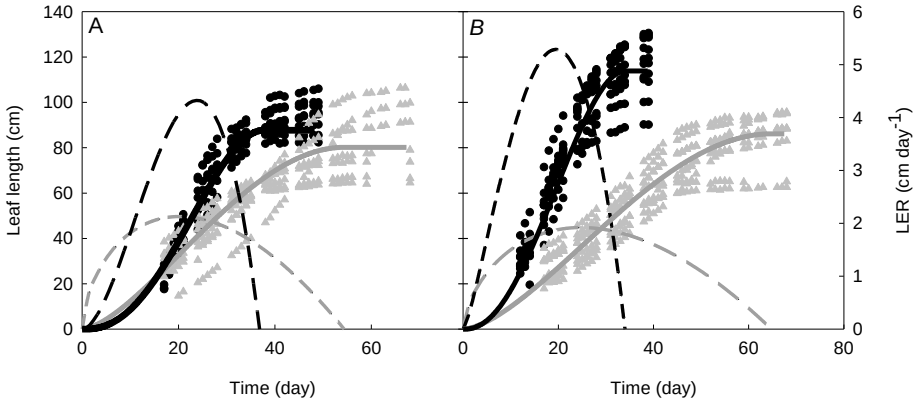


Figure 5.3: Growth of the fourth leaf of IL10 (A) and IL11 (B) at 20°C (black) and at 12°C (grey). Full lines show the average growth curve per treatment (n = 10), calculated using LEAF-E. Leaf elongation rates (LER) are shown in dashed lines. The actual measurements are represented by symbols.

Capacity for carbon assimilation under chilling stress

IL10 showed slightly higher P_N than IL11 when both grew at 20 and 12°C (Fig. 5.4A). The relative decrease due to a lower temperature at 1,000 µmol (photon) m^{-2} s^{-1} was 67 and 73%, respectively, indicating that IL11 was slightly more affected by the lower temperature. This is in accordance with Purdy et al. (2013) who found that when IL10 and IL11 were transferred from 28 to 12°C PN declined by 65% in both genotypes over the course of 12 h, but IL10 also retained a higher P_N than IL11 at both temperatures. In a second experiment, where IL10 and IL11 were grown and measured at 12 and 20°C (data not shown), similar results were obtained. Moreover, while IL11 had a significantly higher Chl content index per leaf area at 20°C than that of IL10 (40.4 ± 2.3 and 28.6 ± 1.8, respectively), at 12°C, the Chl content in IL11 became lower and inter-genotype 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 IL10 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. x giganteus* is exposed to prolonged chilling stress, the expression of genes coding for photosynthetic proteins and proteins protecting PSII increases (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. x 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 C4 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 IL11.

Stomatal conductance (g_s) for both species was similar at 20°C, but at a growth temperature of 12°C, IL10 was able to maintain a higher g_S in comparison to IL11, 0.064 ± 0.003 and 0.025 ± 0.002 mol m^{-2} s^{-1}, respectively. These values are in the same range as those measured by Głowacka et al. (2015b) on several *Miscanthus* genotypes at 15°C. The lower g_S found for IL11 grown at 12°C compared to IL10 was probably not 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. (2014a) 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 chilling stress. Taken together, the photosynthesis measurements demonstrated that IL10 was capable of higher P_N at optimal temperatures and displayed a lesser decline after exposure to chilling stress.

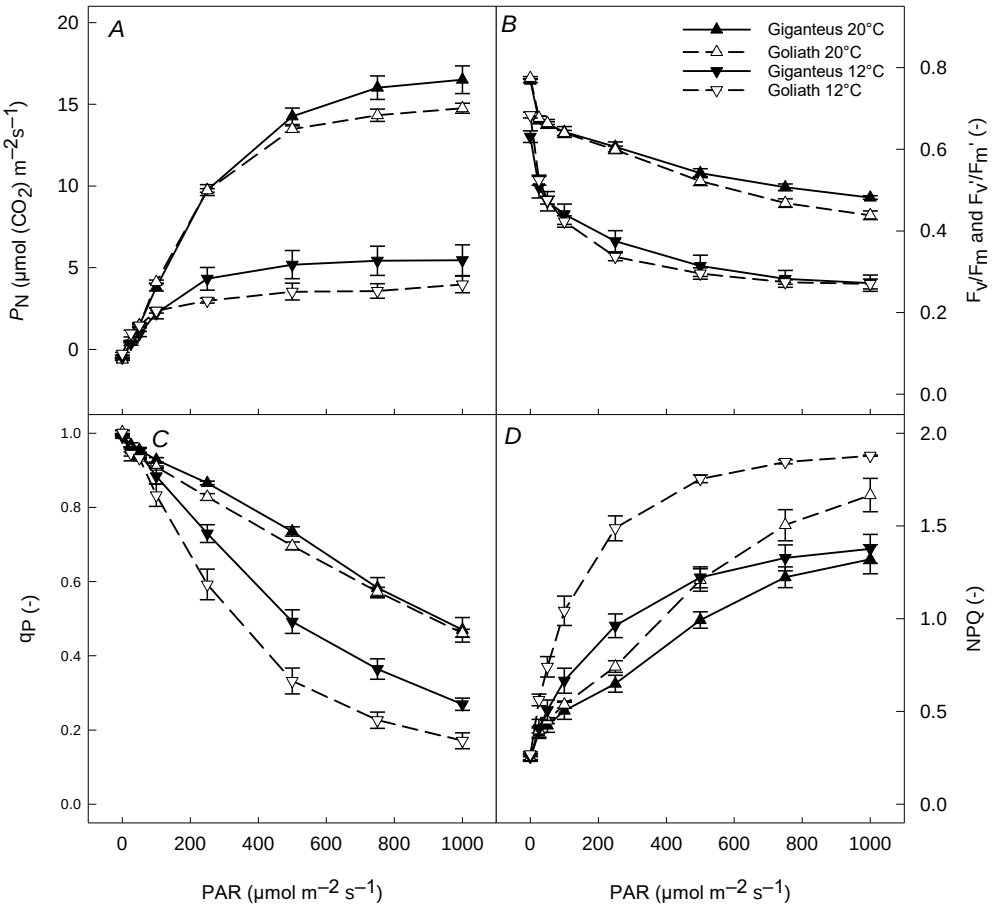


Figure 5.4: Net photosynthetic rate (P_N) (A), maximum (F_v/F_m, at PAR of 0 µmol m^{-2} s^{-1}) and effective (F_v'/F_m') efficiency of PSII (B), photochemical quenching (q_P) (C), and nonphotochemical quenching (NPQ) (D) of Miscanthus x giganteus IL10 and M. sinensis IL11 grown and measured at 20°C and at 12°C at different photosynthetic active radiation levels (PAR). Error bars show standard errors (n = 6).

Efficiency of the photosynthetic apparatus under chilling stress

Chl fluorescence revealed that plants grown at 12°C suffered from photoinhibition due to chilling stress. The maximum quantum efficiency of PSII (Fv/Fm) was significantly lower under prolonged chilling stress in IL10 than that in IL11 contrary to the light adaptive maximum quantum efficiency (F_v'/F_m') (Fig. 5.4B). 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 IL10 and IL11 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 IL10 and IL11, 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 IL10 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 season growth) 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 IL10 showed lower F_v/F_m but also higher growth and photosynthesis than IL11 under chilling stress. Similarly, Friesen et al. (2014) and Głowacka et al. (2015b) 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 mean 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 IL10 and IL11, 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 IL10.

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. 5.4*C*, *D*). For IL10, 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 IL11. 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.* x *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.* x *giganteus* grown at low temperature, which was associated with higher zeaxanthin and carotenoid levels. 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 IL10 and IL11 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 chlorophyll 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. 5.5). 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 IL11 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.* x *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, IL10 exhibited the higher assimilation rate than IL11, even at 12°C. This seems to be related to a more efficient use of light energy. The lower NPQ and higher q_P in IL10 showed that this genotype dissipates less light energy as heat and is able to utilize more light energy for photochemistry.

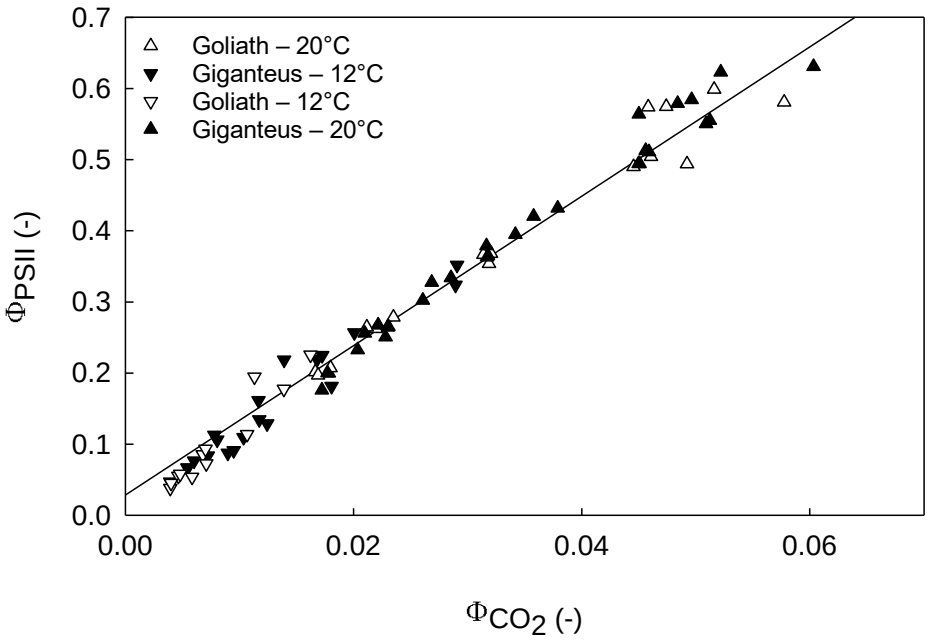


Figure 5.5: Relationship between PSII operating efficiency (Φ_{PSII}) and quantum yield of CO2 (Φ_{CO2}) assimilation in the plants grown at 20°C (upward triangles) and 12°C (downward triangles) of IL10 (black symbols) and IL11 (white symbols). assimilation. Regression line for both genotypes and temperatures (R^2=0.983).

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. 5.6). 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 any of the temperatures investigated. Within both genotypes, raffinose concentration and total sugar concentration were significantly higher at 12°C. Although the relationship 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 IL11 happens faster than that in IL10 (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 IL10 and IL11, respectively. There are no other 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 IL10 under chilling stress.

The accumulation of soluble sugars can be the 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 tolerance (Nägele and Heyer 2013), indicating that a certain sucrose threshold value should be passed to initiate sugar-mediated signaling 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 the 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 in 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).

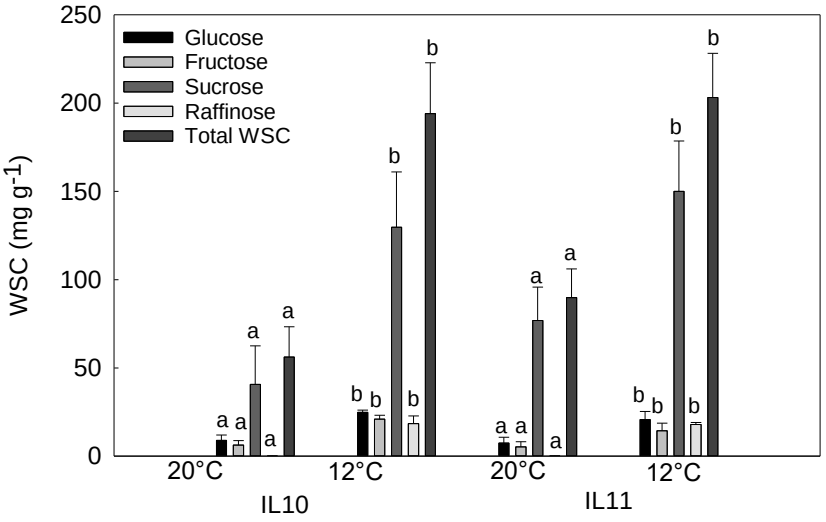


Figure 5.6: Water soluble carbohydrate concentrations (WSC) in mature leaves of IL10 and IL11 grown at 12 and 20°C. Error bars show standard errors (n = 4). Letters indicate significant differences between genotypes and temperatures for a specific WSC. DM – dry mass.

Conclusions

Although IL10 produced higher biomass yields than IL11, IL10 did not start growing earlier than IL11 but rather had a higher growth rate early in the spring under field conditions. The higher growth rate of IL10 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 IL10 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 IL10 was relatively more photoinhibited when growing at low temperatures, but could use more light energy than IL11. Under field conditions in the spring, IL10 can have a higher photosynthetic capacity than that of IL11. 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. Based on our results, this screening can be both performed by measuring growth under controlled conditions or in the field. Screening under controlled conditions has the advantage that it can be performed all year long and that the results are standardized, but field conditions are difficult to imitate in growth chambers, a validation in the field is thus necessary. Photosynthesis measurements yielded good results to distinguish the genotypes, but proved too time consuming to be of practical use in a large scale screening, especially under field conditions. Analysis of WSC concentrations showed interesting effects of chilling stress and was fast to perform. A more detailed analysis of WSC under chilling stress might show if this method is useful for screening.

Chapter 6: Development of a methodology to quantify early-season growth in a large miscanthus collection

Introduction

It was one of our purposes to screen a large germplasm collection for early-season growth and the ability to cope with low temperatures. This requires the use of efficient and informative field-evaluation approaches. In the work presented in chapter five, we compared the relationship between photosynthesis and growth at chilling temperatures and early-season growth in the field. However, the evaluation of growth in the field was restricted to easy to determine parameters. While shoot length and shoot numbers are easy to measure, they are not necessarily the best indicators of growth and canopy formation, since this also depends on the number of leaves formed and their characteristics. Several other methods have been proposed to estimate early-season growth in miscanthus. For example, Robson et al. (2013a) estimated canopy development visually, by measuring light interception and using digital images to estimate ground cover. LAI determined by measurements of light interception was best correlated with final yield, but also laborious to determine, weather-dependent and not well suited for small plots. Image analysis and visual scoring were easier to perform, quicker and less dependent on weather conditions. However, image analysis required substantially more effort due to data processing and visual scoring was subjective and rather imprecise.

Vargas et al. (2002) suggested the use of spectral reflectance measurements as a fast and reliable method for the determination of light interception and biomass accumulation in miscanthus. They measured reflectance in the visible and near infra-red part of the spectrum and calculated spectral vegetation indices, such as the normalized difference vegetation index (NDVI). A good relationship was found between LAI, NDVI and biomass accumulation in the genotype *M. sinensis* 'Goliath', indicating that NDVI is potentially a useful approach for the rapid screening of early-season growth in miscanthus, similarly to what has been demonstrated in wheat and maize by Verhulst et al. (2011a). In these studies, a Greenseeker NDVI sensor was used, as it allows the rapid measurement of spectral reflectance on large numbers of plots, potentially allowing to screen non-invasively early-season growth quickly in a large numbers of genotypes.

Therefore, here we present a preliminary study analyzing the relevance of the measurements taken in the field plots to estimate early-season growth, and to get insights into the interrelationship between parameters such as shoot number, shoot length, leaf number and biomass accumulation early in the growing season. Given its more general use in different kinds of crops, we also included NDVI

measurements in this analysis. We explore the interrelationships between parameters that describe early-season growth, and in relationship to biomass accumulation. A schematic representation of the experiments performed can be found in Figure 6.1.

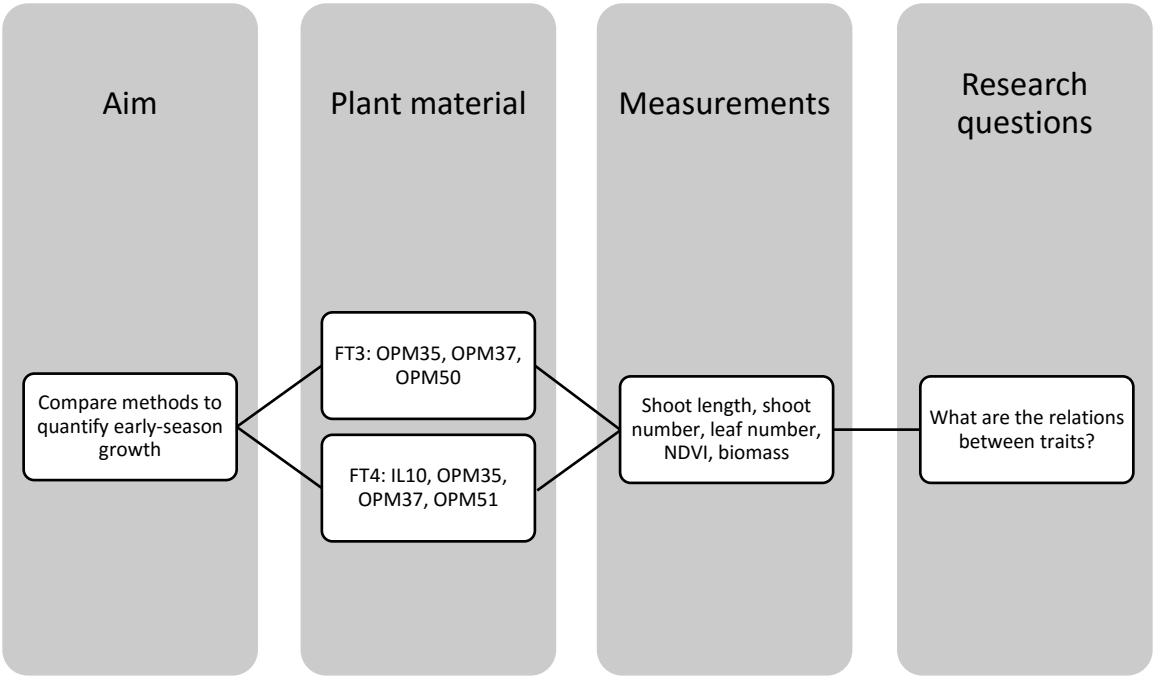


Figure 6.1: Schematic representation of the performed experiments.

Materials and methods

Measurements

Two field trials, FT3 and FT4, were used for this study. The trials were harvested on 20/03/2014 (FT4) and on 20/03/2015 (FT3) and the regrowth of the genotypes IL10, OPM35, OPM37, OPM50 and OPM51 was monitored. As FT3 was planted late in 2013 it could not yet be used for measurements in the spring of 2014. Therefore, FT4 was used for measurements in 2014, and FT3 was used for similar measurements in 2015. In FT4 at each measurement point, three plants located at neighboring positions in the row were selected, generating data that could be compared to those recorded in FT3 in 2015. Note that genotypes OPM35 and OPM37 are common to both trials, and are therefore part of the 2014 and of the 2015 datasets.

The length of the longest shoot per plant (from soil level to the highest leaf tip), the length of the longest stem per plant (from soil level to the highest ligule), the number of shoots per plant and the number of leaves on the longest shoot per plant were measured weekly on three plants per genotype (from 7/04/2014 until 16/05/2014 and from 10/04/2015 until 1/06/2015). After each measurement the

aboveground biomass of each measured plant was harvested, dried in an air-ventilated oven and weighed to determine total plant above-ground dry weight.

NDVI was determined from weekly spectral reflectance data obtained using a GreenSeeker™ Handheld Optical Sensor Unit (NTech Industries, Inc., USA), which measures reflectance in the red (650 nm) and near infra-red (770 nm) bands of the light spectrum. The device has a self-contained light source and is thus not dependent on sunlight. The sensor takes readings in an area of 0.6 by 0.01 m at a rate of approximately 1000 measurements per second. The sensor averages these readings and outputs data at a rate of 10 readings per second. The measuring protocol was based on Verhulst and Govaerts (2010). Measurements were performed by moving the sensor head over the three measured plants at a steady walking pace (approximately 1 m s^{-1}), with the sensor head held horizontally 1 m above the canopy. NDVI was calculated using the formula:

$$NDVI = \frac{NIR-VIS}{NIR+VIS} \quad \text{(eq. 6.1)}$$

were NIR is the fraction of reflected near infra-red light and VIS is the fraction reflected visible red light. About 20 readings were obtained per plot (set of three plants). These values were then averaged to obtain the mean NDVI value per plot. Measurements were performed in the afternoon to avoid the effect of morning dew.

Statistical analysis

The relationship between the measured parameters and dry weight was tested using generalized linear models using the 'glm' function of the *stats* package in R 3.1.0 (R core team, Vienna, Austria). Correlations were calculated and tested for significance using the 'cor.test' function from the *stats* package. Correlations were plotted using the 'corrgram' function of the *corrgram* package.

Results

Early-season growth of the five genotypes

In the both years investigated (2014 and 2015) shoot growth started immediately after the start of the measurements and increased quasi linearly from then on (Fig. 6.2A, B). In 2014 NDVI started to increase immediately after shoot emergence, while in the colder conditions of 2015 NDVI only started to rise about 20 days later (Fig. 6.2C, D). NDVI increased throughout the measuring period until a maximum of around 0.8 was reached (Fig. 5.2C), and corresponded to a shoot length of approximately 1 m (Fig. 6.2A), with shoots of 6-7 leaves (Fig. 6.2E). In both years shoot number increased rapidly during the first weeks of the measuring period and remained more or less constant thereafter. One plant of genotype OPM50 had a larger than average shoot number, leading to the

extreme value in Fig. 6.2D. Shoot length was still low at that moment and this plant did therefore not have a markedly high biomass. Leaf number increased to a maximum of around 6-7 leaves per plant in both years (Fig. 6.2E), following similar patterns between years and genotypes.

Biomass increased gradually (Fig. 6.2F). Because of the later start of the growing season in 2015, plant dry mass was initially lower in 2015, but by mid-May (DOY 130) plant biomass was similar to that recorded in 2014 for OPM35 and OPM37. The dry matter content was on average 15% of the fresh biomass for all genotypes at all time points (data not shown).

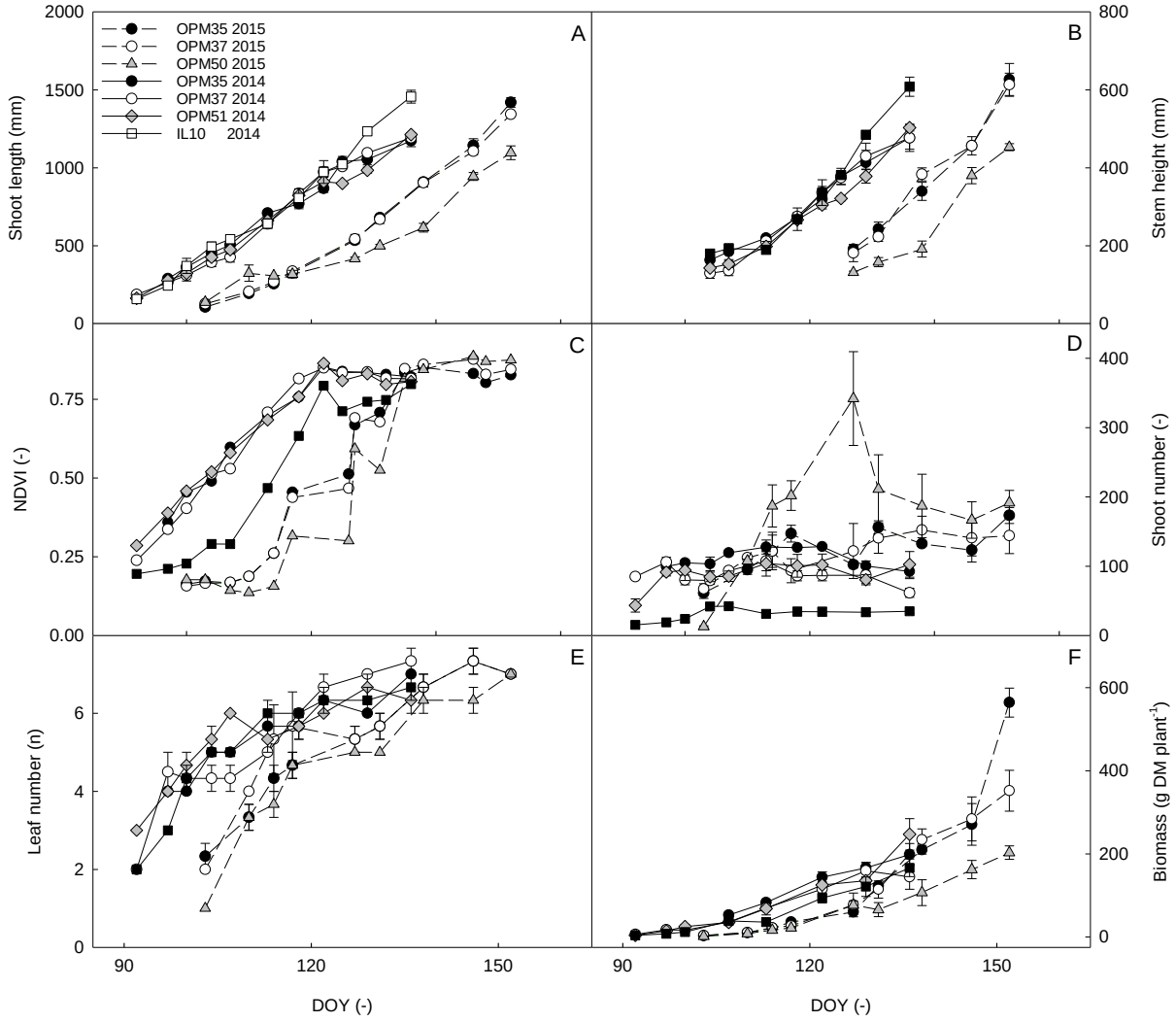


Figure 6.2: Early-season growth parameters measured in 2014 in FT4 (full lines) and in 2015 in FT3 (dashed lines) in function of the day of the year (DOY). Symbols depict mean values per genotype and date. Error bars show standard error (n=3). A: Shoot length (mm); B: Stem height (mm); C: NDVI per plot (-); D: Shoot number (n); E: Leaf number and F: biomass (g DM plant^{-1}).

Relation between early-season growth parameters

The relationship between plant biomass and most parameters was non-linear (results not shown), therefore a log-transformation was applied, which resulted in linear relationships (Table 6.1). NDVI increased from a minimum of about 0.2 until a maximum was reached of about 0.8, when canopy closure occurred, while biomass continued to increase after NDVI values surpassed 0.8. Therefore, NDVI values below 0.2 and above 0.8 were not used to calculate correlations. Shoot length on the other hand continued to increase with biomass, and after canopy closure, indicating that these are probably better indicators of plant biomass.

There was a strong correlation between all parameters (except shoot number) and biomass, with the highest value for shoot length (r = 0.94). NDVI displayed also a very high correlation with biomass (r=0.84), and seemed to be completely independent of shoot number (r = 0.31). In general, shoot number had the lowest correlations with all other parameters probably because shoot number only changed in the beginning of the growing season until about DOY 120 and remained relatively constant afterwards. This indicates while NDVI is a good proxy for biomass, early in the season the length of the shoot is also an even better option, given the high correlation with biomass and the fact that shoot length continues to increase as biomass accumulates, while NDVI becomes constant once the canopy closes. Note also the high correlation between shoot length and stem height (r = 0.98), indicating an almost complete redundancy.

Table 6.1: Correlations based on log-transformation between the early-season growth measurements, measured in FT4 in 2014 and in FT3 in 2015. All correlations are significant, except for the correlation between shoot number and stem height and between shoot number and NDVI (Pearson's product moment correlation, $p < 0.05$).

	Shoot length	Stem height	Leaf number	Shoot number	NDVI
Dry weight	0.94	0.84	0.89	0.47	0.90
Shoot length		0.98	0.84	0.20	0.89
Stem height			0.80	-0.04	0.67
Leaf number				0.39	0.80
Shoot number					0.31

Discussion

Except for shoot number, all the performed measurements proved to be good indicators of biomass accumulation early in the growing season and thus of early-season growth. Shoot counts can be indicative of the moment of plant emergence and give complimentary information to shoot length measurements, but are not strongly indicative of early-season growth, given the low correlation with biomass accumulation. In addition, these measurements were extremely time-consuming.

The best correlation with biomass was obtained for shoot length, which had an almost linear relationship with biomass over the complete measuring period. Stem height yielded almost identical information. As shoot length is easier to measure, we decided to use shoot length measurements to follow up plant growth in the field trials described in the following chapters. NDVI was also a good proxy for biomass, but became insensitive to biomass chances once the canopy had closed. NDVI measurements are thus mainly useful for the estimation of biomass accumulation in the beginning of the growing season. The main advantage of NDVI is the speed at which measurements are recorded. Shoot length measurements were more time consuming than NDVI measurements but were better correlated with biomass. Furthermore, NDVI provides information on the approximate moment of canopy closure and thus light interception, while shoot length measurements allow to determine growth rates and growth duration (Zub et al., 2012) and can be used over a longer period in the growing season to estimate biomass accumulation.

Additionally, measuring NDVI throughout the whole growing season as reported by Verhulst et al. (2011) could yield information on flowering, end of the growing season and senescence. However, due to the large size of the plants and impenetrability of the field trials this is not practical with a handheld sensor, as done here. The use of above-canopy sensors would be a more practical option (Jørgensen et al. 2003), or the use of sensors mounted on drones.

Taking these results into consideration, in the following chapters emphasis is put on variables derived from shoot length measurements when describing early-season growth. In FT1, comprising miniplots for each single genotype, additional NDVI measurements were carried out.

Chapter 7: Genotypic diversity of early-season growth and low temperature response in the miscanthus germplasm

Introduction

As in any other lignocellulosic biomass crop, one of the main targets in miscanthus breeding is improvement of the above-ground yield, a highly complex, multifactorial trait. Previous work has demonstrated that early-emergence and fast canopy closure may contribute significantly to higher yields in miscanthus (Robson et al., 2013a; Sage et al., 2015). This suggests that genotypes that emerge early and take a fast start early in the season are potentially good germplasm to breed high-yielding varieties. On the other side, Zub et al. (2012) concluded that higher final canopy height (as a proxy of yield, as shown in Zub et al. (2011) and Clifton-Brown et al. (2001a)) was mainly associated to late emergence and high growth rate during summer. This apparent contradiction might be due to differences in the germplasm set used (different number of genotypes and even different species between the studies). Moreover, while Robson et al. (2013a) focused on early-season canopy formation and canopy duration, Zub et al. (2012) modeled whole-season shoot growth, which may have masked the effects of early-season growth characteristics on final yield. When striving to define adequate selection targets in miscanthus, these examples illustrate the importance of separately considering the different secondary traits that influence biomass yield. There is thus a need to increase our understanding of emergence and early-season growth in miscanthus, before the relationship with biomass yield is investigated.

Information on early-season growth characteristics recorded in the field can be combined with high-resolution measurements of growth responses at low temperature under controlled conditions - if the focus is to identify genotypes suited for cultivation in regions where chilling stress is a risk. By comparing growth potential under optimal and chilling temperatures in controlled environments, the influence of temperature on growth can be estimated independent of any other factor. Such controlled experiments are necessary, as early-season growth in the field can be influenced by other factors such as frost or drought stress (Saint Pierre, 2012), masking temperature-related responses. When observing growth at low temperatures under controlled conditions, substantial variation has been reported in miscanthus (Clifton-Brown and Jones, 1997; Głowacka et al., 2014a, 2015b).

We investigated the genetic variation available for emergence and early-season growth in a germplasm collection consisting of *M. sinensis*, *M. sacchariflorus*, *M. sinensis x sacchariflorus* hybrids and *M. x giganteus* representative of the collections of European miscanthus breeders. The

results of field evaluations over two growing seasons and multi-site evaluation in contrasting environments were combined to identify genotypic and GxE (genotype x environment) responses. This information was interpreted together with results from growth chamber experiments regarding growth responses under chilling stress. The specific aims of this study were to: (i) evaluate the extent of variation in early-season growth in miscanthus, (ii) determine GxE interactions using a multi-location field trial in contrasting environments in Europe and Turkey, (iii) evaluate growth-responses under chilling temperatures in controlled environments and (iv) get insights into the interrelationships among these characteristics for a common set of genotypes.

Materials and methods

Three experiments were conducted: (i) a common garden experiment in Merelbeke, Belgium, (ii) a multi-location field trial at five locations in Europe and Turkey, and (iii) a growth chamber experiment. The measurements performed and the derived early-season growth parameters are summarized in Table 7.1.

Table 7.1. Overview of experiments, measurements and derived parameters.

Experiment	Measurement	Frequency of measurements	Derived parameters	Abbreviation	Units
Common garden exp.	Length of longest shoot	2x per week	Absolute shoot growth rate	AGR	mm GDD^{-1}
			TT* at which the longest shoot reached a length of 30 cm	L30	GDD
			TT at which the longest shoot reached a length of 50 cm	L50	GDD
	Number of leaves on longest shoot	2x per week	Leaf formation rate	LFR	Leaves GDD^{-1}
			TT at which the fourth leaf emerged	Leaf4	GDD
	Number of shoots	2x per week	TT at which 50% of maximum shoot number was formed	S50	GDD
			Maximum shoot number	N_{max}	n
Multi-location field trial	Length of longest shoot	6x in early growing season	TT at which the longest shoot reached a length of 30 cm	L30	GDD
Growth chamber exp.	Growth of fourth leaf	2-3x per week	Maximum leaf elongation rate	LER_{max}	mm GDD^{-1}

*TT: Thermal time

Plant material

A total of 108 miscanthus genotypes were used. Most genotypes were obtained through the OPTIMSC project (*www.optimisc-project.eu*)(Lewandowski et al., 2015), i.e., 56 genotypes from the collection of Wageningen University (The Netherlands) and 33 genotypes from the collection of Aberystwyth University (UK). The 9 genotypes also used in the EMI project (Clifton-Brown et al., 2001a) were kindly provided by Uffe Jørgensen (Aarhus University, Denmark). The remaining genotypes were acquired from commercial suppliers. The specific set of genotypes included in each

of the three experiments described in Table 1 is shown in Table 3.1. In short, 102 genotypes were used for the field trial in Merelbeke, Belgium (10 *M. x giganteus,* 14 *M. sinensis x sacchariflorus* hybrids, 17 *M. sacchariflorus* and 61 *M. sinensis*). Eleven genotypes and four seed-based *M. sinensis* populations (1 *M. x giganteus,* 7 *M. sinensis x sacchariflorus* hybrids, 4 *M. sacchariflorus* and 3 *M. sinensis*) were included in the multi-location field trial. Finally, 54 genotypes were tested in the growth chamber (3 *M. x giganteus,* 7 *M. sinensis x sacchariflorus* hybrids, 14 *M. sacchariflorus* and 30 *M. sinensis*).

Most of the plant material was derived from crosses between genotypes of unknown origin. As a result, no data about the climatic conditions at the location of origin were available for most of them, except for 13 of the *M. sacchariflorus* genotypes (see below).

Common garden experiment

The experiment was performed in field trial FT2. Measurements (Table 7.1) were taken twice per week from 18/03/2014 until 27/05/2014 and from 18/02/2015 until 28/05/2015. The *M. sacchariflorus* genotypes were removed from the trial in May 2014 because their rhizomes spread too extensively. For these genotypes, only data from the 2014 growing season are available.

Every time measurements were taken, the number of shoots per plant were 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. L30 was chosen as a parameter to quantify the first phases of growth. For most genotypes, L30 represented the section that precedes the linear phase in the shoot growth curve (data not shown). Therefore, we also estimated L50, as it represents the linear part of the growth curve and is expected to be more repeatable than L30. The accumulated thermal time until the fourth leaf appeared on the longest shoot (Leaf4), and the leaf formation rate (LFR) were chosen as indicators of canopy formation. A linear regression of leaf count in function of thermal time was used to calculate Leaf4 for each plant. The slope of this regression line was used as LFR. Finally, as the total number of shoots formed is strongly influenced by plant base diameter, the accumulated thermal time until 50% of the shoots present at the last measurement (in May each year) had been formed (S50) was chosen to quantify earliness of shoot formation. S50 was calculated by fitting a three parameter Verhulst logistic function to the data (Verhulst, 1838). The maximum of this function (N_{max}) was used as the maximum shoot number per plant. Differences between species and genotypes were found using a mixed models approach in Statistica 12.0 (Statsoft, Tulsa, OK,

USA). As significant species by year interactions were observed for most parameters, data were analyzed separately per year using the following mixed model:

$$Y_{ijkl} = \mu + S_i + G_j + R_k + e_{ijkl} \quad \text{(eq.7.1)}$$

Where Y is the effect of one of the early-season growth parameters described above, μ is the overall mean, S is the effect of the species group *i*, G the effect of the genotype *j*, nested in the species group, R is the random block effect *k* and e the first residual term. Genotype and block were considered random effects. The model was used to calculate adjusted means values for the traits per genotype and per year. These adjusted means were used to calculate correlations and in the principal component analysis.

Pearson's product-moment correlations were calculated using 'corr.test' from the *psych* package in R 3.1.0 (R core team, Vienna, Austria). Principal component analysis was performed using the 'PCA' function in the *FactoMineR* package (Lê et al., 2008). Variables were scaled to unit variance. Correlations between principal components and early-season growth parameters were calculated using the dimdesc function of the *FactoMineR* package.

Broad sense heritability was calculated according to the following formula (based on Barre et al. (2015):

$$H^2 = \frac{\sigma^2{}_G}{\sigma^2{}_G + \frac{\sigma^2 GY}{2} + \frac{\sigma^2 GB}{6} + \frac{\sigma^2 R}{12}} \quad \text{(eq.7. 2)}$$

Where σ^2_G is the variance due to genotype, σ^2_{GY} is the variance due to genotype by year effects, σ^2_{GB} is the variance due to genotype x block effects and σ^2_R is the residual variance. Variances were calculated using the following mixed model using the 'lmer' function from the *lme4* package in R.

$$Y_{ijkl} = \mu + G_i + J_j + B_k + G_i \times J_j + G_i \times B_k + e_{ijkl} \quad \text{(eq. 7.3)}$$

Where Y is the effect of one of the early-season growth parameters described above, μ is the overall mean, G the effect of the genotype *i*, J is the effect of year *j*, B is the random block effect *k* and e the first residual term.

Multi-location field trial

In the multi-location field trials the shoot length of five plants per plot was determined as described above at six time points during the growing season in 2014 and 2015 (Table 7.1). These six time points were distributed from the day of first emergence until the plants had reached a height of approximately 50 cm. L30 was estimated as described above. The data did not allow to estimate L50 values accurately due to poor resolution in the late measurements in spring. To compare L30 across

locations, the percentage difference of each genotype or population from the mean L30 for each site-year was calculated. Adjusted means were calculated using the following model:

$$Y_{ijkl} = \mu + G_i + S_j + B_k + G_i \times S_j + G_i \times B_k + e_{ijkl} \quad \text{(eq.7.4)}$$

Where Y is the effect L30, μ is the overall mean, G the effect of the genotype i, S is the effect of site-year j, B is the random block effect *k* and e the first residual term.

Growth chamber experiment

Two runs were set up in growth chambers. The first run was initiated on 11/03/2014 and the second one on 30/06/2014. Each run included 31 genotypes, two of which (OPM09 (*M. x giganteus)* and OPM50 (*M. sinensis*)) were common to both to enable combination of the results. Not all genotypes emerged well, resulting in a total of 54 genotypes with sufficient data for comparison. Rhizomes of field-grown plants were used. Before planting, the rhizomes were soaked in thiophanate-methyl (4 ml l^{-1}) (Bayer Crop Science, Germany) for 1h to prevent *Fusarium* infection. Twenty rhizomes per genotype were planted in potting soil (Saniflor beroepspotgrond, Van Israel N.V., Belgium) in 20 1L plots. Ten pots per genotype were placed in a growth chamber at 20°C and ten in a growth chamber at 14°C, with each growth chamber divided into 4 blocks to account for potential variability in temperature. Each block was surrounded by a border row. Light intensity was set to 130 μmol PAR m^{-2} s^{-1} at 10 cm above soil level and the vapor pressure deficit to 0.6 kPa.

In each pot, the first emerging shoot was marked and its growth was monitored by measuring the length of the fourth leaf of this shoot at regular intervals. At 20°C measurements were carried out three times per week, while at 14°C two measurements were done per week due to slower growth. The leaf length was measured with a ruler from soil level to leaf tip. A growth curve was fitted to data of each individual leaf using the LEAF-E Excel macro (Voorend et al., 2014) to estimate the maximum leaf elongation rate (LER_{max}, mm day^{-1}). The resulting models were visually inspected for a correct fit, and considering R^2 values (the minimum value of R^2 was 0.980 in this experiment). The two genotypes repeated in the two runs (OPM09 and OPM50) were used to adjust the results of the second run. First a general linear mixed model was used to analyze the data:

$$LER_{max} = \mu + G_i + T_j + R_k + R_k\,(B_l) + G_i \times R_k + T_j \times R_k + e_{ijklm} \quad \text{(eq. 7.5)}$$

Where G is the effect of genotype *i,* T is the effect of temperature *j*, R is the effect of run *k,* B is the effect of block *l,* nested in R and e is the first residual term. Only block was assumed to be a random factor. The regression model showed that for the two reference genotypes LER_{max} was 0.0139 mm day^{-1} higher in the second run with a 95% confidence interval of ± 0.06725 mm day^{-1}. The LER_{max} values of the second run were therefore corrected by subtracting 0.0139 mm day^{-1}.

Results

Genotypic diversity for early-season growth

Climatic characteristics of the seasons investigated

Temperatures in Merelbeke (Belgium) during winter and spring 2014 were above the 25-year mean (Fig. 3.2). The mean temperature from January 1st until May 31st 2014 was 9.7°C, while the 25-year mean over the same period was 8.1°C. In 2015, the average temperature over this period was 8.1°C, but in late spring temperatures were above the 25-year mean. Accumulated thermal time for this period, calculated using a base temperature of 0°C, was 1553 growing degree days (GDD) in 2014, while the 2015 growing season (1307 GDD) was not different from the 25-year average of 1299 GDD (Fig. 7.1). As a result of the warmer weather in 2014, emergence started markedly earlier than in 2015. Both measuring periods were relatively dry, with a total precipitation of 329 and 326 mm for 2014 and 2015, respectively, compared to an average for the last 25 years of 477 mm.

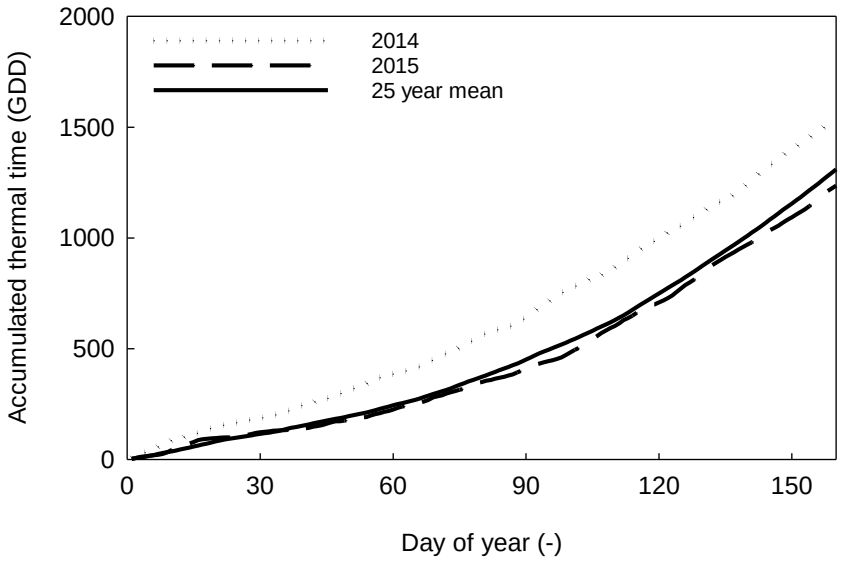


Figure 7.1: Accumulated thermal time in the 2014 and 2015 growing seasons compared to the 25-year mean for the period January 1st until May 31st, weather data were obtained from a meteorological station located approximately 1 km from the field trial in Merelbeke, Belgium.

Extent of variation in the germplasm

In both years, early-season growth varied widely among genotypes and species (Fig. 7.2A-F). For all parameters investigated (L30, L50, AGR, S50, Leaf4 and LFR) significant species x year and genotype x year interactions were found. Therefore, the data were analyzed separately per year. These interactions might have been caused by the differences in weather during both measuring periods or might be a result of differences in plant age between years. The within species variation was also substantial, except in the *M. x giganteus* group, and the genotype effects explained the

largest part of the variation in the data (Supplementary table 7.1). In 2014 there was a significant difference between the species for all traits except for S50, while in 2015 there were no significant differences on a species level. The hybrid group was most similar to the *M. sinensis* group, which is to be expected as these were all diploid hybrids with *M. sinensis* mothers. In general, the hybrids did not emerge earlier or grow faster than the other species. The *M. sacchariflorus* genotypes had on average lower AGR and higher L30 and L50 than *M. sinensis*. However, the intra-species variation was remarkably large for most parameters in *M. sacchariflorus*, even though there were only 17 genotypes of this species in the dataset. A number of extreme genotypes and outliers were observed in the *M. sinensis, M. sacchariflorus* and hybrid groups with especially good early-season growth, which were consistent between both years. These genotypes might be good candidates for breeding improved varieties.

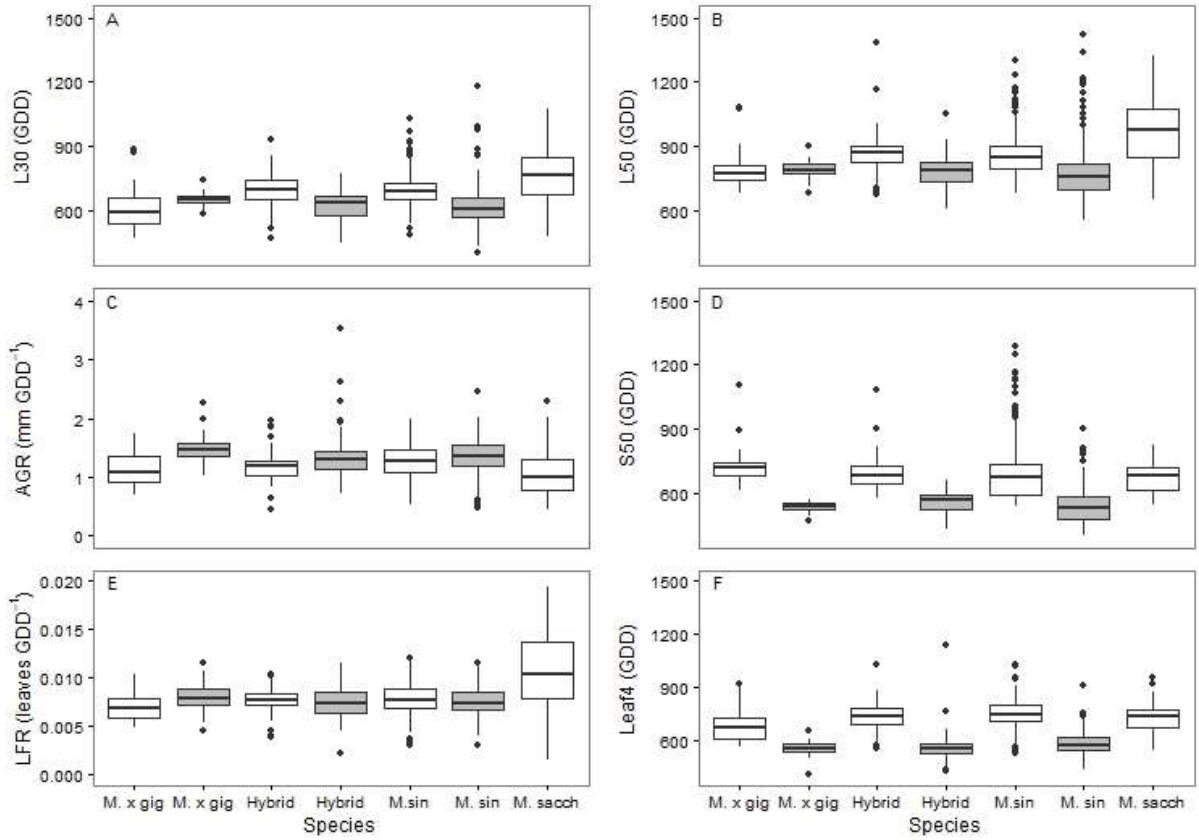


Figure 7.2: Box and whisker plots of genotypic adjusted means of early-season growth parameters, grouped per species and per growing season. The bottom and top of the box indicate the first and third quartiles, and the line inside the box indicates the median. White boxes show 2014 data and grey boxes show 2015 data. M. x gig: M. x giganteus (n = 10); Hybrid: M. sinensis x sacchariflorus hybrids (n = 14); M. sacch: M. sacchariflorus genotypes (n = 17, only in 2014) and M. sin: M. sinensis genotypes (n = 61). A: mean thermal time until shoot length 30 cm reached; B: mean thermal time until shoot length 50 cm reached; C: shoot growth rate; D: mean thermal time until 50% of shoots formed; E mean thermal time until fourth leaf emerges; F: leaf formation rate.

Overall growth patterns in 2014 and 2015

At the genotype level there was a significant correlation for growth parameters between the 2014 and 2015 seasons (Table 7.3). This is striking, considering that 2014 was the first year after establishment, while in 2015 more mature plants were evaluated. AGR and L50 displayed the highest between-year correlation for shoot development, while for L30 the correlation was lower. For plant development we also found a strong between-year correlation for N_{max}, although the correlation was rather low for S50. For leaf development, a strong correlation for Leaf4 was found between years, but not for LFR. This low correlation in LFR between both years might have been a result of the small variation for this parameter and the relatively large measuring error due to the experimental setup (the number of leaves was always counted on the longest shoot, but this was not the same shoot at all times that measurements were taken). Correlations were higher for most parameters at species level, except for the *M. x giganteus* group, for which only a limited number of genotypes was investigated.

Table 7.3: Correlations of early-season growth variables (absolute growth rate of the longest shoot (AGR, mm GDD^{-1}), thermal time until the longest shoot reached 30 cm (L30, GDD), thermal time until the longest shoot reached 50 cm (L50, GDD), leaf formation rate (LFR, leaves GDD^{-1}), thermal time until the fourth leaf emerged on the longest shoot (Leaf4, GDD), thermal time until 50% of the shoots had emerged (S50, GDD), Maximum shoot number (N_{max}, number of shoots) determined both in 2014 and 2015 at the field trial in Merelbeke, Belgium. Results at genotype and at species level are presented. All correlations are significant, except for correlations marked with ^{NS} (Pearson's product-moment correlation, $p < 0.01$, $n_{all\ genotypes} = 85$ $n_{M.\ sinensis} = 61$, $n_{M.\ x\ giganteus} = 10$, $n_{M.\ sinensis\ x\ sacchariflorus} = 14$).

	All genotypes n =85	*M. sinensis* *n = 61*	hybrid n = 14	*M. x giganteus* *n =10*
Shoot development				
AGR (mm GDD^{-1})	0.63	0.79	0.71	-0.08^{NS}
L30 (GDD)	0.38	0.53	0.77	0.50^{NS}
L50 (GDD)	0.74	0.83	0.76	0.63^{NS}
Leaf development				
LFR (leaves GDD^{-1})	0.27	0.40	0.26^{NS}	0.04^{NS}
Leaf4 (GDD)	0.54	0.55	0.65	-0.42^{NS}
Plant development				
S50 (GDD)	0.42	0.45	0.25^{NS}	0.43^{NS}
N_{max} (n)	0.79	0.78	0.84	0.80

The interrelationships between parameters were investigated further using principal component analysis (PCA) for the two years separately (Fig. 7.3A, B). In both cases, the first component explained more than 50% of the variation. Both years, the first component represented the contrast between L30, L50, S50, and Leaf4 versus AGR. In both years, genotypes with low values for the first component thus had high absolute growth rates and early emergence; genotypes with low values for this component therefore had vigorous early-season growth. Genotype loadings for the first component were highly correlated between years (r=0.64; Fig. 7.4). Genotypes located in the lower-

left sector in Fig. 7.4 (mostly *M. sinensis*, one hybrid and two *M. sacchariflorus* genotypes as far as results could have been confirmed in a second year for these genotypes) display strong early-season growth in both years, and are promising germplasm for breeding improved varieties. All species groups, except *M. x giganteus*, showed substantial variation in early-season growth. Hybrid genotypes tended to have lower early-season growth than *M. x giganteus*, though one genotype (OPM109) had the highest early-season growth of all genotypes in the trial. The *M. sacchariflorus* genotypes (only evaluated in 2014) had the lowest early-season growth, but this group also contained two genotypes (OPM25 and OPM19) with strong early-season growth.

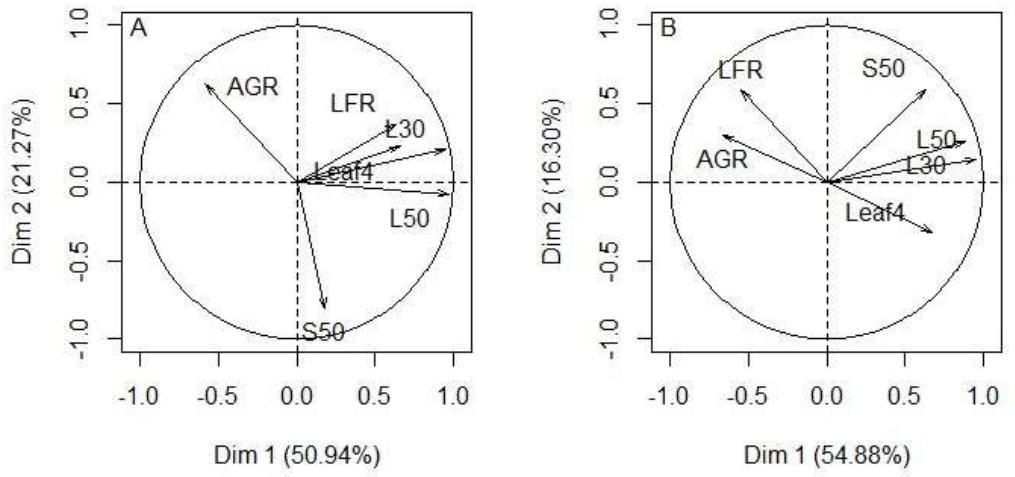


Figure 7.3: Principal component analysis of the early-season growth parameters measured in the common garden experiment in 2014 (A) and 2015 (B). variables. AGR: absolute growth rate of the longest shoot, L30: thermal time until the longest shoot reached 30 cm, L50: thermal time until the longest shoot reached 50 cm, LFR: leaf formation rate, Leaf4: thermal time until the fourth leaf emerged on the longest shoot, S50: thermal time until 50% of the shoots had emerged.

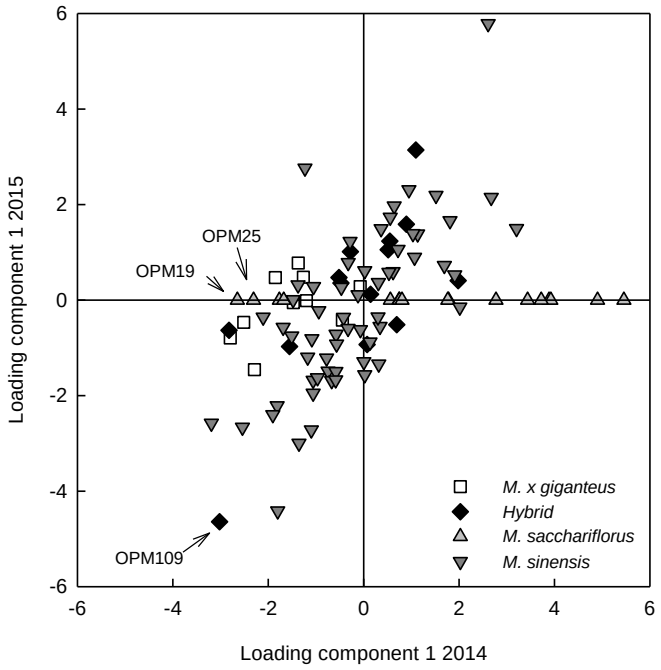


Figure 7.4: Genotype loadings of the first component of the 2014 and 2015 principal component analyses. Genotypes in the bottom left corner have strong early-season growth. M. sacchariflorus genotypes were not investigated in 2015; data from 2014 is displayed for completeness. Genotypes in the lower left part of the graph exhibited strong early growth. Different symbols indicate different species.

Table 7.4: Broad sense heritability of traits measured in the common garden experiment. Heritability for *M. sacchariflorus* was only calculated for 2014 because only data for 2014 were available for this species.

	All genotypes	*M. sinensis*	*M. sacchariflorus**	*M. x giganteus*	hybrid
Shoot development					
AGR (mm GDD^{-1})	0.80	0.86	0.87	0.30	0.68
L30 (GDD)	0.66	0.66	0.95	0.25	0.80
L50 (GDD)	0.88	0.89	0.91	0.08	0.73
Leaf development					
LFR (leaves GDD^{-1})	0.71	0.76	0.71	0	0.64
Leaf4 (GDD)	0.81	0.60	0.83	0	0.15
Plant development					
S50 (GDD)	0.56	0.59	0.92	0.45	0.38
N_{max} (n)	0.73	0.75	0.68	0.37	0.79

Broad sense heritability was high over all species, indicating that early-season growth is largely genotypically determined (Table 7.4). On a species level heritability was high in *M. sinensis, M.*

sacchariflorus and in the hybrids. As was to be expected in the M. x giganteus group, heritabilities are low, as there is little variation in this group. The *M. x giganteus* group was not combined with the hybrid group due to the different ploidy levels. Over all genotypes, the highest heritabilities were found for AGR, L50 and Leaf4, while heritabilities for S50 and L30 were lower.

Overall, L50 appeared to be the most reproducible parameter between both years, with a high correlation between both years as well as high heritability. This parameter also displayed a large variation between both years and appears to be the most suitable parameter to measure to select genotypes for further breeding. The *M. sinensis* and *M. sacchariflorus* genotypes showed particularly large variation for this trait among the genotypes (Fig. 7.5A, B) enabling selection for this trait.

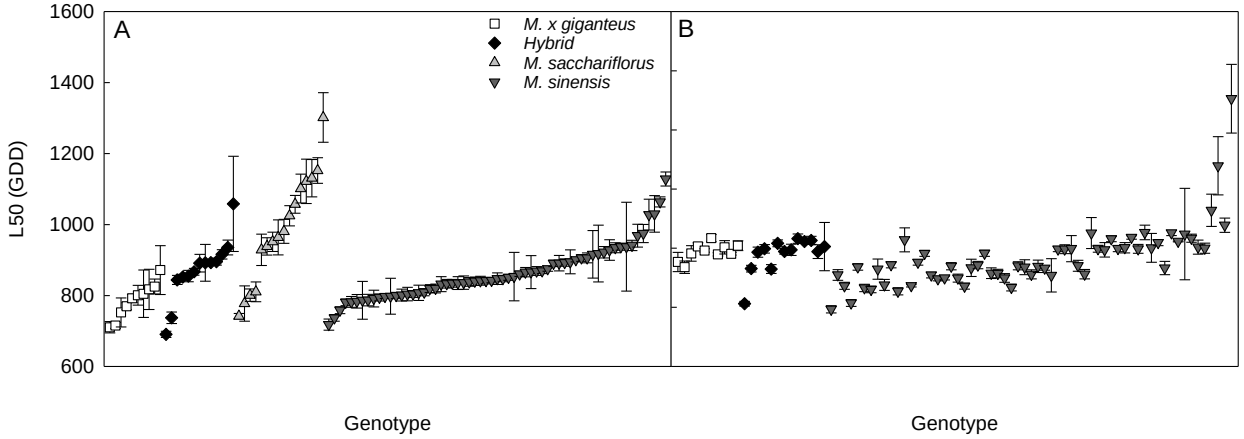


Figure 7.5: L50 per genotype and species in 2014 (A) and 2015 (B) measured in the common garden experiment in Merelbeke, Belgium. Genotypes are ordered, per species group, according to 2014 L50.

Early-season growth in contrasting regions

The multi-location trial covered a wide range of climates, and consequently showed a large range in L30 values (Fig. 7.6). In 2015, for example, the earliest genotype in Adana, Turkey (OPM01) reached L30 on 25/03, while the earliest genotype in Moscow, Russia (OPM10), reached L30 almost two months later, on 20/05. L30 was reached in 2014 on average after 570, 677 and 762 GDD in Ukraine, Germany and Wales respectively, while in Turkey this occurred only after 1072 GDD. Similarly, in 2015 L30 was reached after on average 574, 618 and 676 GDD in Ukraine, Russia and Wales, respectively, but 994 GDD in Turkey. This indicates that L30 in most locations was probably determined by temperature, while in Turkey temperature was not the main driver. As a result, L30 was not correlated on a genotype basis between both years in Turkey (r = 0.04), but was strongly correlated between both years in Wales (r = 0.52) and Ukraine (r = 0.60). In the European trials the overall variation among genotypes was the largest in the maritime climate of Wales, while in the other sites (all of which have a continental climate with colder winters) the differences were less pronounced.

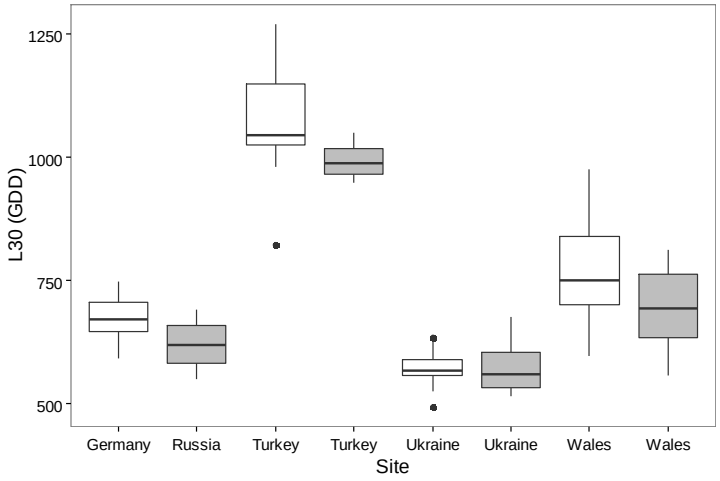


Figure 7.6: Box and whisker plot of mean L30 per genotype per year and location. The bottom and top of the box indicate the first and third quartiles, and the line inside the box indicates the median. White boxes show 2014 data; grey boxes show 2015 data.

On average over all locations, the hybrid OPM05 and M. sacchariflorus OPM01 were the first to reach L30, while the M. sacchariflorus genotypes OPM02, OPM03 and OPM04 were latest in almost every location (Fig. 7.7). However, there were strong genotype x environment interactions, even after exclusion of the site-years from Adana, Turkey. No genotype was the latest or the earliest in all locations, showing the need for locally adapted varieties. The seed-based populations also contained both early types, such as OPM12 and OPM14, and late populations such as OPM15. However, there were also strong genotype x environment interactions in these populations, even when excluding the site-years from Adana, Turkey. The variation over the site-years reveals that at L30, OPM06, OPM11 and OPM14 showed much less variation with more stable performance over different locations and years compared to the other genotypes.

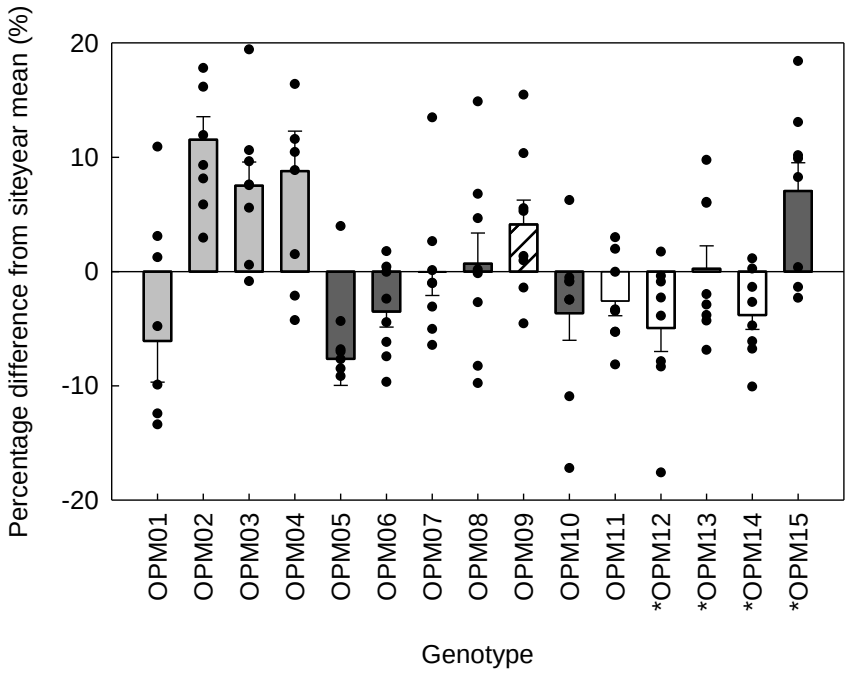


Figure 7.7: Percentage difference from the site-year mean of L30 per genotype and site-year. Bars give the mean and standard error per genotype over all locations. Points depict the site-year means per genotype relative to the site-year mean. Genotypes marked with * are seed based populations; other genotypes are clonal varieties. Light grey bars: *M. sacchariflorus,* White bars: *M. sinensis;* Tilted stripes*: M. x giganteus,* Dark grey bars*: M. sinensis x sacchariflorus* hybrids.

Early-season growth in relation to response to chilling

Screening under controlled conditions in the growth chamber revealed the availability of genotypic variation in the response to chilling stress in the germplasm investigated. The maximum leaf growth rate (LER_{max}) was significantly reduced in all genotypes at 14°C compared to 20°C on a calendar day basis (results not shown). On a thermal time basis, LER_{max} was on average 10% lower at 14°C, indicating some effect of chilling stress on growth, but most genotypes showed a good capacity for growth at both temperatures (Fig. 7.8), with some of them performing better at 14°C than at 20°C (points above the diagonal in Fig. 7.8). Chlorophyll fluorescence measurements indicated that for none of the genotypes the photosynthetic capacity was significantly reduced at 14°C (data not shown). LER_{max} was on average 1.4 mm GDD^{-1} at 20°C and 1.2 mm GDD^{-1} at 14°C. The LER_{max} of OPM09 (reference *M. x giganteus* genotype) was above the average of the 54 genotypes at both temperatures (1.5 mm GDD^{-1} at 20°C and 1.3 mm GDD^{-1} at 14°C). Although LER_{max} was significantly different between the *M. sinensis* and *M. sacchariflorus* genotypes at both temperatures, no marked difference in relative change between the species was observed. Both species groups contained genotypes in which LER_{max} was similar at both temperatures as well as more stressed genotypes. There was a strong correlation between LER_{max} at 20°C and at 14°C (r = 0.79), indicating a strong

genotypic determination of growth rate. Nevertheless, growth rate in the growth chamber (LER_{max}) was not significantly correlated with early-season growth in the field. The correlation between LER_{max} at 14°C and AGR was -0.08 and -0.18 in 2014 and 2015, respectively, while for LER_{max} at 20°C, these correlations were -0.01 and 0.01, respectively.

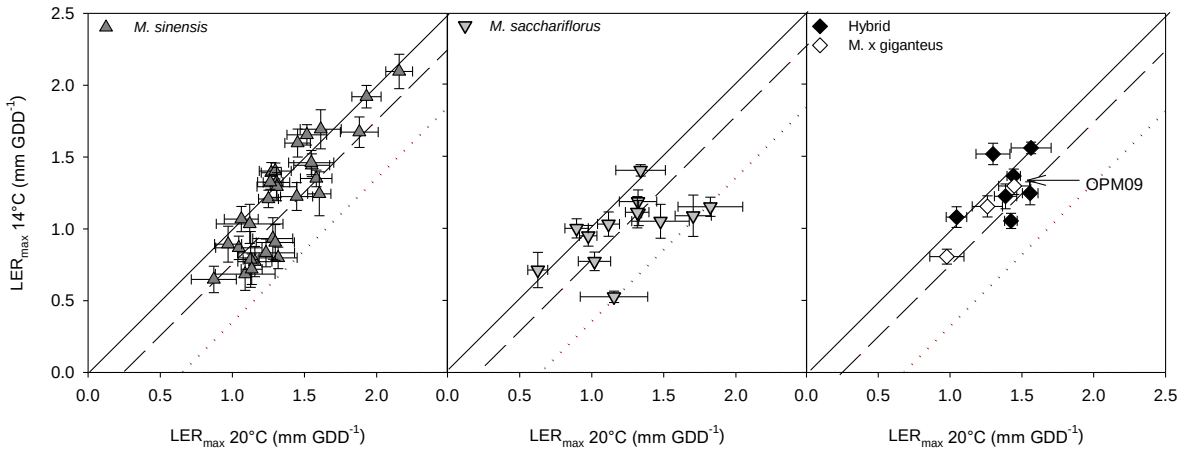


Figure 7.8: Average LER_{max} per genotype at 14°C and at 20°C. Symbols depict averages per genotype. Different symbols indicate different species groups. Error bars show standard error (n = 10). The solid lines represent the 1:1 relationship, the dashed lines indicate a reduction in LER_{max} of 10% and the dotted line indicates a reduction of 25%. A: M. sinensis, B: M. sacchariflorus and C: M. sinensis x sacchariflorus and M. x giganteus genotypes.

Early-season growth in relation to origin

For 13 *M. sacchariflorus* genotypes, the location of origin was known. For these genotypes, L30, L50, Leaf4 and LFR were strongly correlated with the latitude at the collection site (Table 7.5). The correlation between AGR and S50 and latitude was not significant. Genotypes from more northerly latitudes emerged later and reached L30, L50 and Leaf4 later than genotypes from more southern latitudes. When grown next to each other at a single location (Merelbeke, Belgium), genotypes from lower latitudes and warmer locations thus emerged earlier and grew faster.

LER_{max} measured in the growth chamber was also significantly correlated with latitude, but here the relationship between origin and growth was inverse compared to the field. In the growth chamber genotypes from colder and more northern locations grew faster at both temperatures. This might be related to the light intensity or day length settings of the growth chamber which were more similar to northern growing conditions and which might have favored the northern genotypes. This inverse reaction between growth chamber and field trial again indicates that the growth chamber experiments did not relate well to field conditions.

Table 7.5: Correlations (r) between early-season growth (only in 2014) and LER_{max} at two temperatures and characteristics of the location of origin of 13 *M. sacchariflorus* genotypes. Correlations with * are significant (Pearson's product moment correlation, $p < 0.05$).

	AGR (mm GDD^{-1})	L50 (GDD)	L30 (GDD)	LFR (Leaves GDD^{-1})	Leaf4 (GDD)	S50 (GDD)	LER_{max} 14°C (mm GDD^{-1})	LER_{max} 20°C (mm GDD^{-1})
Latitude (°N)	-0.52	0.82*	0.85*	0.79*	0.88*	0.50	0.38	0.62*
Maximum monthly temperature (°C)	0.53	-0.79*	-0.82*	-0.74*	-0.76*	-0.69*	-0.48	-0.68*
Annual GDD (GDD)	0.49	-0.65*	-0.62*	-0.59*	-0.60*	-0.23	-0.30	-0.36

Discussion

Screening miscanthus for early-season growth

Our study demonstrated considerable variation in the miscanthus germplasm with regards to early-season growth and growth at low temperatures. This is in accordance with the studies of Zub et al. (2012b) in northern France and Robson et al. (2013a) in Wales, who observed considerable variation in emergence and canopy formation in their field trials, and with the studies of Clifton-Brown and Jones (1997) and Głowacka et al. (2014a), who found substantial variation in growth at low temperatures in growth chamber experiments. Our study is the first to combine field trials with growth chamber experiments and to study early-season growth in multiple locations.

The obtained growth rates in the growth chamber did not correlate well to early-season growth parameters in the field. This is probably due to the large differences in growing conditions between the field and the growth chamber. While in the growth chamber experiment temperature and light intensity were stable and light intensity relatively low, in the field large fluctuations in both light intensity and temperature were observed. Plant growth responses under variable environments and presumably in reaction to multiple stresses that occur simultaneously probably induce different responses than a single abiotic stress (Mittler, 2006). It is therefore possible that the chosen treatments in the growth chamber were not representative enough of field conditions. Indeed, chlorophyll fluorescence measurements in the growth chamber revealed no considerable stress symptoms at 14°C (data not shown). During a first run at 12°C, nearly no growth was observed; this precluded the use of lower temperatures in the experiment. In other studies, discrepancies are often found between growth chamber and field experiments. Friesen et al. (2014) found that the least chilling tolerant miscanthus genotype in their study under field conditions was one of the least affected by chilling stress under controlled conditions. In other crops, growth chamber experiments have been used for the identification of genotypes with high early-season growth, but results have not always

been consistent with field trials. For example, Rodríguez et al. (2007) and Revilla et al. (2000) found no correlation between early season growth traits in the field and the growth chamber in European maize germplasm. Menkir and Larter (1985) also found no relationship between emergence traits in inbred maize lines but found significant correlation in post emergence growth between growth chamber and field conditions. In sorghum, both Franks et al. (2006) and Tiryaki and Andrews (2001) found a significant correlation of emergence under chilling stress under controlled and field conditions. The inconsistent results in literature regarding the use of growth chamber experiments to predict early-season growth in field trials demonstrate the need to design growth chamber experiments to better simulate field conditions and the need for validation of growth chamber experiments in field trials when the experiments are used for germplasm screening. Furthermore, screening under field conditions has additional benefits, as it allows for more genotypes to be evaluated simultaneously under realistic conditions and allows for the simultaneous screening of other relevant traits in the same experiment. In the common garden experiment in our study, traits derived from shoot length measurements were the most consistent between years and were the fastest to measure. In a large scale germplasm screening shoot length would thus be the most advisable traits to measure.

Relationship between environmental factors and early-season growth

Although only a limited number of genotypes came with data relating to their origin, it appears that *M. sacchariflorus* genotypes from northern latitudes and colder regions start to grow later in the spring. Of course, latitude and temperature are only rough estimators of climate, but similar observations have been made in miscanthus (Yan et al., 2011) and in common reed (*Praghmites australis*) (Clevering et al., 2001). The later emergence of the northern accessions might have evolved to avoid cold damage in early spring. The *M. sacchariflorus* genotypes in our collection are rather susceptible to frost and had the highest reduction in LER_{max} at 14°C in our growth chamber experiment. Late emergence could thus have been selected for in regions where frost temperatures are likely to occur until late in spring. Planting genotypes from warmer locations in more northern locations might lead to higher yields, as they would emerge earlier and grow faster. Zhao et al. (2013), for example, also found that miscanthus accessions of lower latitudes yielded better at their trial site in Wuhan, China. However, damage due cold stress, either by winter mortality or by damage to newly emerged shoots, might threaten the possible gains in yield by earlier emergence. Crossing southern genotypes (early emergence and high growth rates) and northern genotypes (higher cold tolerance) might be an option to produce new varieties that can form a canopy earlier and optimize their use of the radiation available during the growing season. However, there might be a certain trade-off between growth rate and cold tolerance (Clifton-Brown and Jones, 1997; Farrell et al., 2006; chapter

5, 8). It is also unclear for the moment if the later emergence of more northern genotypes is specific to *M. sacchariflorus* or is a general tendency in miscanthus.

Although Hastings et al. (2009a), included photoperiod sensitivity as a main driver of emergence in their *M. x giganteus* yield model MISCANFOR, it appears that emergence in miscanthus is mainly temperature regulated, rather than photoperiod sensitive. In both 2014 and 2015 some genotypes started emerging before the spring equinox in the trial in Belgium, while in 2016 no emergence was observed before March 21st due to lower temperatures (personal observation). In the trial in Adana, Turkey, all genotypes emerged even earlier in 2014 and 2015, reaching L30 around the spring equinox. The relationship between emergence and latitude in *M. sacchariflorus* suggests a role of photoperiod sensitivity for emergence in that species. For other phenological traits, such as flowering, *M. sacchariflorus* has been shown to be photoperiod sensitive, while *M. sinensis* is day neutral (Jensen et al., 2011).

The multi-location trials indicated strong genotype x environment interactions for early-season growth parameters. No single genotype was the earliest in all locations. This suggests that the best way to maximize yield potential is to develop varieties adapted to local environmental conditions. The field trials set up across Europe during the European Miscanthus Improvement (EMI) project confirm this: a marked effect of location on miscanthus productivity was observed. The highest yielding genotypes in the more northern locations gave below average yields in the warmer locations and vice versa (Clifton-Brown et al., 2001a). The genotypes in our study responded markedly differently to temperature in Turkey compared to the other locations, with considerably less growth per GDD. Lack of water, rather than temperature, appeared to be the driving factor for early growth in the Turkish field trial. Further research is required to confirm this finding.

Implications for breeding

Breeding for an extended growing season is one of the options to develop high yielding miscanthus varieties, as it would theoretically allow to increase the intercepted radiation in the growing season (Sage et al., 2015; Zhu et al., 2010). In miscanthus yield models, earlier emergence does indeed lead to predicted yield increases through increased radiation interception (Davey et al., 2015; Farrell et al., 2006; Kandel et al., 2016). However, these modeling studies did not take into account other phenological traits. Early emerging genotypes could also flower and senesce earlier, which would counteract the advantages of early emergence (Jensen et al., 2011; Robson et al., 2011). By emerging earlier, the plants can form a canopy earlier and get maximum benefit from the long days in late spring and early summer, growing best when water availability is higher. Nevertheless, relatively slower growth later in the season or an earlier senescence in the early emerging genotypes

can offset the gains made by early emergence. Contrasting observations about the relationship between growing season duration and final biomass yield have been reported. Robson et al. (2013a) concluded that earlier emergence is related with higher biomass yields in miscanthus, while Zub et al. (2012) concluded from another field trial that genotypes with late emergence combined with high growth rates in summer yield the most biomass. In other biomass crops, such as willow (Salix spp.) an earlier budburst and canopy formation is also associated with a higher biomass yield (Cannell et al., 1987; Weih, 2009), but this topic has to date not been studied extensively in perennial biomass crops. More research is required to establish the relationship between early-season growth and final biomass yield.

In our study, the *M. x giganteus* genotypes had moderate early-season growth compared to the other genotypes. Some *M. sinensis* and *M. sacchariflorus* genotypes and *M. sinensis x sacchariflorus* hybrids showed stronger early-season growth than *M. x giganteus* in the tested collection. *M. sinensis* is sometimes assumed to be more tolerant of low temperatures than *M. sacchariflorus* (Lewandowski et al., 2003), while others reported *M. sacchariflorus* to be more cold tolerant (Głowacka et al., 2014a; Jiao et al., 2016b; Yan et al., 2011). We found that both species have genotypes with strong early-season growth. When *M. sinensis* is crossed with *M. sacchariflorus* to develop new hybrid varieties, either parent could pass on early-season growth superior to *M. x giganteus*. The high genotypic correlations between years for early-season growth parameters and their high broad sense heritabilities indicate strong genotypic determination of early-season growth. This is in agreement with Slavov et al. (2013) and Gifford et al. (2015) who reported high broad sense heritabilities for flowering related phenological traits (e.g., heading date) in miscanthus, as well as in agreement with Presterl et al. (2007) and Strigens et al. (2013) who found high broad sense heritability for phenological traits in maize. Because early-season growth is strongly correlated between the second and third growing season, selection of valuable genotypes could already be performed in the second year after planting. Identifying traits in the second year would greatly increase breeding efficiency (Gifford et al., 2015). The substantial variation in early-season growth observed in our studies, combined with the high correlation of growth traits between years and the high heritability of these traits, indicates that breeding for an extended growing season should be possible. The best genotypes from our study can be used to develop new varieties with increased early-season growth.

Conclusions

Large variation in early-season growth was found in the germplasm collection investigated and genotypes with high early-season growth were found in all species groups. It should thus be possible to develop improved varieties compared to *M. x giganteus*. Growth in the growth chamber did not correlate very well to early-season growth in the field. But at the species level common trends were

found. When evaluating new germplasm, it is thus better to use field trials than growth chamber experiments, although there can also be a large variation between field trials in different locations.

Chapter 8: Physiological basis of chilling tolerance and early-season growth in miscanthus

This chapter is based on: Fonteyne S., Muylle H., Lootens P., Kerchev P., van den Ende W., Staelens A., Reheul D., Roldán-Ruiz I. (submitted) Physiological basis of chilling tolerance and early-season growth in miscanthus. Annals of Botany.

Introduction

M. x giganteus, the most commonly planted miscanthus type, has been reported to be more chilling tolerant than other phylogenetically related C4 species such as maize, sorghum or sugarcane (Long and Spence 2013; Sage et al. 2015). Compared to these crops, *M. x giganteus* is capable of higher photosynthetic activity at lower temperatures (Naidu et al. 2003; Głowacka et al. 2014a). 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 (chapter four; Friesen et al., 2014; Kaiser and Sacks, 2015; Long and Spence, 2013). Chilling stress does influence negatively photosynthetic efficiency and causes a temporal growth reduction (Clifton-Brown and Jones 1997; Głowacka et al. 2014a; 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; Friesen et al., 2014; Głowacka et al., 2014a, 2015b; Purdy et al., 2013, previous chapters), and genotypes that perform even better than *M. x giganteus* have been identified (Głowacka et al., 2014a, 2015b) 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. x 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, Portis, et al. 2008; 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. x giganteus* when exposed to chilling temperatures. This agrees with the observation of a lower decrease in chlorophyll content and

photosynthesis activity in *M. x giganteus* than in the more chilling sensitive *M. sinensis* 'Goliath' at low temperatures (chapter five).

Under field conditions, low temperatures combined with high light intensities induce photobleaching in chilling sensitive miscanthus genotypes, indicating oxidative stress (chapter four). In the few miscanthus genotypes in which this has been investigated, the ratio of quantum efficiency of photosystem II (Φ_{PSII}) to the quantum efficiency of CO2-fixation (Φ_{CO2}) seems to remain constant until temperatures drop below 12°C (Naidu and Long 2004; Friesen and Sage 2015). At 10°C this ratio increases, indicating the channeling 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 have also received some attention in miscanthus, as they are not only indicative of photosynthesis and growth, but also provide protection against damage by chilling stress and serve as stress signaling 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. x 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 overall characterization of 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, Naidu, et al. 2008; Purdy et al. 2013; Głowacka et al. 2014a). 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, with not only 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 chapter photosynthesis and several biochemical traits putatively related to chilling tolerance were investigated under field conditions in a diverse set of five miscanthus genotypes. The main objectives were (i) to characterize the response of these five genotypes to changes in temperature throughout the growing season and (ii) to identify traits and responses that distinguish genotypes classified as chilling tolerant and chilling sensitive. Leaf samples of field-grown plants were taken on five dates between late April and early June 2015, representing different weather conditions. Traits indicative of photosynthesis, redox homeostasis and carbohydrate metabolism responses were investigated. Photosynthesis was studied by determination of chlorophyll and carotenoid contents, PPDK activity and chlorophyll fluorescence measurements. To investigate the influence of low temperature on redox homeostasis, we monitored the water soluble antioxidants ascorbate and glutathione, the activity of the H_2O_2-removing enzyme catalase and the levels of malondialdehyde (MDA), which is frequently used as a marker of ROS-induced lipid peroxidation (Hodges et al. 1999; Foyer et al. 2002). Carbohydrate metabolism was studied by analyzing glucose, fructose, sucrose, raffinose and maltose contents.

Materials and methods

Growth conditions

Chlorophyll fluorescence, plant growth measurements and biomass sampling were performed in April and May 2015 in field trial FT2. Weather data were collected in a meteorological station located at about 1 km from the trial.

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: (i) cold stress symptoms after a cold spell in March 2014 as reported in chapter four (ii) early-season shoot growth in 2014 as described in chapter seven. 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 8.1. Two genotypes were chosen due to indications of chilling tolerance: *M. sinensis* ‘OPM66’ and *M. sinensis x sacchariflorus* hybrid ‘OPM06’. Two genotypes were chosen as more chilling sensitive: *M. sinensis* ‘OPM51’ and *M. sinensis x sacchariflorus* hybrid ‘OPM35’. *M. x giganteus* ‘OPM09’ was included in the study because it is the most widely used, both in scientific research as in commercial production.

Table 8.1. Early-season growth parameters of five miscanthus genotypes (OPM06, OPM66, OPM09, OPM35 and OPM51) in 2014 and 2015. Growth parameters for these five genotypes and overall median, maximum and minimum values in 2014 for a larger collection of genotypes as described in chapters four and seven are shown to illustrate how this set of five genotypes was chosen. Trait values higher (stress score and AGR) or lower (L50, L30, Leaf4) than the median are indicated in bold and underlined. Data for 2015 refer to the season in which the investigation presented here was carried out. 'stress score': damage to plants scored after a cold spell on 28/3/2014 (chapter four); 'AGR': absolute growth rate, 'L50' thermal time at which a length of 50 cm was reached, 'L30': thermal time at which a length of 30 cm was reached, 'Leaf4': thermal time at which the fourth leaf on the longest shoot emerged.

Genotype	Year	Stress score (-)	AGR (mm GDD^{-1})	L50 (GDD)	L30 (GDD)	Leaf4 (GDD)
Median	2014	5.9	1.2	801.4	696.1	747.8
Maximum		8.0	1.9	1243.6	936.7	927.9
Minimum		2.8	0.6	690.6	532.2	593.0
OPM06	2014	**6.7**	1.2	852.3	**680.2**	**711.0**
OPM66		**7.5**	**1.5**	**718.0**	**587.6**	**630.0**
OPM09		4.7	1.1	825.3	**628.4**	**689.7**
OPM35		4.8	**1.5**	866.7	721.2	758.1
OPM51		4.5	**1.3**	895.0	738.1	752.0
OPM06	2015	/	1.3	798.2	633.8	560.2
OPM66		/	1.5	593.4	455.2	506.7
OPM09		/	1.5	781.5	651.5	564.9
OPM35		/	1.5	816.6	679.6	571.4
OPM51		/	1.6	798.7	674.6	563.8

Growth measurements and sampling in 2015

From 18/02/2015 to 28/05/2015, the length of the longest shoot, the number of leaves on that shoot and the number of shoots per plant were measured twice weekly to determine growth parameters as described above. The trial was harvested on 12/01/2016 and the total aboveground part of each individual plant was cut and weighed individually. A subsample of approximately 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 (28/04, 7/05, 12/05, 27/05 and 9/06, referred to as T1 till T5) (Table 8.3). On each sampling day, 5 – 10 young, fully expanded leaves 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 MDA, glutathione and ascorbate contents and catalase activity. Additionally, several leaf discs 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.

Chlorophyll fluorescence measurements

The quantum efficiency of photosystem II (Φ_{PSII}) was measured on four dates close to the sampling dates (29/04, 8/05, 13/05 and 21/05/2015, referred to as t1 till 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 PAM2100 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 x 3 measurements per leaf) was repeated sequentially over a period of three hours, rendering approximately five measurement points per leaf at slightly differing light and temperature conditions on each measurement date.

Biochemical analyses

An overview of the traits investigated is provided in Table 8.2. 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.

Table 8.2: Overview of biochemical traits determined in this study.

Trait	Abbreviation	Unit	Reference
Chlorophyll a+b content	Chl	mg g^{-1} DW	Lichtenthaler and Buschmann (2001)
Carotenoid content	Car	mg g^{-1} DW	Lichtenthaler and Buschmann (2001)
PPDK activity	PPDK	µmol m^{-2} s^{-1}	Wang et al. (2008)
MDA content	MDA	nmol g^{-1} FW	Hodges et al. (1999)
Catalase activity	Cat	µmol H_2O_2 mg^{-1} protein	Aebi (1984)
Glutathione content	GSH	nmol g^{-1} FW	Queval et al., (2007)
Ascorbate content	Asc	µmol g^{-1} FW	Queval et al., (2007)
Glucose content	Glc	mg g^{-1} DW	Zhang et al. (2015)
Fructose content	Fru	mg g^{-1} DW	Zhang et al. (2015)
Sucrose content	Suc	mg g^{-1} DW	Zhang et al. (2015)
Raffinose content	Raf	mg g^{-1} DW	Zhang et al. (2015)
Maltose content	Mal	mg g^{-1} DW	Zhang et al. (2015)
Total carbohydrate content	Tot	mg g^{-1} DW	Zhang et al. (2015)

Chlorophyll a+b and carotenoid content

40 mg 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 24h in the dark and turned around periodically. Subsequently the samples were centrifuged at 10000 rpm at 4°C for 10 min. Two hundred microliters of twice-diluted supernatant was then pipetted in triplicate in a microtiter plate. Chlorophyll was then estimated by measuring the absorption at 663, 647 and 470 nm and calculated using the formulas reported in Lichtenthaler and Buschmann (2001).

PPDK activity

The protocol for PPDK activity analysis was adapted from Wang et al. (2008). Two buffers were used. The extraction buffer contained 50 mM Hepes-NaOH, pH 8.0, 10mM MgCl2, 5mM DTT, 1 mM EDTA, 1% casein, 1% PVP, 0.05% Triton-X-100, 20 mM NaF, 2µM orthovanadate and 1 protease inhibitor cocktail tablet per 10 mL buffer. The assay buffer contained 100 mM Hepes-NaOH, pH 8.0, 15 mM MgCl2, 0.15 mM EDTA, 5 mM NaHCO3, 0.3 mM NADH, 5 mM NH4Cl, 2.5 mM K2PO4, 5 mM DTT, 1 mM Glc-6-P, 1.5 mM ATP and 10 U ml-1 malate dehydrogenase. Two leaf discs of were ground in a Retsch tissue lyzer for 15 s at 20 Hz in an 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 4 times (3 technical repeats and 1 blank). The samples were then incubated at 30°C for 5 min in an Eppendorf

thermomixer block. Then, 5 µl enzyme mix (0.75 µl (1 U µl-1) mPEPc, 3.125 µl pyruvate (100 mM) and 1.125 µl assay buffer) was added to 3 of the 4 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, Portis, et al. 2008).

Malondialdehyde (MDA) content

The protocol by Hodges et al. (1999) was followed. Frozen leaf powder (100 mg) was homogenized in 1 mL 80% (w/v) ethanol solution with 0.02% butylated hydroxytoluene (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% TCA) and another 400 µL of the supernatant was added to 800 µL of TBA+ solution (20%TCA and 0.65% (w/v) 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 10min 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 nm, 532 nm and 600 nm. The amount of MDA equivalents was calculated using the formula by 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 PMSF) on ice. The tubes were centrifuged for 15 min at 14,000 rpm at 4°C and the supernatant was transferred to a fresh 1.5 ml reaction tube. A five-fold diluted subsample 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 MQ-H_2O and 270 µL CBB solution. Absorption at 595 nm was then measured using an iMARK spectrophotometer (BIORAD, Hercules, CA, USA). The concentration of protein was determined using a BSA standard curve. A total of 250µl 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 microtiter 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). One milliliter 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 minutes at 4°C at 14,000 rpm. Five hundred microliters of supernatant were neutralized by adding 50 µL sodium phosphate buffer 0.2 M (pH 5.6) and 420 µl 0.2 M NaOH to a final pH of 5. Glutathione: 20 µl of the neutralized supernatant and 50 µl water were added in triplicate to wells in a microtiter plate. A mixture of 100 µl 0.2 M sodium phosphate buffer (pH 7.5, 10 mM EDTA), 10 µl 10 mM NADPH, 10 µl 12 mM DTNB and 10 µl 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, GSH standards with consecutive dilutions of 0 nM, 0.2 nM, 0.4 nM and 1 nM were run. Ascorbate was measured after reduction of DHA to ascorbate. One hundred µL neutralized supernatant was mixed with 140 µl sodium phosphate buffer (0.12 M, pH 7.5) and 10 µl 25 mM DDT then incubated at room temperature for 30 min. Each sample was then measured in triplicate using 50 µl 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 MQ 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 6 times with 200 µL of MQ water; the water was collected together with the sample. The soluble sugar content of the samples was then analyzed 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). Samples were analysed with HPAEC-PAD on an ICS3000 system (Thermo Scientific Dionex). Analysis and detection were perfomed at 32 °C and the flow rate was 250 µL per minute. 15µL of sample was injected on a Guard CarboPac PA100 (2 x 50 mm) in series with an analytical CarboPac PA100 (2 x 250mm) equilibrated for 9 minutes with 90 mM CO2-free NaOH. Sugars were eluted in 90 mM NaOH, with an increasing NaAc-gradient: from minute 0 to 6, the NaAc-concentration increased linearly from 0 to 10 mM; from minute 6 to 16 the concentration increased linearly from 10 to 100 mM; from minute 16 to 26, the concentration increased linearly from 100 to 175 mM, then the columns were regenerated with 500 mM NaAc for 1 min and equilibrated with 90 mM NaOH for 9 minutes for the next run.

Data analysis

The chlorophyll fluorescence measurements were analyzed 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:

$$Y = \mu + G_i + D_j + L_k + T_l + G_i \times D_j + G_i \times L_k + G_i \times T_l + L_k \times T_l + B_m + e_{ijklm} \quad \text{(eq. 8.1)}$$

Where Y is the quantum efficiency of photosystem II (ΦPSII), μ the overall mean, Gi the effect of genotype i, Dj the effect of measuring date j, L the effect of light intensity k, Tl the effect of temperature l, B the effect of block m and eijklm the first residual term. Light intensity (L), Temperature (T) and Block (B) were considered random effects. Significance of the differences between genotypes at a given time point and between time points were 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 analyzed using generalized linear models with the 'glm' function of the stats package. Data were analyzed according to the model:

$$Y = \mu + Gi + Dj + Gi \times Dj + Bk + eijk \quad \text{(eq. 8.2)}$$

Where Y is a biochemical trait, μ the overall mean, Gi the effect of genotype i, Dj the effect of sampling date j, B the effect of block k and eijk the first residual term. Samples of six plants of each genotype were analyzed 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 analyzed per genotype and per sampling date separately. Significance of the differences between genotypes at a given time point and between time points were determined by post-hoc least square means calculation using the 'lsmeans' function of the lsmeans package.

Principal component analysis of a dataset 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. 8.1. Table 8.3 summarizes the main characteristics of the dates chosen for leaf sampling (T1 till T5) and chlorophyll fluorescence (t1 till 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 likely not experiencing chilling stress at the time of sampling (16:00). Air temperature was the lowest in the 24 h period before T1 and highest before T3 (Table 8.3, Fig. 8.2). The highest temperatures during the whole sampling period were reached in June, but in the days before sampling on 9/06 (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 5/06. Regarding the chlorophyll fluorescence measurements, the coldest time point was t1 and the warmest t2, but note that 7/05 (the day preceding the t2 chlorophyll fluorescence measurements) had a maximum temperature of only 15.3°C.

Table 8.3: Mean, minimum and maximum air temperatures in the 24 h before leaf sampling for biochemical analyses and chlorophyll fluorescence measurements. The temperature at sampling (16:00) or at the start of the chlorophyll fluorescence measurements (08:00) is also provided. Calculations based on data recorded by a weather station located at approximately 1 km from the field trial.

	Leaf sampling						Φ_{PSII}			
Date	28/04	7/05	12/05	27/05	9/06		29/04	8/05	13/05	21/05
Code	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
T_a at 16:00 (°C)	12.1	14.7	18.5	18.9	15.3	T_a at 8:00 (°C)	4.9	11.6	8.5	7.9

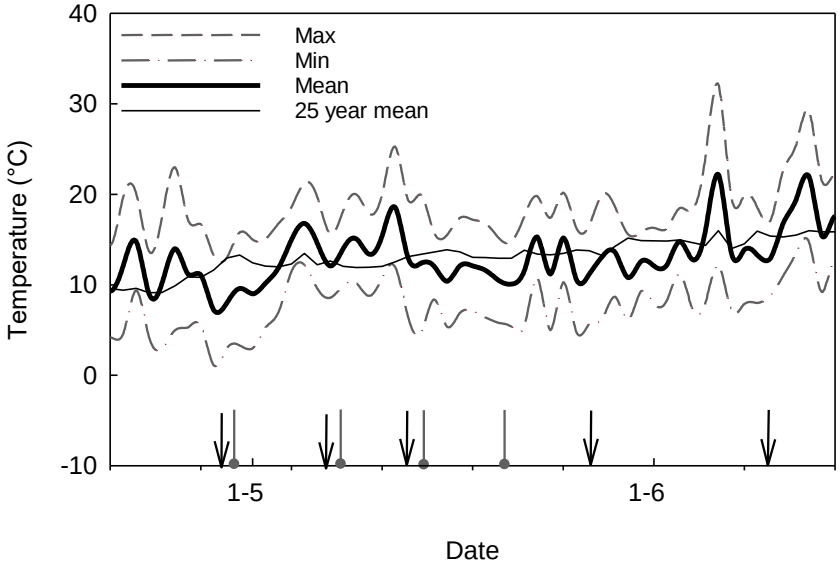


Figure 8.1: Maximum, minimum and mean daily air temperature during the study period. The thin solid line shows the 25-year mean. Arrows indicate the leaf sampling dates; vertical lines indicate days when chlorophyll fluorescence was measured.

Growth characteristics of the five genotypes in 2015

In general, the early-season growth characteristics of the five genotypes determined in 2014 (see Materials and Methods section) 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. OPM06 also emerged about two 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 (Fig. 8.2). OPM66 reached a length of 50 cm on 17/04, while OPM06 and OPM09 reached this length on 3/05 and OPM35 and OPM51 on 5/05. By the beginning of June, *M. x giganteus* OPM09 had overtaken the other genotypes in height and remained the tallest genotype throughout the rest of the growing season. At harvest in January 2016, the highest yielding genotype was OPM09 with 4.1 kg DM plant^{-1}, followed by OPM35 and OPM51 with 1.3 kg DM plant^{-1} and by OPM06 and OPM66 with 1.0 and 0.8 kg DM plant^{-1} respectively.

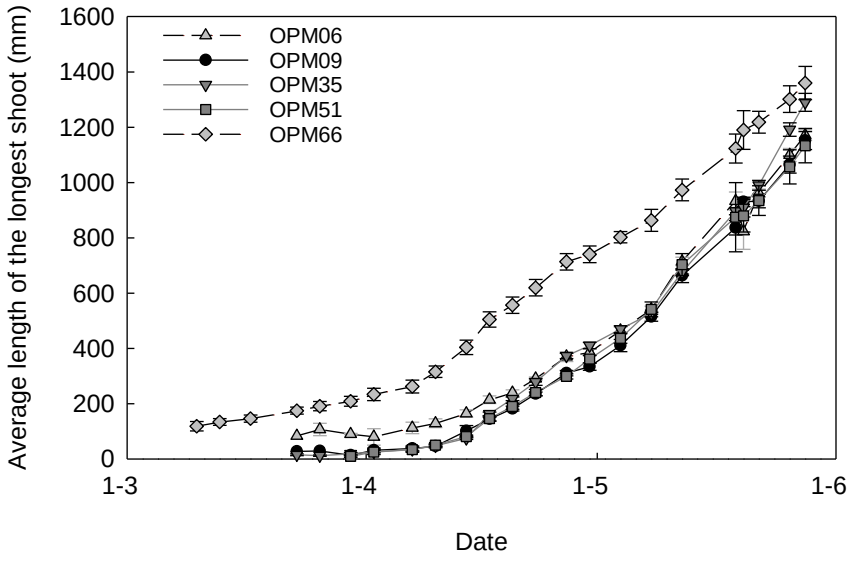


Figure 8.2: Evolution of the length of the longest shoot per genotype in spring 2015. Symbols show mean value per genotype and sampling date; bars represent standard error (n=6).

Traits related to photosynthetic activity

The quantum efficiency of photosystem II (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} PAR on four dates (Supplementary Fig. 8.1, 8.2). 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. 8.3), and considering temperature and light intensity (Supplementary Fig. 8.1, 8.2), 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 the 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.

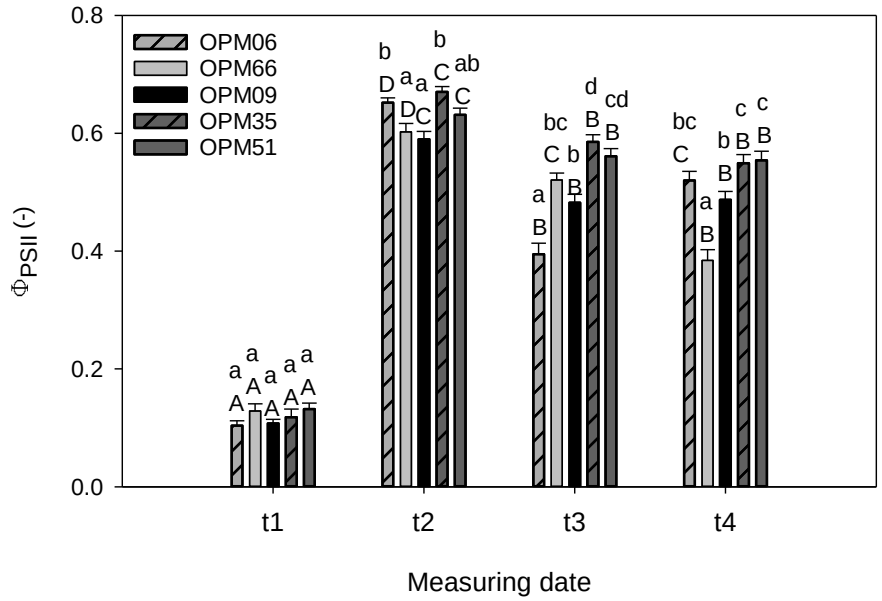


Figure 8.3: Bar plots of mean Φ_{PSII} per genotype and measuring date. Error bars show standard errors. Different patterns of the bars indicate different genotypes. Uppercase letters refer to homogenous groups of sampling dates for a specific genotype, lowercase letters refer to homogenous groups of genotypes for a specific sampling date ($p < 0.05$).

Overall, the chlorophyll content was lower on T1 than at other sampling dates (Fig. 8.4A). 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. 8.4B). With some exceptions, inter-genotype differences were not significant at 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. 8.4B).

The in vitro activity of photosynthetic enzymes, such as PPDK, is indicative of the in vivo carbon assimilation rate (Usuda et al. 1984). PPDK activity was not significantly higher on T1 than on subsequent dates for any of the genotypes (Fig. 8.5). 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). OPM51 had the highest PPDK activity on all sampling dates, indicating the highest photosynthetic activity for this genotype.

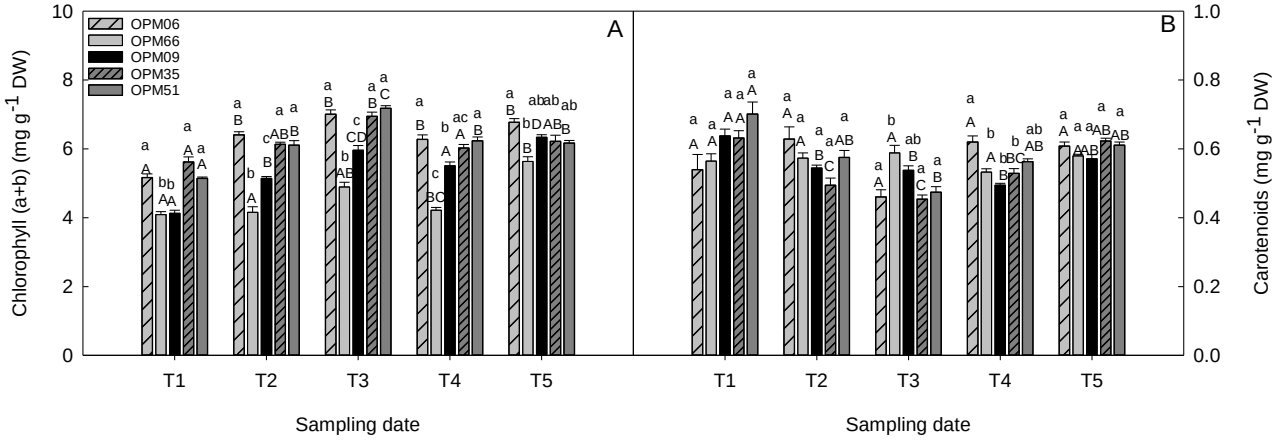


Figure 8.4: A: Content of chlorophyll a and b in the leaves (mg chlorophyll g-1 DW), B: Leaf carotenoid content (mg chlorophyll g^{-1} DW), sampled on T1, T2, T3, T4 and T5. Each bar depicts the mean value per genotype and sampling point. Error bars show standard errors (n=6). Different patterns of the bars indicate different genotypes. Uppercase letters refer to homogenous groups of sampling dates for a specific genotype, lowercase letters refer to homogenous groups of genotypes for a specific sampling date ($p < 0.05$).

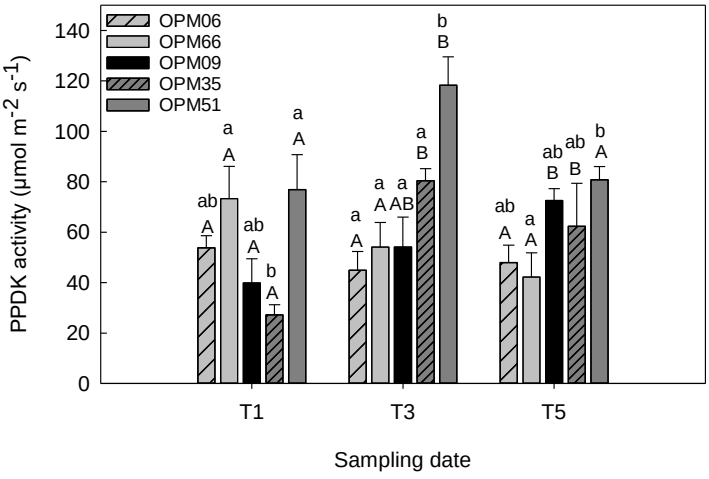


Figure 8.5: PPDK activity (µmol m^{-2} s^{-1}) per genotype and sampling date in the leaves sampled on T1, T3 and T5. Each bar depicts the mean value per genotype and sampling point. Error bars show standard errors (n=6). Different patterns of the bars indicate different genotypes. Uppercase letters refer to homogenous groups of sampling dates for a specific genotype, lowercase letters refer to homogenous groups of genotypes for a specific sampling date ($p < 0.05$).

Traits related to redox homeostasis

Redox homeostasis (MDA, ascorbate and glutathione contents, and catalase activity) was studied for three time points (T1, T3, 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:

- MDA content did not vary over time and was consistently higher in OPM06, OPM66 and OPM09 (Fig. 8.6A). In OPM09 it was significantly lower at T1 compared to T3, and in OPM35 and OPM51 MDA was significantly lower at T3 compared to T5. Inter-genotype differences for MDA became less pronounced at T5.
- OPM06 and OPM66 had higher catalase activity than OPM35 and OPM51 at T1 and T3 (Fig. 8.6B). 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.
- 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. 8.6C)
- Glutathione content was significantly higher at T1 compared to T3 and T5 in all genotypes (Fig. 8.6D). 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, redox homeostasis appeared to be different between the group OPM06, OPM66 and OPM09 versus 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 (for example, reduction of light capture). Correspondingly, the catalase activity was also lower in OPM35 and OPM51 than in OPM06 and OPM66 at T1 and T3. In contrast, OPM35 and OPM51 displayed less 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.

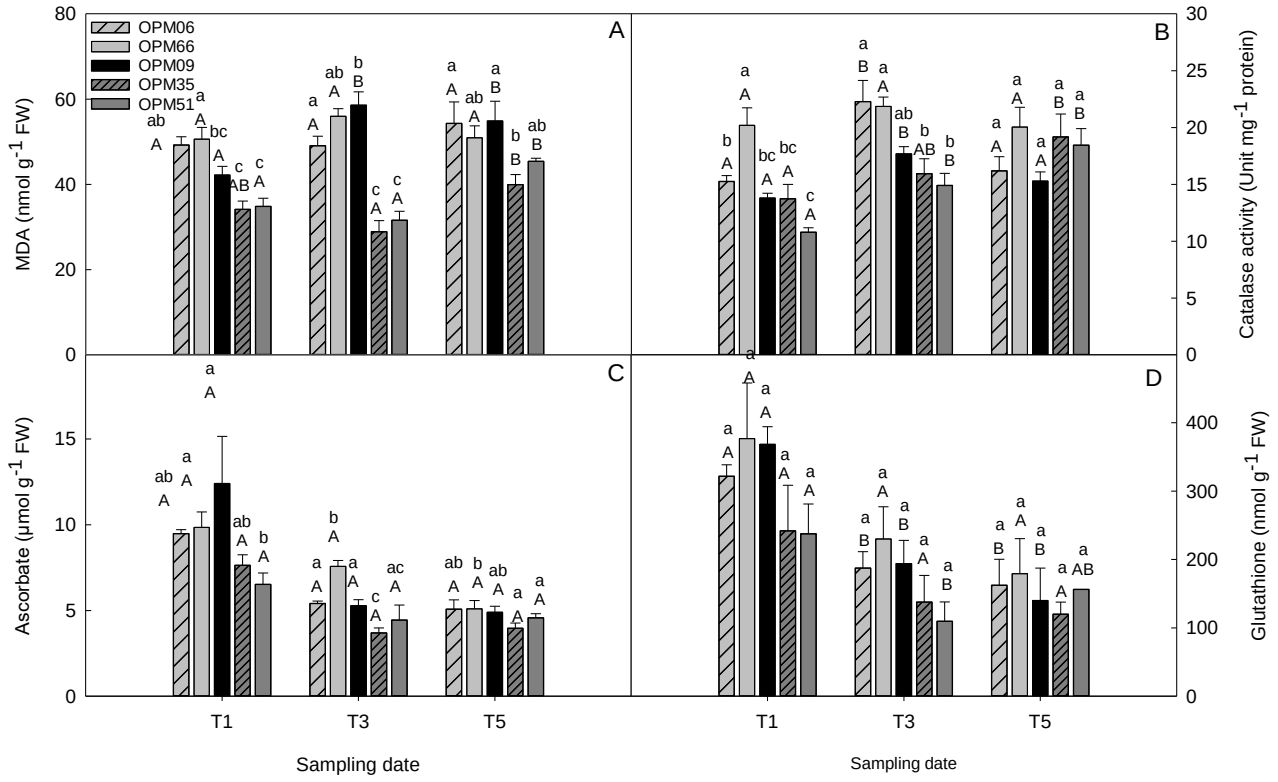


Figure 8.6: Contents of malondialdehyde and antioxidants in the leaves of the five miscanthus genotypes sampled. A: Malondialdehyde content; B: Catalase activity; C: Ascorbate content; D: Glutathione content. Each bar depicts the mean value per genotype and sampling point. Error bars show standard errors (n=6 for MDA, catalase activity, ascorbate; n=4 for glutathione). Different patterns of the bars indicate different genotypes. Uppercase letters refer to homogenous groups of sampling dates for a specific genotype, lowercase letters refer to homogenous groups of genotypes for a specific sampling date ($p < 0.05$).

Carbohydrate concentrations

Different water soluble carbohydrates (WSC) profiles were observed in the five genotypes (Fig. 8.7A-F). OPM35 and OPM51 were characterized by significantly higher levels of glucose and fructose compared to OPM06 and OPM66 throughout the measuring period (Fig. 8.7A, B). Interestingly, *M. x 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. 8.7C). 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. 8.7D), 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 to the other sampling days, after which the concentration of maltose decreased strongly in all genotypes except in OPM66 (Fig. 8.7E).

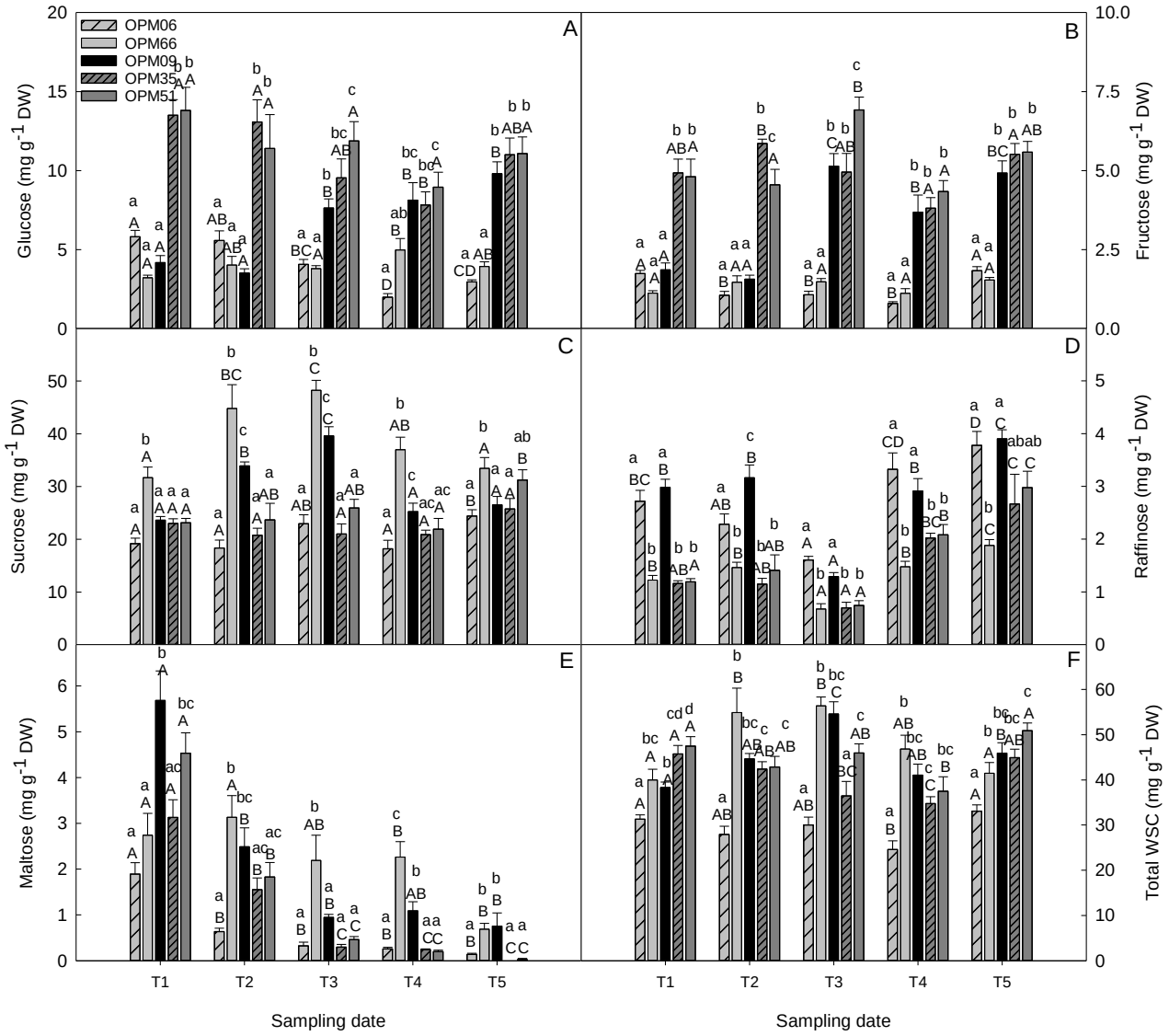


Figure 8.7: Content of water soluble carbohydrates in the leaves of the five miscanthus genotypes. A: glucose content; B: fructose content; C: sucrose content; D: raffinose content; E: maltose content and F: total water soluble carbohydrate (TWC) content. Symbols show mean values per genotype and sampling date, error bars show standard error (n=6). Different patterns of the bars indicate different genotypes. Uppercase letters refer to homogenous groups of sampling dates for a specific genotype, lowercase letters refer to homogenous groups of genotypes for a specific sampling date ($p < 0.05$).

The ratio of glucose to sucrose was lower than 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 Fig. 8.3), indicating 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 indicates a metabolic change in this genotype as temperature increased during the season. While the relative proportions of WSC 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 to 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.

Overall inter-genotype and sampling date patterns for biochemical traits

Correlations between biochemical components and similarities between genotypes in metabolic response were analyzed using principal component analysis. The first component, which explained 29.5% of the variation in the dataset was positively correlated with ascorbate, glutathione, MDA and carotenoid contents and negatively correlated with PPDK activity and glucose, fructose and chlorophyll content (Fig. 9A, B). The second component, which explained 19.4% of the variation, was positively correlated with glucose, fructose, maltose, carotenoids, total WSC, 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 to T3 and T5, which were similar (Fig. 8.8A). 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. 8.8B). This indicates that OPM35 and OPM51 were mainly characterized by higher concentrations of glucose, fructose, and total WSC and higher PPDK activity, while OPM06 and OPM66 were characterized by high levels of MDA, raffinose, sucrose and high catalase activity. *M. x 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 versus OPM35 and OPM51 also stood out when PCAs were calculated per sampling day (Supplementary Fig. 8.4-6).

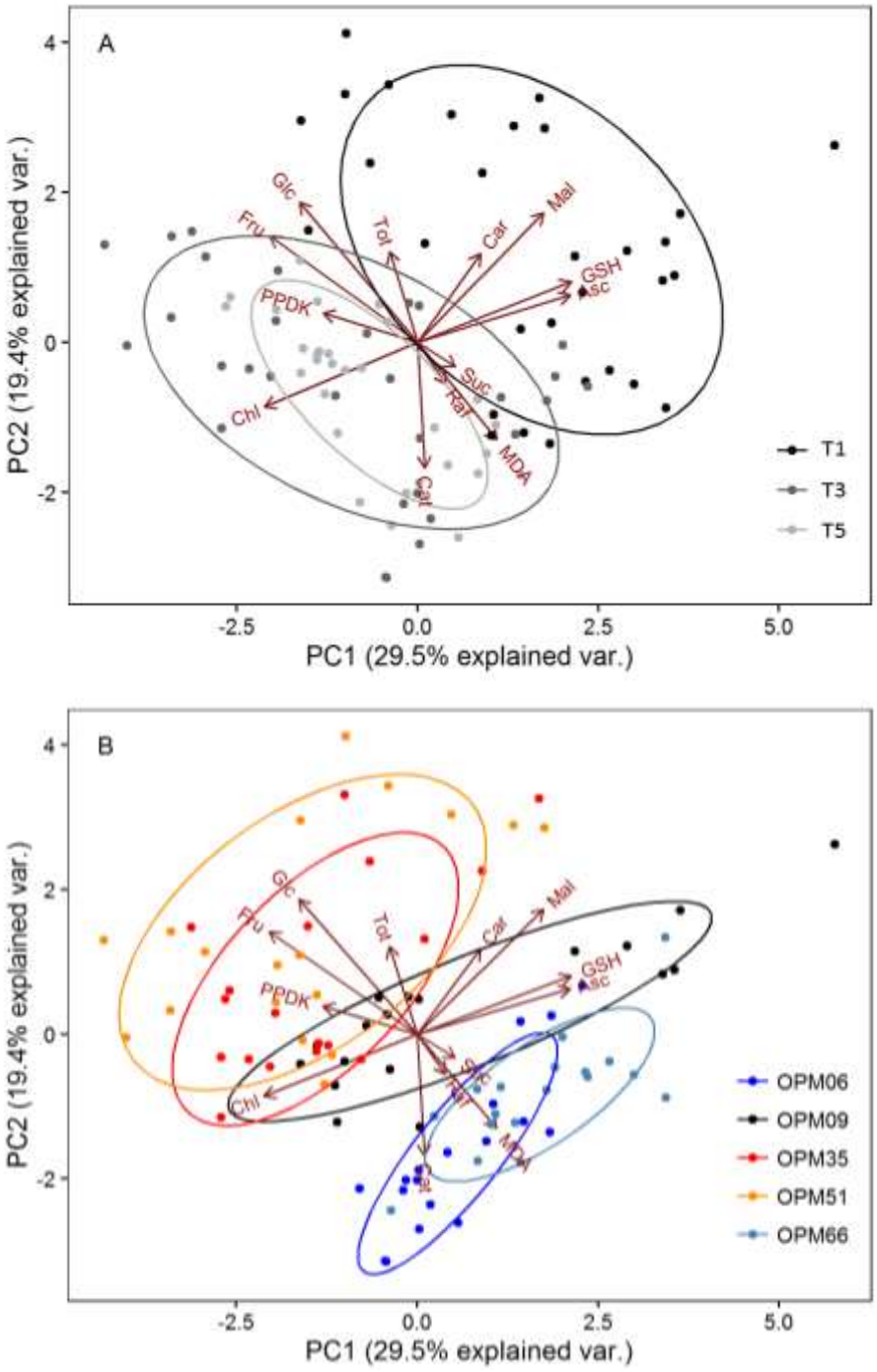


Figure 8.8: Principal component analysis of the biochemical parameters at T1, T3 and T5. Dots show values of the first and second component for individual plants. Different colors indicate different sampling dates (A) or genotypes (B). Ellipses show confidence intervals per sampling date and genotype.

Discussion

To our knowledge, this is the first study in which a whole battery of physiological and biochemical traits related to chilling tolerance was analyzed in a common set of field-grown miscanthus plants. In contrast to most studies available where young 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. Alternatively, the difference between T1, T3 and T5 could be a result of the difference in plant age, to counteract this possible effect sampling was always done on the youngest fully matured leaf. Because no influence 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 to 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 were evaluated in five miscanthus genotypes. Temperatures shortly before sampling dates T1 and T2 were as low as 1.7°C and 7.8°C, respectively, with maximums below or around 15°C. We can thus assume that at 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; Farage et al., 2006; Głowacka et al., 2014a; Naidu and Long, 2004; Purdy et al., 2013; Wang et al., 2008a). 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, redox homeostasis and WSC content. OPM35 and OPM51 seem 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 OPM06 was more similar to OPM35 and OPM51 than to OPM66 for shoot growth rate and chlorophyll content. *M. x 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. 2014a) 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. 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 set of genotypes should be investigated before any definitive conclusions are drawn.

What are the differences at photosynthesis level?

The PPDK activity values recorded in this study were of the same order of magnitude as reported in Wang et al. (2008) and Friesen and Sage (2015). However, while Wang et al. (2008) found higher PPDK activity values in cold-grown than in warm-grown *M. x giganteus* in growth chamber experiments, in 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 larger 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 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 is in agreement with the observation that, although *M. x giganteus* has a high photosynthetic capacity compared to most other miscanthus accessions, even higher CO_2 assimilation rates are possible (Głowacka et al. 2014a, 2015b). 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 RuBisCo and other photosynthetic enzymes. Similarly, we did not observe any permanent damage in OPM35 or OPM51 plants but rather observed a lower PPDK activity on T1 in these two genotypes (note that the ‘stress score’ values shown in Table 8.1 refer to damage by frost temperatures in 2014). Once the temperature increased, these genotypes were capable of high photosynthetic rates.

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, in chapter five we observed lower chlorophyll contents in the two investigated miscanthus genotypes when grown at chilling 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 are lower and carotenoid concentrations increase with chilling stress in *M. x 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. x giganteus* OPM09. This was also the case for OPM35 and OPM51, but not for OPM06 and OP66. In addition, under chilling stress, *M. x 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 (Spence et al., 2014; Wang et al., 2008b). Carotenoids, such as beta-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 contents 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.

Is redox homeostasis an indicator 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 of 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), this 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 compared to 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 indicate that MDA and antioxidants 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 defenses 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. x giganteus* and the chilling tolerant genotypes, indicating that the chilling sensitive genotypes either actually suffer relatively less oxidative stress or invest less in protection against oxidative stress. The latter is in agreement with the observation of higher levels of antioxidants in chilling tolerant maize genotypes than in sensitive ones (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 lower than 0.4. OPM09 was similar to OPM06 and OPM66 at the beginning of the season and similar to OPM35 and OPM51 later on. OPM09 had also high raffinose contents throughout the measurement period, in agreement with de Souza et al. (2013), who observed high levels of raffinose throughout the growing season in *M. x giganteus*, indicating that this is a characteristic of this genotype.

According to available literature, maltose and raffinose are the sugars induced most highly 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) and play a role as stress signaling molecules (Tarkowski and Van den Ende, 2015; Van den Ende and El-Esawe, 2014). In our study, raffinose concentrations were the lowest in all genotypes at T3, the warmest sampling point, but raffinose concentration increased again on T4 and T5, resulting in no clearly observed relationship between raffinose and temperature. In contrast, maltose concentrations were inversely related to temperature, with a clear tendency to decrease throughout the study period.

OPM06 and OPM09 had higher concentrations of raffinose than other genotypes throughout the sampling period. This agrees with knowledge available in rice (*Oriza 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). Raffinose plays a role in the stabilization of membranes, the protection of photosystem II and as an antioxidant (Nishizawa et al. 2008; Janská et al. 2010; Knaupp et al. 2011). Correspondingly, when grown at 12°C, *M. x giganteus* was found to accumulate raffinose, while at 20°C it did not (chapter five). However, raffinose concentration was also high at T4 and T5 in OPM09. Furthermore, raffinose concentrations were not significantly higher in OPM66 at colder sampling dates, indicating other responses in this genotype. This was also the case for maltose concentrations: in all genotypes except OPM66, maltose concentrations were the highest at T1. Like raffinose, maltose has been shown to protect the photosynthetic electron transport chain (Kaplan and Guy 2005), and the higher concentrations of maltose on T1 could be a protective measure against chilling stress. We can thus conclude 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.

Implications for breeding?

The insights gained in this study show 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. It seems that the two chilling sensitive genotypes investigated here had higher photosynthesis, less oxidative stress responses and higher monosaccharide concentrations. This is also evident in the PCA analysis, where chilling sensitive and tolerant genotypes clustered separately. Interestingly, OPM09 seemed to have characteristics of both tolerant and sensitive genotypes. While in the beginning of the growing season this genotype was most similar to the chilling tolerant genotypes, after the warm weather around 12/05/2015, it was more similar to the chilling sensitive genotypes, with higher fructose and glucose contents and higher PPDK activity. *M. x giganteus* OPM09 seems to be well-adapted to low temperatures at the beginning of the growing season and shows good growth capacity once temperatures raise. The high productivity reported for *M. x 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 WSC was also fast and relatively easy to perform. Recently, Purdy et al. (2015) studied the applicability of WSC as marker trait for the detection of high yielding genotypes in breeding programs. 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 strongly during the growing season. Sampling at midsummer, as done by Purdy et al. (2015) is most likely 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 in other studies this ratio could be a good marker trait to select chilling tolerant genotypes.

Conclusions

Chilling tolerant and sensitive miscanthus genotypes can be distinguished by a number of photosynthesis related biochemical traits. There appears to be a trade-off between high and efficient photosynthesis and chilling stress tolerance. *M. x giganteus* seems to be able to overcome this trade-off, and while it is more similar to the chilling sensitive genotypes in early spring, its carbohydrate composition is similar to the chilling tolerant genotypes later on. It thus appears to be possible to combine both chilling tolerance and strong growth. Water soluble carbohydrates appear to be suitable for screening a larger population for chilling tolerance.

Topic III:
Relationship between early-season growth and final biomass yield

Introduction to topic III

Biomass yield is one of the main breeding goals in miscanthus (Clifton-Brown et al., 2016; Lewandowski et al., 2016). Compared to traditional crops, miscanthus breeding is still in an early phase and there is little knowledge available about the contribution of specific plant traits to total biomass yield. In this sense, it is difficult to define breeding targets. Several studies (and results presented in previous chapters) have identified traits regarding plant morphology, such as number of stems, stem diameter, tuft diameter, and plant or canopy height as important contributors to biomass yield in miscanthus (Jeżowski, 2008; Kaiser et al., 2015; Robson et al., 2013b; Zub et al., 2011). Other studies have highlighted the possible importance of optimizing the duration of the growing period in order to optimize biomass yield (Robson et al., 2013a; Sage et al., 2015; Song et al., 2015). In previous chapters of this thesis we focused on the variation available for early-season growth and tolerance to chilling temperatures, assuming that these characteristics could potentially contribute to higher biomass yields in miscanthus. In this part we investigate the variation for biomass yield in a broad collection of miscanthus genotypes over several seasons and estimate the relative importance of early-season growth as determinant of final biomass yield. To get a more complete picture of growth patterns, we also determined traits that describe miscanthus growth throughout the growing season, from emergence to senescence. Plant morphology and phenology over the whole growing season is interpreted in combination with data on early-season growth presented in previous chapters.

We make use of two of the field trials introduced in previous chapters to study the variation in biomass yield in miscanthus and its relationship with the growing period. In FT1, which was planted to study genotype behaviour in an environment similar to a commercial plantation, observations of early-season growth, flowering and senescence were used to describe the growing period. This trial was harvested after the dry matter content of the shoots of the reference *M. x giganteus* genotype OPM09 surpassed 80%, similarly to what is done in commercial plantations. This point was reached in 2014 and 2015 in late March. In FT2, which was established with the specific aim of studying cold tolerance and early-season growth in high detail on a spaced plant basis as described in chapter seven shoot length measurements were continued during the whole growing season. FT2 was harvested in early January, in order to facilitate observations of emergence and early growth.

As these two trials had different designs and were harvested at different moments, marked differences in biomass yield were observed when the common set of genotypes was considered. Note however, the strong correlation observed in between years comparisons (Table III.1). Several factors might have contributed to these between-trial differences. First, the March harvest in FT1 might have had a negative impact on total biomass yield (compared to values in FT2), and have eliminated the growth advantage of early emerging genotypes. Indeed, by late March, the moment at which FT1 was harvested, the early emerging genotypes had already reached shoot heights over 50 cm (observations in FT2). This was reflected in lower average biomass yields in FT1 than in FT2, and lower inter-genotype differences. Second, the early harvest in FT2 might have influenced the microclimate in the trial. With the removal of the aboveground biomass at harvest, the shading effect of the stems produced the previous year was removed. In addition, as the fallen leaves were removed in FT2 to facilitate observation of emerging shoots, the soil may have warmed earlier in FT2, leading to earlier emergence and a longer effective growing period. Third, in FT1 leaves had mostly dropped by harvest, while in FT2 most genotypes the standing biomass contained still a high proportion of leaves at harvest. The harvest in FT1 was thus most similar to the standard practice of winter harvest and the harvest in FT2 was more akin to an autumn harvest. Autumn harvest yields are generally higher than winter harvests by 30 to 50% (Lewandowski et al., 2000). Fourth, differences with regards competition might also have impacted yield. While in FT1 the plants used for measurements were surrounded by plants of the same genotype, in FT2 each plant was surrounded by other genotypes. As a consequence, in FT1 individual plants experienced a more homogenous competition than in FT2.

Because of the differences in design and management between both trials and because of the differences regarding biomass yield, the results of these trials are discussed in separate chapters. Chapter nine focusses on FT2, investigating the relationship between the early-season growth parameters investigated in chapter seven, complemented by parameters that describe the whole season shoot growth, and biomass yield. When available, and to get a more complete overview, flowering and senescence traits measured in FT1 were also included in the analysis of genotype performance in Chapter ten. This was done because, as these are highly heritable traits (Slavov et al., 2013) they are likely very similar between both trials. Chapter ten focusses on the relationship between biomass yield and phenology, assessed on a plot level in FT1.

Table III.1: Correlations between biomass yield in 2014 and 2015 for all plants planted in May 2013. (All correlations are significant, except those marked with ^{NS}, Pearson's product-moment correlation, $p<0.05$). Genotype indicates correlations between adjusted means values per genotype (n = 71 in FT1 and 85 in FT2), plant indicates correlations between individual plants (n = 383 in FT1 and 372 in FT2) and plot indicates correlations between individual plots in FT1 (n = 196).

	FT1			FT2	
	Genotype	Plant	Plot	Genotype	Plant
All genotypes	0.60	0.55	0.55	0.95	0.89
M. sinensis	0.51	0.70	0.72	0.93	0.86
M. sacchariflorus	0.68	0.15^{NS}	0.20		
Hybrid	0.65	0.55	0.57	0.96	0.92
M. x giganteus	0.63	0.57	0.64	0.91	0.84

Chapter 9: Relationship between plant growth characteristics and final biomass yield

Introduction

Early emergence and long canopy duration (referring here to the duration of growth from shoot emergence to senescence within a growing season) are considered important characteristics for high yield in miscanthus (Sage et al., 2015; Song et al., 2015). Theoretically early canopy formation mediated by early emergence, would extend the canopy duration, allowing the plant to intercept more solar radiation and thus produce more biomass. This idea was supported by results of Farrell et al. (2006) and Davey et al. (2015), who concluded, using modeling approaches and assuming that all other growth parameters remained unchanged, that genotypes with earlier canopy formation and lower base temperature for growth would be higher yielding than *M. x giganteus*. However, it is possible that adaptation for early emergence and vigorous early-season growth are inversely related with the ability for fast growth and high photosynthesis rates at higher temperatures later in the season, when the potential gain in intercepted radiation is larger (chapter seven, Zub et al., 2011). Evidence for this other view was provided by Farrell et al. (2006) and Clifton-Brown and Jones (1997), who reported that the genotypes with the highest growth rates at low temperature did not have the highest growth rates at higher temperature, when evaluated under controlled conditions. This indicates that across a broad miscanthus germplasm collection, early-season growth effects might become of low relevance for biomass yield, compared to the capacity to accumulate biomass later in the growing season. Under these circumstances, extension of the canopy duration through a delayed heading date and senescence might be a more appropriate approach to attain a high biomass yield.

The results of the two field trials published so far have not been conclusive regarding the growth traits contributing to high biomass production in miscanthus either. While Robson et al. (2013a) concluded from a field trial of 244 genotypes in Wales that early canopy formation, late senescence and a long canopy duration are associated with high yields, Zub et al. (2012b) reported from a trial of 21 genotypes in northern France that late emergence, rapid growth and short canopy duration are the most important characteristics of high yielding genotypes. Evaluation in different environments and the use of a different set of genotypes might lay at the basis of these contradicting outcomes. To the extent of our knowledge, no other studies regarding the relationship between growing season traits and biomass yield have been published. There is thus a clear need for more research to determine which are the ideal characteristics of high yielding miscanthus genotypes. Our study adds to the knowledge available by reporting on an extra field trial in another location, by directly studying biomass yield, instead of using height as a proxy as in Zub et al. (2012b) and by studying the different

stages in the growing season in greater detail than in Robson et al (2013), who only quantified canopy formation and canopy senescence.

However, yield is a complex trait determined not only by the aspects discussed above, but also by morphological traits (Robson et al. 2013b). Specific morphological characteristics such as number of stems, stem height, stem diameter or tuft diameter (a product of number of stems and stem diameter) impact biomass yield, as a high biomass can be attained either by a high number of shoots of short stature or a relatively lower number of longer shoots. It is therefore essential to consider also these aspects when investigating the most relevant traits affecting biomass yield in miscanthus.

Therefore, in this chapter we investigate the relationship between biomass yield on the one side and early-season growth, morphological traits, heading date and senescence, on the other side using a broad miscanthus collection. Specific aims of the work presented in this chapter were (i) to estimate the variation available in this collection for biomass yield, (ii) to estimate the variation available for growth and phenological traits, and (iii) to determine how early-season growth and/or traits related to plant morphology, flowering and senescence relate to final biomass yield in miscanthus.

Materials and methods

Field trial and plant measurements

For a complete description of the design of FT2 and the results for early-season growth traits we refer to chapters three and seven. As the *M. sacchariflorus* genotypes were removed in May 2014, they were not included in the analyses of yield, rendering a total of 85 genotypes (61 *M. sinensis, 14 M. sinensis x sacchariflorus* and 10 *M. x giganteus*) for this investigation. For each single plant, the number of shoots, the length of the longest shoot and the number of leaves on that shoot were recorded twice weekly from 18/03/2014 until 27/05/2014 and from 18/02/2015 to 28/05/2015 as described in chapter seven. Thereafter the length of the longest shoot was measured from soil level to leaf tip once per month until the end of November.

The biomass harvest was performed in early January in 2015 and 2016. Individual plants were first bound together with rope and labelled. Each plant was then cut using a hedge trimmer and weighed. Thereafter a subsample of about 300 g fresh weight was chopped in a forage maize chopper and weighed, dried in an air-ventilated oven at 70°C for at least 72 h and weighed again to determine dry matter content (DM%) and plant dry weight (Yield). The diverse set of traits describing biomass yield, growth and morphology used in this study is presented in Table 9.1.

Absolute growth rate in spring (AGR), the day of the year or the accumulated thermal time until a length of 30 or 50 cm was reached (L30 and L50), leaf formation rate (LFR) and the day of the year

or the accumulated thermal time until the fourth leaf became visible (Leaf4), the day of the year or the accumulated thermal time until 50% of the maximum number of shoots was formed (S50) and the maximum shoot number (N_{max}) were calculated as described in chapter seven.

Growth traits estimated using shoot length data over the whole season such as L_m, t_e, t_m, MGR, $t_{10\%}$, $t_{50\%}$, $t_{90\%}$ and GD were calculated using the LEAF-E Excel macro (Voorend et al. 2014). This macro was used to automatically fit beta sigmoid growth curves and the first derivative thereof (Yin et al., 2002) to the shoot length data. The duration of shoot growth (GD) was calculated as the time between 10% shoot length and te.

Heading date (HD) and senescence (50%Sen) were not determined in FT2. They were however determined on FT1 as described in chapters four and ten. Since these traits are highly genotype dependent and the trials are located in the same field, the HD and 50%Sen were used for the genotypes that were present in both trials.

All traits were calculated in function of calendar days (DOY) or thermal time (GDD), while the data in function of thermal time was used for further analysis, data in DOY are included for easier reference.

Competition indices

FT2 contained a large number of genotypes of differing morphologies and growth potential. As each of the six blocks was completely randomized, each individual plant of a given genotype was surrounded by a different set of plants from different genotypes and experienced different kinds and levels of competition. Therefore, here we estimated several of the competition indices proposed by Zub et al. (2012a) to reduce statistical variability and improve comparison of genotypes.

Competition indices were calculated for each individual plant by the summation of the number of plants (CN), maximum length per plant (CL), maximum number of shoots per plant (CS) or the biomass yield (CY) of the eight plants that surrounded it (eq. 9.1). For plants located at the border of the trial the average values for the genotype used as border (OPM50) were used where necessary in the calculations. Competition indices were calculated using the following formula:

$$C = \sum_{i=1}^{8} x_i \qquad \text{(eq. 9.1)}$$

where C is the competition index and x_i is the basis on which the competition index was calculated. The parameter x_i was either the number of plants surrounding the plant at position *i* (CN; $x_i = 0$ if no neighboring plant or $x_i = 1$ if neighboring plant present), the maximum length (Lm) of each surrounding plant *i* (CL), the maximum number of shoots Nmax of each surrounding plant *i* (CS) or the biomass yield of each surrounding plant *i* (CY).

Table 9.1: Description of traits used in this chapter. 'Early-season' refers to trait values derived from measurements carried out until 27/05/2014 or 28/05/2015. 'Whole season' refers to trait values derived from shoot length measurements taken over the whole season.

	Trait	Unit	Description
	Yield	Kg DM plant^{-1}	Final biomass yield
	DM%	% DM in biomass	Dry matter content at harvest
Early-season	AGR	mm DOY^{-}1/mm GDD^{-1}	Absolute shoot growth rate in spring
	L30	DOY/GDD	Time until a shoot length of 30 cm was reached
	L50	DOY/GDD	Time until a shoot length of 50 cm was reached
	LFR	Leaves DOY/ Leaves GDD^{-1}	Leaf formation rate
	Leaf4	DOY/GDD	Time until fourth leaf appeared on longest shoot
	N_{max}	Number of shoots	Maximum shoot number
	S50	DOY/GDD	Time until 50% of maximum number of shoots was formed
Whole season	t_e	DOY/GDD	Time until end of shoot growth
	t_m	DOY/GDD	Time until maximum shoot growth rate achieved
	MGR	mm DOY^{-}1/mm GDD^{-1}	Maximum shoot growth rate
	$t_{10\%}$	DOY/GDD	Time until 10% of shoot length reached
	$t_{50\%}$	DOY/GDD	Time until 50% of shoot length reached
	$t_{90\%}$	DOY/GDD	Time until 90% of shoot length reached
	L_m	mm	Maximum shoot length
	GD	DOY/GDD	Canopy duration
Phenology	HD	DOY/GDD	Heading date
	50%Sen	DOY/GDD	Day of the year less than 50% of the plant was green
Competition indices	CN	Number of plants	Competition index based on number of surrounding plants
	CL	mm	Competition index based on length of surrounding plants
	CS	Number of shoots	Competition index based on number of shoots of surrounding plants
	CY	Kg DM	Competition index based on yield of surrounding plants

Statistical analysis

All statistical analyses were performed using R version 3.1.0 (R core team, Vienna, Austria) or Statistica 12.0 (Statsoft, Tulsa, USA). The yields of both years were compared using ANOVA using the 'glm' and 'aov' functions of the *stats* package in R. The effect of competition on biomass yield was examined by analysis of variance using the 'glm' and 'aov' functions of the *stats* package. The following model was used to evaluate the effect of competition for each of the four indices of competition.

$$Yield = \mu + G_i + C_j + B_k + e_{ijk} \quad \text{(eq.9.2)}$$

Where G_i is the effect of genotype *i,* C_j the effect of competition index *j,* B_k the effect of block *k,* and e_{ijk} the first residual term.

All traits were analyzed per year separately using the following mixed model:

$$Y_{ijkl} = \mu + S_i + G_j + R_k + e_{ijkl} \quad \text{(eq.9.3)}$$

Where Y is the effect of one of the whole season traits described in Table 9.1, μ is the overall mean, S is the effect of the species group *i*, G the effect of the genotype *j*, nested in the species group, R is the random block effect *k* and e the first residual term. Genotype and block were considered random effects. The model was used to calculate adjusted means values for the traits per genotype and per year. These adjusted means were used to calculate correlations and in the principal component analysis.

Correlations were calculated using the cor.test function in R, using Pearson's product moment correlation coefficient.

Broad sense heritability (H^2) was calculated according to the following formula, based on Holland et al. (2003) and Barre et al. (2015):

$$H^2 = \frac{\sigma^2_G}{\sigma^2_G + \frac{\sigma^2_{GY}}{2} + \frac{\sigma^2_{GB}}{6} + \frac{\sigma^2_R}{12}} \quad \text{(eq. 9.4)}$$

Where σ^2_G is the variance due to genotype, σ^2_{GY} is the variance due to genotype by year effects, σ^2_{GB} is the variance due to genotype x block effects and σ^2_R is the residual variance, two is the number of years, 6 is the number of repetitions and 12 is the number oy years multiplied by the number of replications. Variances were calculated using the following mixed model using the 'lmer' function from the *lme4* package in R.

$$Y_{ijkl} = \mu + G_i + J_j + B_k + G_i \times J_j + G_i \times B_k + e_{ijkl} \quad \text{(eq. 9.5)}$$

Where Y is the effect of one of the early-season growth traits described above, μ is the overall mean, G the effect of the genotype *i*, J is the effect of year *j*, B is the random block effect *k* and e the first residual term.

Principal component analysis was performed using the 'PCA' function from the *FactoMineR* package. Analysis was based on the adjusted means per genotype in 2014 and 2015. All variables were scaled to unit variance. Missing data were imputed using the 'imputePCA' function from the *missMDA* package.

Results

Competition indices

The extent of morphological variation in the germplasm was considerable. Some genotypes such as the giant *M. x giganteus* reached lengths over 3 m, while dwarf genotypes such as OPM47 reached only a height of 1.3 m. Due to this large variation in genotype morphology, in combination with the differences in planting dates (plants planted in May 2013 outcompeted plants planted in August and

September 2013) and the removal of the spreading *M. sacchariflorus* genotypes in May 2014 (see chapter seven), the trial became rather heterogeneous after 2014. To test for a possible effect on yield caused by competition by neighboring plants, we estimated four competition indices based on Zub et al. (2012a) as indicated in Table 9.1. There was a significant effect on yield of all competition indices except for CN in 2014 (Table 9.2). The competition index based on the yield of the surrounding plants (CY) explained the largest percentage of variation in the data. Adding competition to the model improved it substantially, explaining up to 19.9% of the residuals in the 2015 dataset. The competition index based on the total biomass yield of the surrounding plants had the strongest impact on yield and was, on a plant basis, significantly correlated with lower biomass yields (Table 9.3).

Table 9.2: Analysis of variance of the effects of competition on biomass yield. Model 1: model without competition. CN: effect of the inclusion of CN in the model, CL: effect of the inclusion of CL in the model, CS: effect of the inclusion of CS in the model, CY: effect of the inclusion of CY in the model. RMSE: residual mean square error, %RMSE: percentage reduction in RMSE by inclusion of the competition index. AIC: Akaike information criterion.

		P	AIC	R^2	RMSE	%RMSE
2014	No competition		905	0.652	149.6	
	CN	0.172	889	0.657	144.9	3.1
	CL	0.031	885	0.660	143.6	4.0
	CS	>0.001	875	0.669	139.6	6.7
	CY	>0.001	860	0.691	132.4	11.5
2015	No competition		866	0.711	140.2	
	CN	>0.001	838	0.734	129.3	7.8
	CL	>0.001	815	0.749	121.7	13.2
	CS	>0.001	811	0.753	120.1	14.3
	CY	>0.001	785	0.768	112.3	19.9

Table 9.3: Correlation between the competition index based on the biomass yield of the surrounding plants (CY) and biomass yield. All correlations are significant (Pearson's product-moment correlation, $p<0.05$, $n_{\text{All genotypes}} = 399$, $n_{M.\ sinensis} = 284$, $n_{M.\ x\ giganteus} = 42$, $n_{\text{Hybrid}} = 73$).

All genotypes		*M. sinensis*		Hybrid		*M. x giganteus*	
2014	2015	2014	2015	2014	2015	2014	2015
-0.28	-0.35	-0.27	-0.37	-0.31	-0.32	-0.33	-0.45

Competition by neighboring plants thus had a significant impact on biomass yield. Ideally, the yield results should be corrected for competition effects. However, it is difficult to appropriately correct for competition due to a number of reasons. First, it is likely that different genotypes have different competition sensitivities, with the same competition level having different effects on different genotypes. Second, as miscanthus is a perennial crop, there are likely effects of competition from the previous year that also affect yield. It is therefore possible that high competition in a given year will affect performance in the following year, further complicating interpretation. Third, each of the

traits listed in Table 9.1 will probably be influenced differently by competition. For example, early-season growth traits are perhaps less sensitive to competition, as there is little shading early in the season. We therefore decided not to correct the data for competition effects and assumed that, since the trial was completely randomized with six replications per genotype, the average competition experienced by all genotypes was similar.

Growth traits

Early-season growth was described extensively in chapter seven. The extra monthly measurements of shoot length from May to November allowed to calculate parameters spanning the whole growing season in 2014 and 2015. Since most genotypes started senescing only in October, the end of shoot growth (te) occurred long before the onset of senescence. In 2014 the plants attained their maximum growth rate (tm) on average on day 157 and reached the maximum shoot length (te) on average on day 207. In 2015 this was on day 164 and day 200 respectively (Table 9.3). In 2014 t_e ranged between day 184 for OMP104 and day 243 for OPM43. In 2015 t_e ranged between day 184 for OPM29 and 220 for OPM43. Correspondingly, the duration of shoot growth (GD), calculated as the time between 10% shoot length ($t_{10\%}$) and the end of growth (t_e) was on average 117 days in 2014 and 95 days in 2015. Similar trends were observed when tm and t_e values were estimated in GDD units.

The average maximum shoot growth rate MGR was 27 mm day^{-1} in 2014 and 34 mm day^{-1} in 2015. In both years, maximum growth rates were on average higher for the *M. x giganteus* genotypes than for hybrid and *M. sinensis* genotypes. The highest MGR was 37 mm day^{-1} for *M. x giganteus* IL9 in 2014 and 54 mm day^{-1} for *M. x giganteus* OPM32 in 2015. The hybrid OPM109 was among the fastest growing genotypes, with MGR of 36 and 48 mm day^{-1} in 2014 and 2015 respectively. In both growing seasons OPM109 was the tallest genotype with an adjusted mean shoot length (Lm) of 3.5 m in 2014 and 3.4 m in 2015.

Biomass yield

On average the biomass yield in 2014 and 2015 was 1.3 kg DM plant^{-1} (Fig. 9.1). Remarkably, there was no significant difference in overall yield between years nor genotype by year interactions, but there was a significant species by year interaction. This suggests that by the end of the second growing season (2014), most plants had already reached maturity. All species groups were significantly different from each other (results not shown). In both years the *M. x giganteus* genotypes had the highest yields (2.7 and 3.2 kg DM plant^{-1} in 2014 and 2015 respectively). Hybrids had on average higher yields than the *M. sinensis* genotypes in the two growing seasons. The average yield of the hybrids was strongly influenced by hybrid OPM109, the highest yielding genotype in both years. With an average yield of 5.4 and 5.8 kg DM plant^{-1} in 2014 and 2015 respectively, this genotype out-

yielded all other genotypes by a large margin (Fig. 9.2). When OPM109 was excluded from the dataset, the yield of hybrids and *M. sinensis* genotypes were not significantly different.

Table 9.3: Adjusted mean values of whole season growth traits per species and per year with standard error, calculated on a day of the year (DOY) and growing degree day (GDD) basis using the method by Voorend et al. (2014). Traits are explained in Table 9.1.

	All genotypes		*M. sinensis*		Hybrid		*M. x giganteus*	
	2014	2015	2014	2015	2014	2015	2014	2015
L_m	2521 ± 19	2420 ± 20	2414 ± 17	2326 ± 19	2523 ± 35	2375 ± 40	3265 ± 41	3125 ± 34
MGR	27 ± 0.3	34 ± 0.4	26 ± 0.3	30 ± 0.4	29 ± 0.6	35 ± 0.8	34 ± 0.9	51 ± 1.0
$t_{10\%}$	105 ± 0	89 ± 0	104 ± 0	89 ± 0	106 ± 1	90 ± 1	92 ± 1	116 ± 1
$t_{50\%}$	152 ± 0	144 ± 0	152 ± 0	144 ± 0	150 ± 1	142 ± 1	148 ± 1	156 ± 1
$t_{90\%}$	183 ± 0	184 ± 1	184 ± 0	185 ± 1	179 ± 1	180 ± 1	189 ± 2	182 ± 1
t_m	157 ± 0	164 ± 0	157 ± 1	164. ± 0	155 ± 1	162 ± 1	161 ± 1	167 ± 1
t_e	207 ± 1	200 ± 1	207 ± 1	202 ± 1	201 ± 2	195 ± 1	212 ± 2	195 ± 1
GD	117 ± 1	95 ± 1	118 ± 1	98 ± 1	111 ± 2	89 ± 2	120 ± 3	79 ± 1
HD	242 ± 2	231 ± 4	243 ± 3	229 ± 4	234 ± 5	223 ± 7	NA	286 ± 2
50%Sen	300 ± 3	304 ± 2	305 ± 3	309 ± 2	281 ± 7	286 ± 7	294 ± 4	295 ± 11
MGR	1.9 ± 0.0	2.2 ± 0.0	1.8 ± 0.0	2.0 ± 0.0	2 .0 ± 0.0	2.3 ± 0.1	2.3 ± 0.1	3.3 ± 0.1
$t_{10\%}$	626 ± 4	555 ± 4	618 ± 4	535 ± 4	637 ± 7	563 ± 7	662 ± 11	674 ± 5
$t_{50\%}$	1309 ± 5	1125 ± 4	1303 ± 7	1122 ± 4	1287 ± 12	1099 ± 7	1381 ± 15	1190 ± 7
$t_{90\%}$	1914 ± 11	1623 ± 7	1915 ± 13	1646 ± 9	1849 ± 22	1553 ± 15	2017 ± 29	1594 ± 12
t_m	1395 ± 6	1203 ± 4	1387 ± 4	1194 ± 5	1380 ± 12	1183 ± 8	1459 ± 18	1291 ± 9
t_e	2287 ± 15	1925 ± 10	2295 ± 18	1964 ± 12	218 6 ± 29	1823 ± 22	2370 ± 45	1822 ± 20
GD	1658 ± 16	1368 ± 12	1673 ± 19	1427 ± 14	1550 ± 30	1261 ± 25	1708 ± 51	1155 ± 22
HD	2973 ± 35	2484 ± 60	2993 ± 38	2446 ± 65	2869 ± 77	2352 ± 120	NA	3294 ± 17
50%Sen	3810 ± 30	3480 ± 25	3862 ± 30	3526 ± 24	3571 ± 88	3284 ± 73	3776 ± 50	3395 ± 122

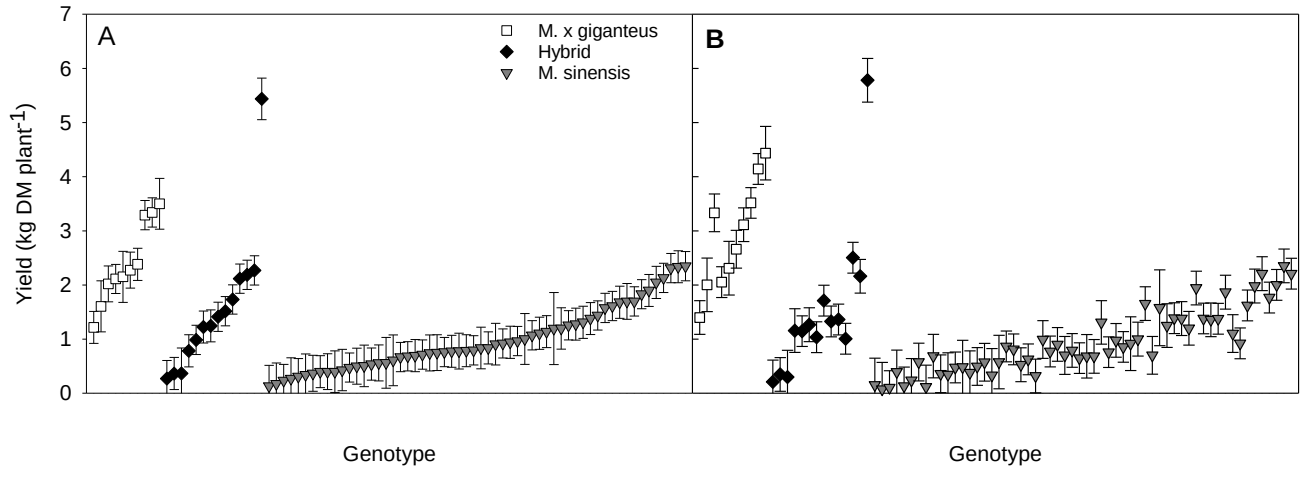


Fig. 9.1: Adjusted mean biomass yield per genotype. A: Yields of 2014, B: Yields of 2015. Genotypes are ranked according to 2014 yield. Different symbols indicate different species. Error bars show standard error.

The highest yielding *M. x giganteus* genotype in both years was IL6, with 3.5 and 4.4 kg DM plant^{-1} in 2014 and 2015 respectively. However, differences among *M. x giganteus* genotypes were not large (Fig. 9.3). The highest yielding *M. sinensis* genotypes were OPM71 in 2014 and OPM79 in 2015, both with 2.3 kg DM plant^{-1}. Average yield per genotype was highly correlated between the two growing seasons (r= 0.95) (Fig. 9.3). Intragenotypic variation in yield was considerable however, especially in the high yielding genotypes. For example, for OPM109 biomass yield ranged between and 3.1 and 8.0 kg DM plant^{-1} in 2014 and between 3.6 and 7.4 kg DM plant^{-1} in 2015. This plant-to-plant differences for clones of the same genotype might be due to competition differences as explained above.

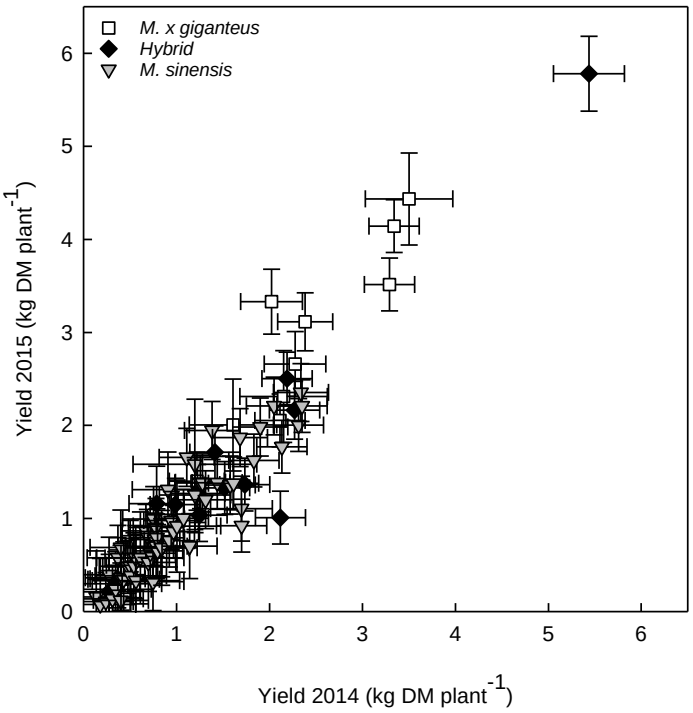


Figure 9.2: Relationship[between biomass yield values in 2014 and 2015. Symbols show mean biomass yield per genotype. Different symbols indicate different species. Error bars show standard error.

Similarity between growing seasons

Most traits were highly correlated between growing seasons, with the exception of the *M. x giganteus* group, which is likely a result of the smaller number of genotypes included and the lower level of variation comprised by this group (Table 9.4). High H^2 values were estimated for most traits, similarly to what has is reported in chapter seven for early-season growth traits. The highest correlations and

H² were found for yield and Lm, but also for most whole season growth traits (and also early-season growth traits, chapter seven), showing that these traits for can be considered for breeding purposes.

Table 9.4: Genotypic correlation between years for the traits measured in FT2 in 2014 and 2015 (r, Pearson's product-moment correlation; all correlations are significant, except those marked with ^{NS} ($p<0.05$); $na_{All\ genotypes} = 82$; $n_{M.\ sinensis} = 59$; $n_{Hybrid} = 13$); $n_{M.\ x\ giganteus} = 10$) and broad sense heritability (H^2). Results are shown for all genotypes and per species separately. Since HD and 50%Sen were not measured in this trial, heritability is not reported here for these traits. The three highest values in each column are underlined. Traits are explained in Table 9.1.

		All genotypes		*M. sinensis*		Hybrid		*M. x giganteus*	
	Trait	r	H^2	r	H^2	r	H^2	r	H^2
	Yield	<u>0.95</u>	<u>0.89</u>	<u>0.95</u>	0.83	<u>0.95</u>	<u>0.94</u>	<u>0.95</u>	<u>0.58</u>
	Dmcontent	0.80	<u>0.90</u>	0.77	<u>0.88</u>	0.85	<u>0.92</u>	0.29^{NS}	0.54
Early-season	AGR	0.63	0.80	0.79	<u>0.86</u>	0.71	0.68	-0.08^{NS}	0.30
	L30	0.38	0.66	0.53	0.66	0.77	0.80	0.50^{NS}	0.25
	L50	0.74	0.88	<u>0.83</u>	<u>0.89</u>	0.76	0.73	0.63^{NS}	0.08
	LFR	0.27	0.71	0.40	0.76	0.26^{NS}	0.64	0.04^{NS}	0
	Leaf4	0.54	0.81	0.55	0.60	0.65	0.15	-0.42^{NS}	0
	S50	0.42	0.56	0.45	0.59	0.25^{NS}	0.38	0.43^{NS}	0.45
	N_{max}	0.79	0.73	0.78	0.75	0.84	0.79	0.80^{NS}	0.37
Whole season	L_m	<u>0.90</u>	<u>0.94</u>	0.73	0.83	<u>0.97</u>	<u>0.94</u>	<u>0.88</u>	<u>0.80</u>
	MGR	0.85	0.83	0.80	0.84	0.92	0.91	0.61^{NS}	<u>0.61</u>
	$t_{10\%}$	0.65	0.71	0.65	0.73	0.47^{NS}	0.85	0.63^{NS}	0.40
	$t_{50\%}$	0.59	0.68	0.46	0.61	0.75	0.73	0.37^{NS}	0
	$t_{90\%}$	0.55	0.61	0.54	0.58	0.83	0.85	0.44^{NS}	0
	t_m	0.68	0.71	0.53	0.66	0.61	0.61	0.58^{NS}	0
	t_e	0.52	0.61	0.56	0.60	0.84	0.87	0.48^{NS}	0
	GD	0.60	0.61	0.59	0.62	0.83	0.89	0.58^{NS}	0
Phenology	HD	0.74		0.72		0.97		NA	
	50%Sen	0.84		0.82		0.84		0.83^{NS}	

Overall correlation between biomass yield and growth traits

When all genotypes were considered together, most traits were significantly correlated with biomass yield (Table 9.5). Overall MGR, L_m, Leaf4 and N_{max} displayed the strongest correlation with yield over the two growing seasons considered. MGR, L_m and N_{max} were positively correlated with yield, indicating that high growth rates over the whole season, final shoot length and the capacity to form a large number of shoots are the main contributors to high yield in this collection of plants. In most cases, traits describing time points in the growing season were negatively correlated with yield. Since lower values for these traits mean that these time points occurred earlier in the year, a negative correlation means vigorous early-season growth and early canopy formation are indeed associated with high biomass yield in miscanthus.

Yield had a markedly higher coefficient of variation than the other traits, except for N_{max}. Most traits did had a CV larger than 10%, and there is thus substantial variation in morphological and

phenological traits among the genotypes. Provided this variation is useful, selection for these traits should thus be possible.

Table 9.5: Correlations between plant traits and biomass yield based on GDD values, (Pearson's correlation coefficient, correlations marked with * are significant ($p<0.05$). The number of observations was: $n_{All\ species}$ = 85, $n_{M.\ sinensis}$ = 62, n_{Hybrid} = 13, $n_{M.\ x\ giganteus}$ = 10. The underlined values indicate the highest and lowest correlation in each column. Correlations with HD and 50%Sen were calculated using only the genotypes for which these data were available from FT1, not flowering genotypes were excluded from the calculations for HD. The highest three absolute values in each column are underlined. Traits are explained in Table 9.1.

		All genotypes				*M. sinensis*				Hybrid				*M. x giganteus*			
	Trait	2014		2015		2014		2015		2014		2015		2014		2015	
		r	CV	r	CV	r	CV	r	CV	r	CV	r	CV	r	CV	r	CV
Morphology	Yield		74		86		64		69		82		93		32		34
	DM%	-0.20	12	-0.19	14	0.11	11	0.01	13	-0.48	14	-0.18	12	-0.17	4	-0.09	6
Early-season growth	AGR	0.05	16	0.56*	21	0.02	16	0.32*	19	0.70*	16	0.86*	31	-0.21	10	0.42	11
	L30	-0.53*	9	-0.09	13	-0.29*	8	-0.21	14	-0.80*	10	-0.67*	7	-0.19	6	-0.73*	3
	L50	-0.47*	9	-0.24*	12	-0.26*	8	-0.28*	13	-0.83*	10	-0.89*	7	-0.13	9	-0.71*	3
	LFR	-0.13	16	0.25*	14	-0.16	16	-0.11	14	0.29	12	0.69*	16	0.05	14	0.18	15
	Leaf4	-0.45*	8	-0.44*	10	-0.32*	7	-0.46*	10	-0.56*	8	-0.63*	7	0.16	8	0.01	5
	S50	0.12	13	0.14	14	0.13	14	-0.03	15	-0.10	8	0.40	14	-0.38	6	0.22	3
	N_{max}	0.46*	76	0.33*	82	0.78*	86	0.66*	94	0.11	52	-0.16	48	0.46	34	0.52	28
Whole season growth	Lm	0.70*	14	0.76*	17	0.40*	9	0.54*	13	0.91*	13	0.90*	15	0.38	4	0.54	4
	MGR	0.73*	17	0.82*	26	0.56*	14	0.77*	19	0.75*	18	0.82*	21	0.56	11	0.21	5
	$t_{10\%}$	0.01	9	0.45*	13	-0.02	10	0.31*	10	-0.42	7	-0.09	7	-0.03	6	-0.48	3
	$t_{50\%}$	-0.18	6	-0.05	5	-0.34*	6	-0.37*	4	-0.43	7	-0.50	5	-0.55	4	-0.33	3
	$t_{90\%}$	-0.22*	8	-0.54*	7	-0.36*	8	-0.68*	6	-0.34	9	-0.42	8	-0.49	6	-0.06	2
	t_m	-0.12	6	0.15	6	-0.25	6	-0.11	5	-0.46	7	-0.41	5	-0.48	3	-0.32	2
	t_e	-0.22*	10	-0.58*	8	-0.34*	10	-0.67*	8	-0.31	11	-0.40	10	-0.46	8	0.05	2
	GD	-0.21*	15	-0.60*	15	-0.29*	15	-0.61*	13	-0.23	15	-0.34	16	-0.40	12	0.30	4
Phenology	HD	0.21	8	-0.05	12	0.07	8	0.06	12	-0.09	7	-0.49	10	0.21	0	/	2
	50%Sen	-0.20	8	-0.41*	6	-0.37*	8	-0.47*	5	0.45	8	-0.42	7	0.31	2	0.03	7

Correlation between biomass yield and growth traits in the different species groups

In the *M. sinensis* group the strongest correlation with yield was found for N_{max}, followed by MGR. High shoot numbers are thus the most determining morphological trait for yield in this group, followed by high growth rates over the whole season. In 2015 t_e, $t_{90\%}$ and GD displayed also a high correlation with yield in this group. Yield in the *M. sinensis group* was negatively correlated with most traits describing time points in the growing season. This indicates that *M. sinensis* genotypes which reached these time points earlier, and thus had lower values for these traits, had higher biomass yields. *M. sinensis* genotypes with strong early-season growth and short growth durations (GD) had the highest yields (Fig. 9.3). Interestingly, te, the time point at which growth ended, was also negatively related to yield, and genotypes that reached their maximum height earlier were thus higher yielding. Furthermore, there was no association between HD and yield, while 50%Sen was negatively correlated with yield. Taken together, these findings indicate that *M. sinensis* genotypes with late

development and extended canopy durations in autumn are not higher yielding and that extending the growing season in autumn may not be a recommendable breeding strategy in this species. In this germplasm early development and the capacity to form a large number of shoots seem to be the more relevant traits.

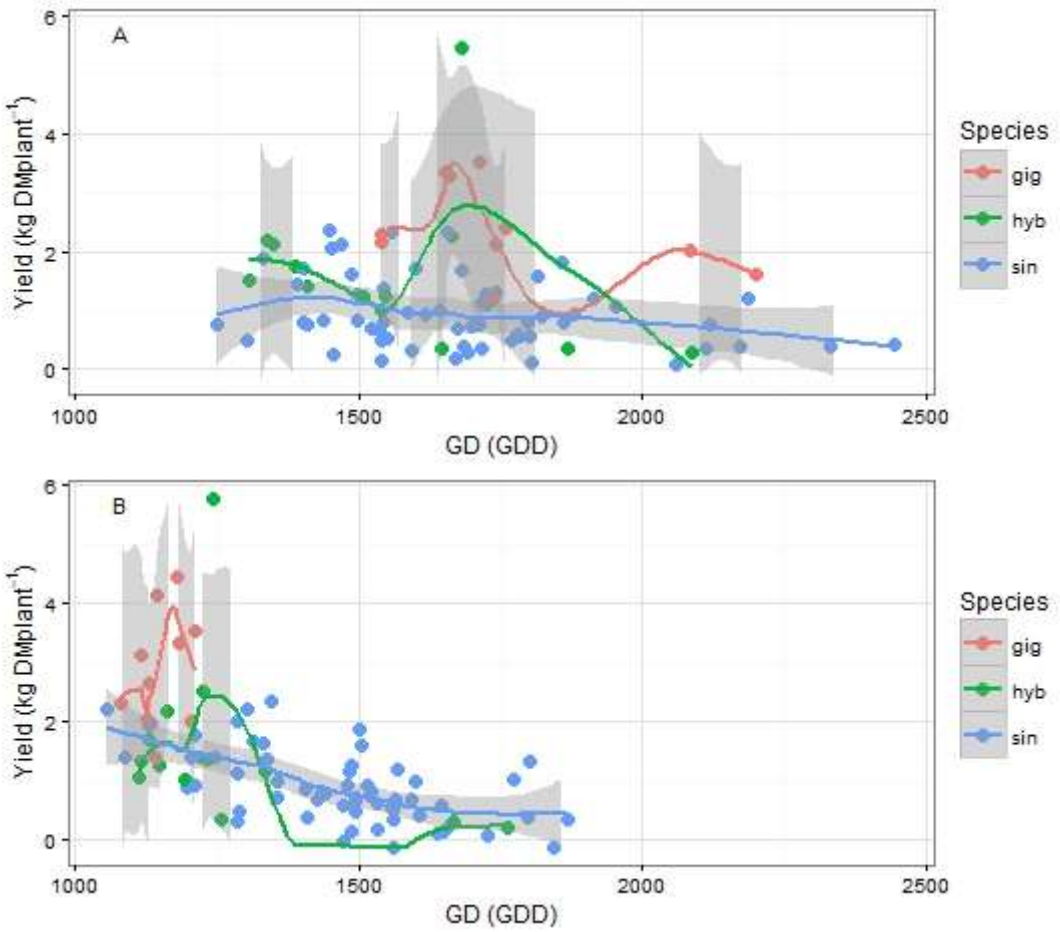


Figure 9.3: Biomass yield in function of growth duration. A: 2014 growing season, B: 2015 growing season. Different colours indicate different species. Lines shows smoother curves and shaded areas show confidence intervals.

In the hybrid group yield was most strongly positively correlated with L_m and MGR. Interestingly, N_{max} was not correlated with yield in 2015 in the hybrid group; this is likely a consequence of the very large variation in shoot number in this group and the heavy influence of OPM109 on all correlations. The traits describing time points in the growing season were also negatively correlated with yield in the hybrid genotypes, indicating that high early-season growth is also associated with high yields. In the *M. x giganteus* group there were no significant correlations, except for L30 and L50 in 2015, the lack of correlation in this group is indicative of the small variation that it comprises.

Interrelationships among variables

The overall relationships among variables were further analyzed using principal component analysis. The first two components explained together over 57% of the variation in all datasets. Over all genotypes, the first component, which explained 38.6% of the variation (Fig. 9.4A), was positively related to all traits describing moments in the growing season and negatively related to L_m, N_{max}, AGR and MGR. The second component was positively related to L_m, N_{max} and MGR. Interestingly, all traits describing moments in the growing season plotted on this axis in a chronological order, starting from the first trait in the growing season, $t_{10\%}$ with the highest positive value, to the latest trait in the growing season, 50%Sen with the lowest negative value. HD plots together with t_e and 50%Sen plots after te, indicating the measurements from FT1 did apply to FT2.

The traits describing moments in the growing season clustered roughly in four groups: the beginning of the growing season ($t_{10\%,}$ L30 and L50), the middle of the growing season (tm, Leaf4, S50 and $t_{50\%}$), the end of growth ($t_90\%$, t_e, HD and GD) and senescence (50%Sen). Further PCA analyses on a species group level (Fig. 9.4B-D) showed similar results. In the *M. sinensis* group (Fig. 9.4B) yield is most strongly positively related with N_{max} and MGR and negatively with the end of growth traits, while less strongly with early-season growth and Lm. In the hybrid and *M. x giganteus* genotypes (Fig. 9.4C, D) yield was most strongly positively related to MGR, AGR and L_m and negatively related with the growing season traits, similar to what is described above.

The plot of the genotype values for PC1 and PC2 showed that the hybrid and *M. x giganteus* genotypes plotted largely within the *M. sinensis* group. These groups were thus not substantially different from the variation within *M. sinensis* (Fig. 9.5). The *M. x giganteus* genotypes formed a small cluster in the plot, indicative of the high similarity between the genotypes in this group. The hybrids also clustered closely together, with the exception of some strongly different genotypes. These exceptions were the very early OPM109 and OPM05 and the later genotypes OPM16 and OPM33.

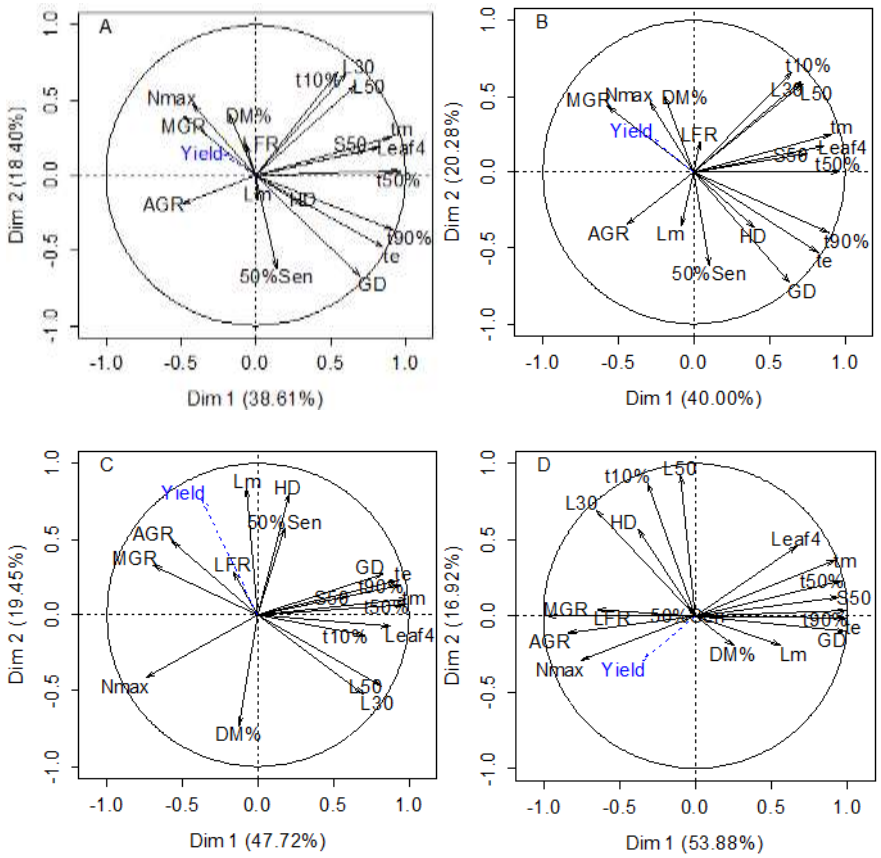


Figure 9.4: Principal component analysis of the data: A: All species, B: M. sinensis, C: hybrids and D: M. x giganteus. Yield was used as a supplementary variable and did thus not influence the outcome of the PCA. Analyses based on adjusted genotypic means in 2014 and 2015.

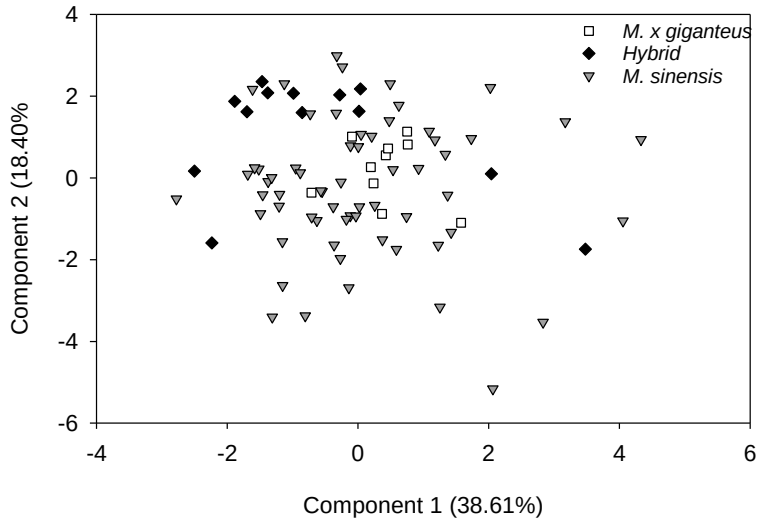


Figure 9.5: Genotype loadings of the PCA of all genotypes. Different symbols indicate different species groups.

Discussion

Yield potential of germplasm

The high biomass yield potential of *M. x giganteus* reported before in many studies was confirmed here. In Western Europe mature yields have been reported to be around 20 t DM ha^{-1} (Clifton-Brown et al., 2001b; Lesur et al., 2013; Lewandowski et al., 2000; Muylle et al., 2015; Zub et al., 2011). The variation in yield potential in larger collections of miscanthus germplasm is however little studied up to now. Most studies so far which compared the yields of *M. sinensis* with *M. x giganteus* based the comparison mainly on ornamental *M. sinensis* varieties (e.g. Kaiser et al., 2015; Larsen et al., 2013; Muylle et al., 2015; Van Hulle et al., 2012; Zub et al., 2011). These varieties were bred for aesthetic characteristics and are not necessarily high yielding, with the remarkable exception of a few genotypes, such as *M. sinensis* 'Goliath' and *M. sinensis* 'Silberfeder' which were among the highest yielding at our location. The trial results reported by Clifton-Brown et al. (2001a) corresponded to *M. sinensis* genotypes collected in the wild in Japan. In that trial *M. sinensis* yields were also lower than those of *M. x giganteus* and *M. sinensis x sacchariflorus* hybrids.

In our trial the *M. x giganteus* genotypes yielded on average more biomass than the *M. sinensis* or hybrid genotypes. Only one genotype, the hybrid OPM109, was higher yielding than *M. x giganteus.* However apart from its high yield it is not an ideal genotype as it was difficult to propagate in vitro and had low establishment success. Furthermore, OPM109 did not flower nor senesce during the whole period investigated, remaining green until late in winter. Its shoots are rather frost tolerant (chapter four), so it is largely green at harvest and consequently, at harvest its biomass contained a high leaf fraction. High leaf contents are associated with high ash contents and low biomass quality (Baxter et al., 2014). The hybrid genotypes in our germplasm are the result of the first breeding efforts in miscanthus, and genotypes such as OPM109, and the one average higher yield of this group compared to *M. sinensis*, shows that breeding for improved biomass yield has been successful to some degree and that it is possible to create varieties with higher yield potential than *M. x giganteus*.

Our trial was harvested in the beginning of January in both years. This is not standard practice in miscanthus, where harvesting is either performed in autumn or later in winter after leaves and moisture content have dropped. In our case the trial was harvested in January to facilitate observations of early emerging shoots after the harvest (chapter seven). In general, *M. x giganteus* biomass yields are 33% lower at winter harvest compared to autumn harvest (Hastings et al., 2009). The early harvest, as done in FT2, benefits early emerging genotypes, which could be damaged by a later harvest in February or March. When earlier emerging varieties compared to *M. x giganteus* would be planted, field management would therefore have to take the earlier emergence into account.

Harvest would have to be performed earlier in order not to damage newly emerging shoots. This earlier harvest in turn would necessitate that early varieties should also senesce relatively early, to insure sufficiently dry biomass at harvest.

Growth traits

The growing period dynamics observed in our study were similar to those reported in literature. In both years plants emerged earlier than early April as is usual at our site (Hilde Muylle personal communication) due to the warm winters and spring in 2014 and 2015. The accumulated thermal time for emergence was similar between both years (chapter seven). This early emergence, high temperatures and long growing season in 2014 might explain why the plants reached full maturity already in the second growing season and why no difference in yield was observed between both years, while generally miscanthus in Belgium only reached full yields in the third growing season (Muylle et al., 2015).

Maximum shoot length was reached around the end of July in both years. In agreement with other studies reporting leaf area index (LAI) data, it reached a maximum of 5 to 6 m^2 m^{-2} around the beginning of August (Clifton-Brown et al., 2000; Dohleman and Long, 2009; Vargas et al., 2002). In these studies, biomass accumulation increased for another month and maximum biomass was reached around the beginning of September. The maximum growth rates observed in our study are similar to the growth rates reported for Wales by Purdy et al. (2015) which were between 0.6 and 3.5 cm day^{-1}. High growth rates might be a sign of high photosynthetic capacity. Genotypes with higher photosynthetic capacity have been shown to have higher growth rates and biomass accumulation (Głowacka et al., 2013; Jiao et al., 2016, chapter five). While the plants emerged later in 2015 and they reached higher growth rates later than in 2014, they attained similar heights at the end of both growing seasons. Maximum height thus appears to be more genotypically determined than dependent on the growing season conditions and might set a limit to the biomass yield of a certain genotype.

Competition

The indices of competition based on the number of plants (CN) and the length of the surrounding plants (CL) had less impact than the indices based on shoot number (CS) or yield (CY) which agrees with Zub et al. (2012a). These latter indices give a more accurate estimation of the competition imposed by surrounding plants, since badly established plants with a small number of shoots can still reach heights of 1-2 m, but given their low number of shoots and biomass will have low impact on CS and CY. There was a significant genotype by competition interaction, which indicates that not all genotypes were impacted to a similar level by competition. Possibly some genotypes are more

tolerant to competition than others, and some genotypes may have been planted, by chance, among less competitive ones. It is also possible that the yield of the lowest yielding genotypes was even further reduced by competition by more competitive, taller neighboring genotypes.

While currently *M. x giganteus* is propagated by rhizomes, future miscanthus fields will also be sown or planted with seed-based non-clonal varieties (Clifton-Brown et al., 2016; Xue et al., 2015). Since miscanthus is self-incompatible and inbred lines are difficult to produce, these seed-based varieties will be more heterogeneous than clonal varieties and differential competition effects will play a role in biomass yields. Perennial crops show higher within genotype variability in fields of the same genotype than cereals, due to for example lower planting densities (Knörzer et al., 2013). Genotypes that suffer relatively less penalty by competition are thus potentially interesting breeding candidates. These genotypes might be planted closer together and produce more biomass, an approach which in maize breeding, for example, has been responsible for major yield gains (Tollenaar and Wu, 1999). Further research on the effects of competition in miscanthus fields will be necessary to determine its relevance for biomass yield.

Relationship between canopy duration and biomass yield

High yielding genotypes in our trial were characterized by tall stems, high number of shoots, rapid growth, early development and short canopy formation periods. The strong link between plant morphology and biomass yield has been reported in a number of studies (Jeżowski, 2008; Kaiser et al., 2015; Robson et al., 2013b; Zub et al., 2011). The underlying causes that allow genotypes to achieve these high shoot numbers and high shoot lengths have not been determined. We found that high growth rates, early development and short canopy formation periods were correlated with higher yields. This both agrees and disagrees with literature. Zub et al. (2012b) reported late emergence, high maximum growth rates and short growth durations are associated with high yields. Robson et al. (2013a) on the other hand reported that early canopy formation and late senescence are associated with high yields. Taken together, from these three studies it thus appears that vigorous early-season growth and fast canopy formation are indeed associated with bigger biomass yield.

The reported differences in outcome between studies might be a result of the different climatological conditions of the trials, different characteristics of the growing seasons investigated, different methodologies to determine the growing season or the use of different sets of genotypes. The inclusion of high yielding, late emerging *M. x giganteus* genotypes in the relatively small set (20 genotypes) of genotypes in the study by Zub et al. (2012b) might explain why in their study early emergence was not correlated with high yield. When Zub et al. (2012b) analyzed only *M. sinensis* genotypes they found no significant correlation between emergence and yield. Similarly, we observed

different relationships between biomass yield and early-season growth depending on the species. While Zub et al. (2012b) and our study defined the end of growth duration as the moment at which the maximum shoot height is reached, Robson et al. (2013a) defined the end of the growing season as the moment the plant had senesced for more than 50%. These studies are thus not necessarily contradicting each other. All genotypes reached their maximum height before August and probably after that period only a limited amount of extra biomass was produced (Clifton-Brown et al., 2000; Dohleman and Long, 2009). In August – September flowers and seeds are formed and carbohydrates are translocated to the rhizomes in most genotypes, and by mid-September starch levels in the rhizomes reach their maximum (Purdy et al., 2014). A longer canopy duration might, on the other hand, be responsible for more nutrient storage in the rhizomes, allowing more growth in the next growing season. Due to the warm autumns in both years investigated here, senescence was late in most genotypes and was therefore not strongly related to the end of shoot growth. It is then possible that in Wales, where the growing season temperatures are colder, it takes longer for the plants to fully develop. The final height of the plants might even be limited by the growing season temperature and duration in Wales, while in Belgium or France this was not the case and miscanthus genotypes could reach their full potential length. Shoot length seems to be genetically determined, and genotypes will not become higher than this maximum even if growing conditions would allow. Later senescing genotypes thus might be able to grow longer and produce more biomass under limiting conditions, but will not have an advantage under more optimal growing conditions.

Since miscanthus is a perennial crop that generally takes multiple years until full establishment, miscanthus breeding will take a long time. It would therefore be useful to find ways to speed up the breeding process and a good understanding of the traits underlying biomass yield are thus important. The highest correlations with yield in all species groups are with shoot height (L_m) and shoot number (N_{max}). Selecting for these morphological traits will be the first thing to do in breeding (Robson et al., 2013b). The main trait to improve will depend on the species, for example, in *M.* sinensis N_{max} had the highest correlation with yield, while in the hybrids this was Lm. Additionally, a focus on selection for high growth rates, fast canopy development and early emergence could help to select high yielding genotypes. In the hybrid group the correlations indicate that early-season growth was the most strongly associated with biomass yield, while in *M. sinensis* the end of the growing season was more strongly associated with biomass yield. However, determining shoot numbers or growth rates is rather labor intensive. Therefore, the most useful traits for breeders would be to measure L_m and the height at a certain date in spring, in order to obtain data on an as large number of genotypes as possible.

Chapter 10: Evaluation of biomass yield potential and phenological traits of a diverse miscanthus collection

Introduction

In the previous chapter we determined the relationship between yield and phenological and morphological traits on a plant basis, in this chapter we evaluate the relationship between phenology and yield in uniform plots in a trial under a management representative of commercial plantations.

This trial is the first to report on the yield potential of almost 100 mostly wild and semi-improved miscanthus accessions grown in uniform plots, comprising *M. sinensis, M. sacchariflorus* and hybrid germplasm, collected in the wild or generated by crosses with the specific aim of improving biomass production.

As discussed in previous chapters, phenological traits might have an impact on biomass yield and quality. Although late senescence might thus be associated with high biomass yield (Robson et al., 2013a), it is also associated with higher moisture content at harvest, lower rates of leaf loss during winter and higher ash contents at harvest and thus a lower biomass quality (Clifton-Brown and Lewandowski, 2002; Robson et al., 2011). There might thus be a certain trade-off between yield and quality. In commercial miscanthus plantations, harvest is generally performed when the standing biomass has dried down sufficiently, in order to avoid drying costs. However, if the moisture content drops only late in winter, new shoots can already be emerging at harvest, which might have an impact on yield. Furthermore, during senescence nutrients and carbohydrates are relocated to the rhizomes (Himken et al., 1997; Purdy et al., 2014) for use in the next growing season. If senescence is initiated too late, this process might be disrupted by frost and growth in the next season might be affected. Flowering time is another key phenological trait and possibly also has an impact on miscanthus biomass production (Jensen et al., 2011). When plants transition from the vegetative to the reproductive phase, stem elongation stops and biomass accumulation is strongly reduced. Genotypes which flower later in the growing season could therefore be higher yielding (Clifton-Brown et al., 2001b) but late flowering can also be associated with delayed senescence and higher moisture content and lower biomass quality at harvest as discussed above.

The aims of the research presented here were i) to evaluate the yield potential of a large collection of genotypes from different miscanthus species grown in plots under a management representative of commercial plantations, ii) to interpret these data in view of differences regarding canopy closure, flowering and senescence. A schematic overview of set-up of this chapter is provided in figure 10.1.

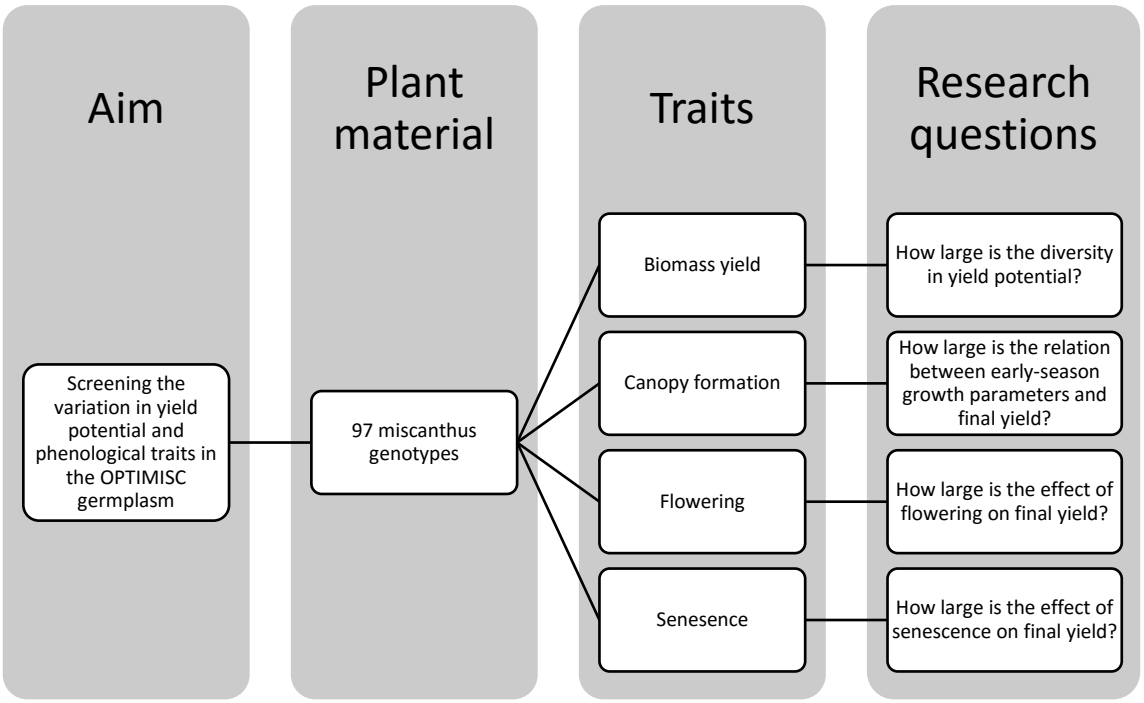


Figure 10.1: Schematic representation of this chapter.

Material and methods

Field trial and Biomass yield determination

All measurements reported here were performed in field trial FT1, which is described in detail in chapter three. The trial was harvest on 16--20/03/205 and 4-8/04/2016, when the dry matter content of OPM09 surpassed 80%. For the biomass yield determination, the two middle plants of each plot were harvested. For *M. sinensis, M. giganteus* and hybrid genotypes, two plants were bound together in the field and cut at 5 cm above soil level with a hedge trimmer. For *M. sacchariflorus* genotypes an area of 50 by 50 cm was harvested because individual plants were difficult to recognize due to the spreading rhizomatous growth of this species. Two such areas were defined in each plot around the position where each plant was planted and all stems within this area were bound together and harvested. Plants were then weighed (Yield) and the length of the longest stem was measured (Stem height). Thereafter a subsample of five stems was taken which was split into leaf and stem fraction to determine the leaf to stem ratio (Stem%), an important indicator of biomass quality. Panicles were considered part of the stem. The rest of the plant was chopped in stationary forage maize chopper. A subsample of approximately 300 g of this was dried at 70°C for over 48 h to determine the moisture content of the biomass (DM%).

Phenology-related traits

Early canopy formation, flowering and senescence were studied to describe the growing season dynamics. An overview of the measured and derived parameters and their abbreviations can be found in Table 10.1. Early canopy formation was measured using spectral reflectance as described in chapter six.

Table 10.1: Overview of parameters used in this study.

	Trait	Abbreviation	Method	Unit
Yield	Final biomass yield	Yield	harvest	kg DM plant^{-1}
	Dry matter content biomass	DM%	harvest	% DM
	Stem content biomass	Stem%	harvest	% stems
	Length longest stem	Stem height	Measured at harvest	cm
Canopy formation	DOY NDVI value of 0.5 reached	NDVI0.5	NDVI - Greenseeker	DOY
	DOY canopy closure	CC	NDVI - Greenseeker	DOY
Flowering	Flag leaf appearance	FL	Visual score	DOY
	Heading date	HD	Visual score	DOY
	Anthesis	ANT	Visual score	DOY
Senescence	DOY 50% plant senesced	50%Sen	Visual score	DOY
	DOY 90% plant senesced	90%Sen	Visual score	DOY
Growing period	Duration of vegetative growth	VegDur	NDVI + Visual score	day
	Canopy duration	CanDur	NDVI + Visual score	day

Flowering and senescence

Flowering was scored visually on the two middle plants in each plot from 25/06/2014 to 21/09/2014 and from 01/07/2015 until 20/11/2015 using a score of 0 to 4 (0: no flowering; 1: flag leaf formed; 2: panicle emergence; 3: anthesis; 4: end of anthesis) (Jensen et al., 2011). The day of the year (DOY) when a certain score was reached was calculated using linear interpolation.

Senescence was scored visually from 25/09/2014 until 11/03/2015 and from 18/09/2015 until 11/03/2016 by estimating on a plot basis the proportion of the plant that was still green, as described in Robson et al. (2011). The DOY when 50% of the plant had already senesced (50%Sen) and when 90% of the plant had already senesced (90%Sen) was then calculated through linear interpolation. Duration of vegetative growth (VegDur) was calculated as the time between the day a NDVI0.5, as indication of early canopy formation, until HD as indicative of flowering. Canopy duration (CanDur) was calculated as the time between NDVI0.5, as indication of early canopy formation, until 50%Sen, as indication of the end of the canopy duration.

Statistical analyses

All statistical analyses were performed in R 3.1.0 (R core team, Vienna, Austria). Correlations were calculated using the corr.test function from the *psych* package. Differences in phenological traits between genotypes and species were determined using the 'glm' function of the *stats* package and the 'lsmeans' function of the *lsmeans* package. Principal components analysis was performed using the 'PCA' function of the *FactoMineR* package. All variables were scaled to unit variance. Biomass yield was included as supplementary variable.

Results

Biomass yield

On average, biomass yields were slightly higher in the second (2014) growing season (0.69 ± 0.38 kg DM plant^{-1}) compared to the third (2015) growing season (0.66 ± 0.29 kg DM plant^{-1}) but there was no significant difference between both years (Fig. 10.2). This indicates that most genotypes had already reached yields in the second (2014) growing season similar to the yields of mature plants, which generally only happens in the third year in Belgium (Muylle et al. 2015), although the variation between genotypes was still large in 2014. In 2014 biomass yield was not significantly different between species but in 2015 the *M. sacchariflorus* genotypes had on average significantly lower yields than the other species groups. The relatively low yields in 2015 compared to 2014, in particular of the *M. sacchariflorus* genotypes, might be a result of higher leaf loss during winter. In 2015 the *M. sacchariflorus* genotypes lost almost all leaves during winter, while in 2014 leaves accounted for on average 7% of the total harvested biomass (Fig. 10.3). Another reason for the generally lower yields in 2015 than in 2014 might be the colder and dryer weather in the 2015 growing season. There was a significant effect of planting date in both years, genotypes planted in May 2013 generally had higher yields in both years than the genotypes planted at later dates, although some of the later planted genotypes were competitive with those planted in May. Biomass yield per plant was highly correlated between both years ($r=0.55$, $p<0.001$).

In 2014 biomass yields ranged between 0.09 ± 0.01 kg DM plant^{-1} for *M. sinensis* OPM89 and 1.70 ± 0.18 kg DM plant^{-1} for *M. x giganteus* OPM32. In 2015 yields ranged between 0.16 ± 0.08 kg DM plant^{-1} for *M. sinensis* OPM64 and 1.28 ± 0.20 kg DM plant^{-1} for *M. x giganteus* OPM32. The highest yielding *M. sinensis* genotype was OPM91 with 1.20 ± 0.16 kg DM plant^{-1} in 2014 and OPM44 in 2015 with 1.38 ± 0.17 kg DM plant^{-1}. The highest yielding *M. sacchariflorus* genotype was OPM19 in both years, with 1.20 ± 0.20 kg DM plant^{-1} in 2014 and 0.85 ± 0.15 kg DM plant^{-1} in 2015. The highest yielding hybrid in 2014 was OPM05 0.96 ± 0.28 kg DM plant^{-1} and OPM109 in 2015 with 0.93 ± 0.27 kg DM plant^{-1}. Although the highest yielding genotype in both years was thus *M. x giganteus* OPM32,

a number of *M. sinensis, M. sacchariflorus,* and *M. sinensis x sacchariflorus* genotypes showed high yield potential, comparable to *M. x giganteus*. These plant based yields extrapolate, at the planting density of the trial of 20.000 plants ha^{-1}, to yields between 3 and 34 t DM ha^{-1}. There is thus a potential in the germplasm to breed new varieties competitive with *M. x giganteus,* though the data show that interspecific hybrids are not necessarily higher yielding and were not significantly higher yielding than *M. sinensis* nor *M. sacchariflorus*.

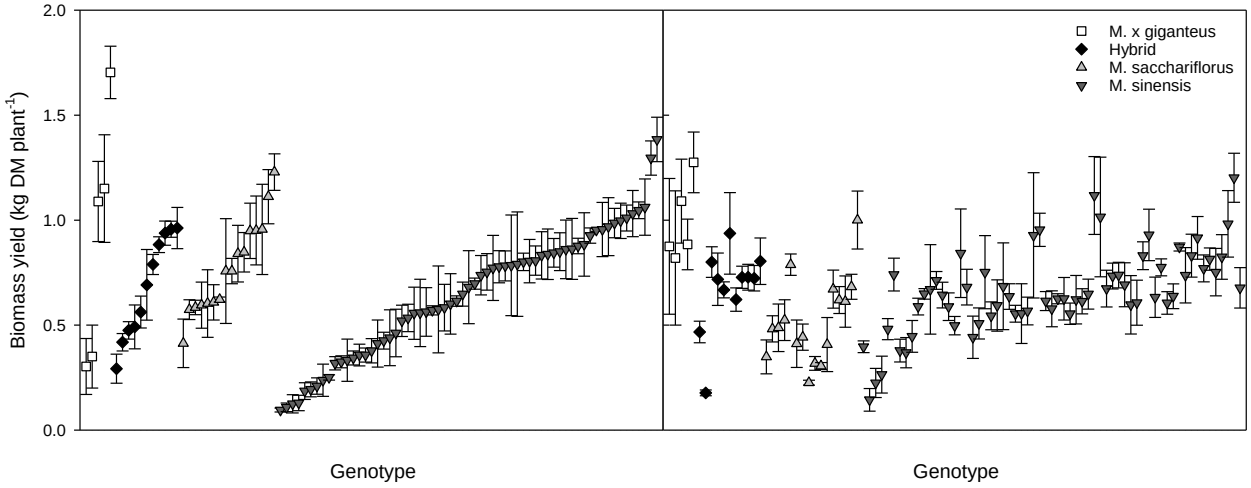


Figure 10.2: Scatterplots per species of biomass yield genotype. Different symbols indicate different species; error bars show standard error (n=4). Genotypes are ordered according to the 2014 yield.

There was no significant difference in dry matter content (DM%) between both years, but *M. sinensis* (average DM% 75.8 ± 0.5*%)* and hybrid genotypes (average DM% 78.0 ± 0.9%) had significantly lower DM% than the *M. sacchariflorus* genotypes (82.0 ± 0.9% DM) (Fig. 10.4). DM% ranged between 55.7 ± 0.7% for *M. sinensis* OPM44 and 90.0 ± 0.3% for *M. sacchariflorus* OPM03 in 2014 and 55.0 ± 0.7% for *M. sinensis* OPM80 and 88.9 ± 3.1% for *M. sinensis* OPM81 in 2015. DM% was significantly correlated with HD ($r = -0.35$, $p < 0.001$) and 50%Sen ($r = -0.51$, $p < 0.001$). Plants that flowered and senesced earlier thus had lower moisture contents at harvest. There was a weak negative correlation over all genotypes between yield and DM%, but not between yield and stem content (Table 10.3). Higher yielding genotypes thus tended to have somewhat lower dry matter contents.

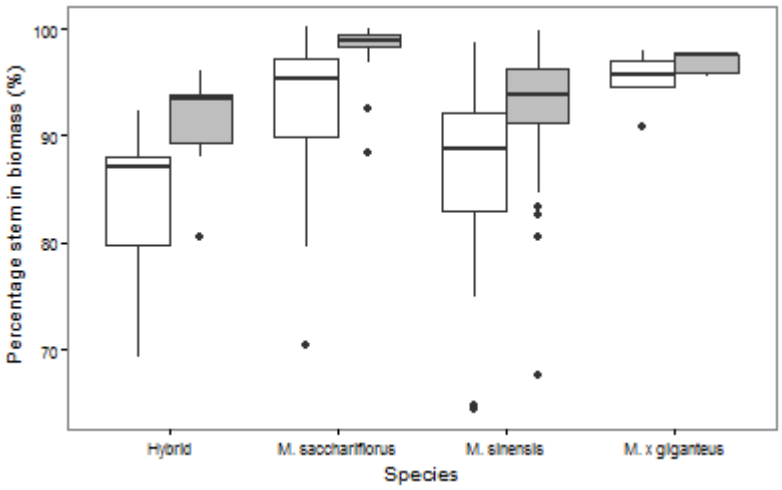


Figure 10.3: Boxplots showing the variation in stem proportion of the harvested biomass within species. White boxplots show the 2014 growing season, grey boxplots show the 2015 growing season.

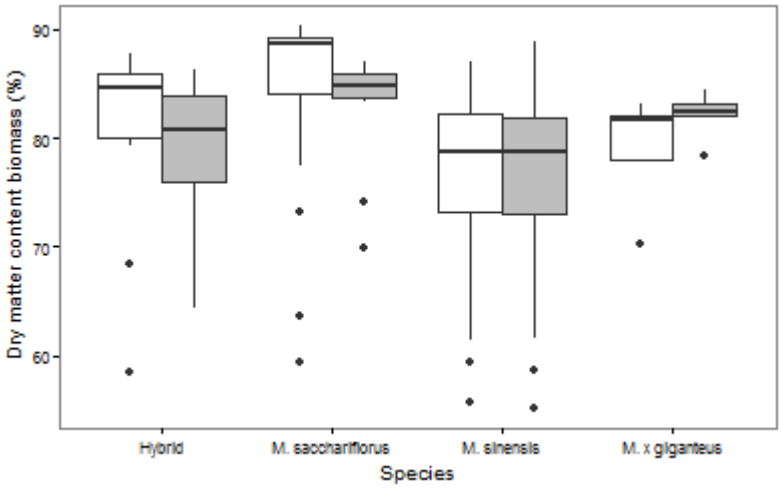


Figure 10.4: Boxplots showing the variation in dry matter content per species. White boxplots show the 2014 growing season, grey boxplots show the 2015 growing season.

Genotypic variation in phenological traits

Early canopy formation

In both years early canopy formation, as measured by NDVI, varied considerably between the genotypes although the differences were smaller in 2015 than in 2014. In 2014 NDVI started increasing immediately after the harvest on 20/03/2014 in some of the genotypes, while NDVI values of other genotypes only increased slowly and did not even reach the maximum by the end of the measuring period. This slow development was partially caused by less complete establishment in the first growing season, leading to a lower growth capacity in spring of the next year. In 2015 NDVI values started increasing almost four weeks later than in 2014, when temperatures started rising.

The differences between the genotypes were less pronounced in 2015 and most genotypes reached maximum NDVI by the end of the measuring period at the end of May.

Table 10.2: The mean and extremes of DOY a NDVI score of 0.5 was reached (NDVI0.5), the DOY of canopy closure (CC), heading date (HD) and the DOY 50% senescence was reached (50%Sen) per species group and year.

		M. sinensis			*M. sacchariflorus*		
		Minimum	*Mean*	*Maximum*	*Minimum*	*Mean*	*Maximum*
2014	NDVI0.5	109	119 ± 0.5	135	107	119 ± 1.2	143
	CC	119	132 ± 2.4	179	118	132 ± 4.6	170
	HD	172	225 ± 1.5	279	-	-	-
	50%Sen	273	312 ± 1.0	401	281	308 ± 2.0	343
2015	NDVI0.5	124	137 ± 0.4	153	122	132 ± 0.7	141
	CC	135	149 ± 1.0	168	132	146 ± 2.1	158
	HD	211	240 ± 0.9	303	-	-	-
	50%Sen	265	304 ± 1.2	399	275	301 ± 1.5	340
		Hybrid			*M. x giganteus*		
		Minimum	*Mean*	*Maximum*	*Minimum*	*Mean*	*Maximum*
2014	NDVI0.5	109	112 ± 0.6	125	114	117 ± 1.1	121
	CC	120	122 ± 0.8	125	127	131 ± 3.8	138
	HD	195	224 ± 3.0	299	279	283 ± 1.2	292
	50%Sen	260	290 ± 2.6	325	278	301 ± 4.8	329
2015	NDVI0.5	120	132 ± 0.8	145	135	140 ± 1.2	145
	CC	137	141 ± 1.0	164	147	153 ± 0.6	160
	HD	213	234 ± 1.8	263	283	286 ± 3.3	303
	50%Sen	268	284 ± 2.5	324	285	294 ± 1.8	300

Early canopy formation differed significantly between species groups (Table 10.2), although the extent of variation was similar among species. In both years the hybrids reached NDVI0.5 and CC earlier than the *M. sinensis* genotypes and were significantly earlier than the *M. sacchariflorus* genotypes in 2014. In 2015 the *M. sacchariflorus* and hybrid genotypes had a significantly earlier NDVI0.5 than the *M. sinensis* genotypes. NDVI0.5 was significantly correlated on a genotype level between both years ($r = 0.44$, $p < 0.01$). There was some variation in the day of approximate canopy

closure (CC) among the genotypes, on average the *M. sacchariflorus* and hybrid genotypes reached CC earlier than the *M. sinensis* genotypes, but within species variation was large (Fig. 10.5). The first genotype to reach CC was *M. sacchariflorus* OPM01 on day 132 and the last was *M. sinensis* OPM31, over a month later, on day 167.

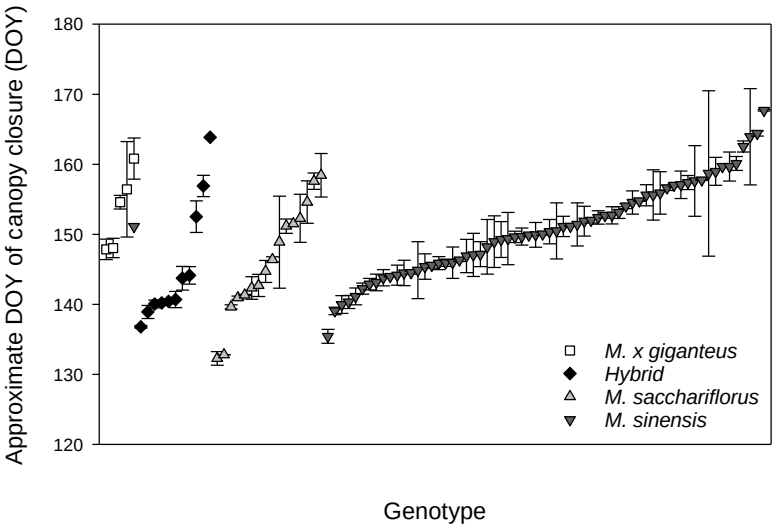


Figure 10.5: Average day of approximate canopy closure, calculated as the day the maximum was reached in the NDVI curve, in the 2015 growing season (determined by fitting sigmoid curves to the data) per genotype, ordered per species group from early to late canopy closure. Symbols show average values per genotype, different symbols indicate different species. Error bars show standard error (n=2).

Flowering

The germplasm displayed a wide variation in timing of flowering between genotypes and among species groups. None of the *M. sacchariflorus* genotypes in the trial flowered in any of the years (Table 9.3). Most, but not all, other genotypes did flower, and even the *M. x giganteus* genotypes initiated flowering both years. Hybrid genotypes had a significantly earlier heading date than the *M. sinensis* genotypes, which in turn had an earlier heading date than the *M. x giganteus* genotypes. Heading date varied between day 157 and 320 (June 6th and November 12th) in 2014 and day 205 and 324 (July 24th and November 20th) in 2015. This was after 232-1161 and 563-1045 GDD with a base temperature of 10°C in 2014 and 2015 respectively. Flowering was initiated under photoperiods ranging between 8.5 and 16.5 h day length. The time from flag leaf emergence to end of flowering was very long in some genotypes and ranged between 19 and 138 days in 2014 and 26 and 98 days in 2015. Flowering started later in 2015, but the same number of genotypes reached anthesis in 2014 as in 2015, in the hybrid and *M. sinensis* group (10 and 63 genotypes respectively). Three *M. sinensis* and hybrid genotypes did not flower, these genotypes also did not senesce properly but stayed green until their leaves were killed by frost. In the *M. x giganteus* group all five genotypes reached anthesis

in 2014, but only one in 2015. Overall, FL, HD and ANT were strongly correlated between both years (Fig. 10.6A).

Senescence

Timing and rate of senescence also varied widely between the genotypes. While some genotypes started to senesce in late summer and had completely senesced by October 1st, other genotypes did not senesce until late in winter or were even still partially green at harvest. Some genotypes senesced early, in September and October and appeared to be influenced by photoperiod, while other genotypes senesced late or over a long period and appeared to be more temperature driven. The exceptionally mild winter in 2014 and 2015 thus led to a slow progress of senescence in these latter genotypes. On average the plants reached 50%Sen around DOY 308 ± 22 in 2014 and DOY 299 ± 30 in 2015 (4/11/2014 and 26/10/2015). The hybrid genotypes senesced significantly earlier than the other species groups, while there was no difference between the other species. The rate at which the genotypes senesced varied considerably, some genotypes changed from more than 80% green to complete senescence in less than 30 days while in other genotypes the same senescence process lasted over 5 months. Two genotypes, OPM43 and OPM44 reached 50%Sen only in January in both years and did not fully senesce by harvest. 50%Sen was significantly correlated between both years (Fig. 10.6B), indicating a strong genotypic determination for this trait. In 2015 50%Sen was significantly correlated with heading date ($r = 0.51$, $p < 0.01$), while in 2014 this correlation was significant but not very strong ($r = 0.12$, $p < 0.05$).

Overall large variation in phenology was observed among the genotypes. There were genotypic differences in emergence, flowering and senescence. As a result, there was a large variation in the duration of vegetative growth and the canopy duration between the genotypes in the trial. The duration of vegetative growth, calculated as the time from NDVI0.5 to heading was on average 110 ± 1 days in 2014 and 107 ± 1 days in 2015. In 2014 canopy duration, calculated as the time between NDVI0.5 and 50%Sen was 183 ± 4 days compared to an average of 165 ± 2 days in 2015 (Fig. 10.7) due to later emergence and earlier senescence in 2015.

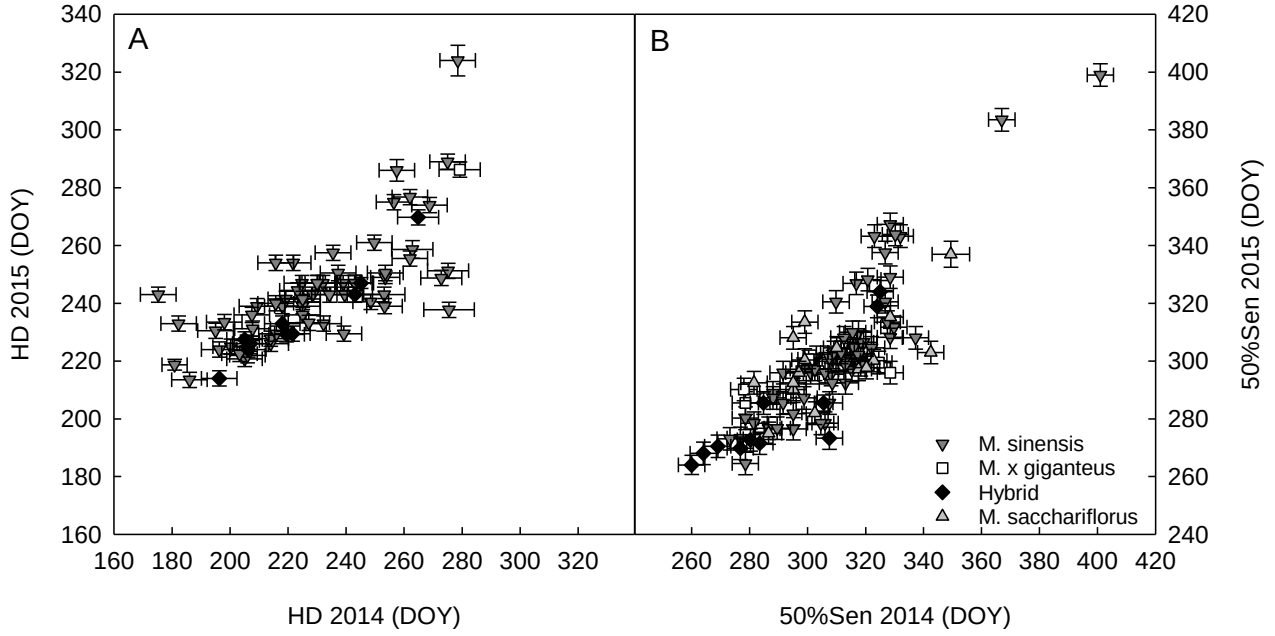


Figure 10.6: A: Correlation between heading date in 2014 (x-axis) and in 2015 (y-axis). B: Correlation in DOY 50% senescence per plot in 2014 (x-axis) and 2015 (y-axis). Each point depicts a genotype, different symbols indicate different species.

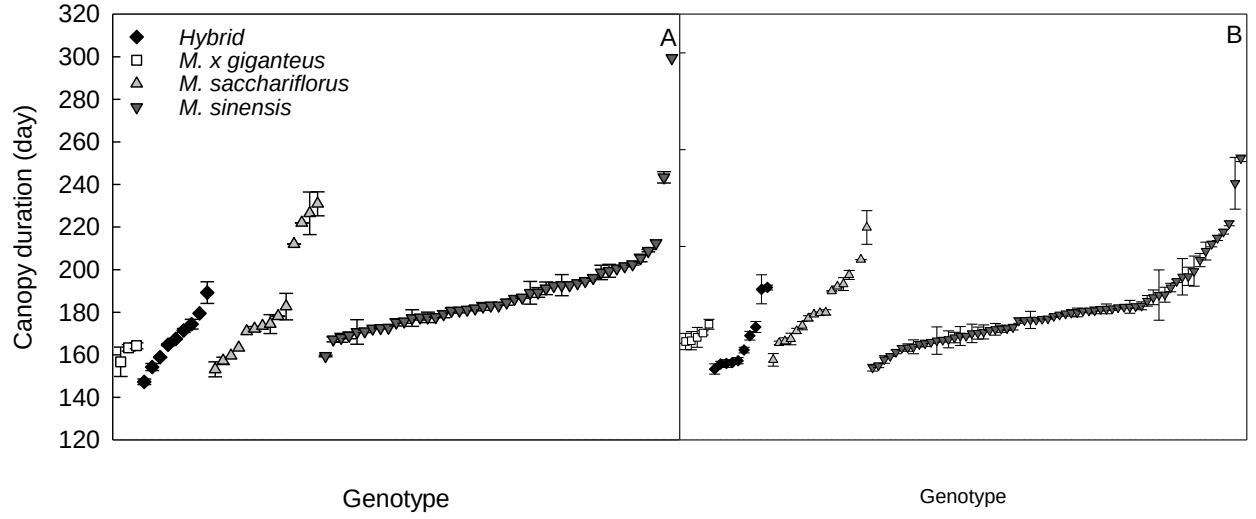


Figure 10.7: Average day of canopy duration in 2014 (A) and 2015 (B) per genotype, ordered per species group from short to long canopy duration. Symbols show average values per genotype, different symbols indicate different species. Error bars show standard error (n=2). In 2014 not all parameters could be measured on all genotypes, resulting in less genotypes shown in A.

Relationship between phenology and yield

Simple correlations of phenology and biomass yield

To avoid the effect of possible differences in establishment, only plants planted in May 2013 were used for further analyses regarding the relationship between morphology and phenology and yield. Consequently, only 69 genotypes were used for further analysis. The correlations between biomass yield and phenology traits were generally rather low (Table 10.3). NDVI0.5 was negatively correlated with yield in both years in *M. sacchariflorus*, but not in the other species groups. Early canopy formation was thus associated with yield in this group. The fact that NDVI0.5 was significantly correlated in *M. sacchariflorus* but not in *M. sinensis* was probably a result of the larger variation in early-season growth in *M. sacchariflorus* (see chapter seven). Of the flowering stages only FL was significantly correlated with yield over all species in 2014. Within *M. sinensis* or the hybrids, the correlations between flowering traits and yield were negative and not significant. There was thus no association between flowering and yield or between the duration of vegetative growth and yield, late flowering was certainly not associated with higher yield. The positive significant correlation of FL and yield over all genotypes in 2014 is a result of the flowering of the *M. x giganteus* genotypes in that year. Since these genotypes are high yielding and late flowering, they heavily influence the correlation. Senescence was not clearly related with yield either, except in the *M. sacchariflorus* genotypes, where late senescence was indeed positively correlated with yield. Canopy duration tended to be positively correlated with yield showing that longer canopy durations might indeed be somewhat associated with higher yields. Especially in the *M. sacchariflorus* genotypes the association between CanDur and yield was marked.

Table 10.3: Correlation between biomass yields per genotype and harvest components. Correlations marked with * are significant (Pearson's product-moment correlation, $p < 0.05$). No correlations were calculated for M. x giganteus as there were only 5 M. x giganteus genotypes.

		All species		*M. sinensis*		*M. sacchariflorus*		Hybrid	
		2014	2015	2014	2015	2014	2015	2014	2015
Morphology	Stem height (cm)	0.50*	0.54*	0.21	0.54*	0.48	0.76*	0.48	0.71*
	Stem% (%)	0.01	-0.10	-0.21	0.02	-0.19	-0.30	0.20	0.50
	DM% (%)	-0.26*	- 0.25*	-0.29	-0.08	-0.57*	-0.46	-0.05	-0.10
Phenology	NDVI0.5 (DOY)	-0.13	0.17	-0.03	0.03	-0.57*	-0.19	-0.21	0.69
	CC (DOY)	-0.00	0.19	0.12	0.14	-0.50	-0.08	-0.46	0.50
	FL (DOY)	0.37*	-0.03	0.00	-0.14	-	-	-0.02	-0.22
	HD (DOY)	0.24	0.01	-0.13	-0.05	-	-	-0.03	0.11
	ANT (DOY)	0.26	0.09	-0.11	0.03	-	-	-0.12	0.15
	Sen50 (DOY)	0.13	0.07	0.23	0.00	0.41	0.78*	0.15	0.06
	Sen90 (DOY)	0.24*	0.17	0.00	0.19	0.65*	0.67*	0.34	0.73*
	VegDur (days)	0.26	-0.03	-0.13	-0.07	-	-	0.06	-0.13
	CanDur (days)	0.20	0.05	0.31*	-0.01	0.46	0.71*	0.25	0.25

Interrelationships between growing season traits

Biomass yield is a complex trait determined by the complex interaction between numerous factors throughout the growing season. The duration of the growing season is determined both by the beginning and the end of the growing season. Both an earlier beginning or a later end can extend the duration of the growing season. Figure 10.8 shows that these factors are not independent from each other, genotypes with a later beginning of the growing season also tended to have a later growing season end. As was to be expected from the simple correlations in the previous sector, no clear trend between growing season duration and final biomass yield could be observed in our trial.

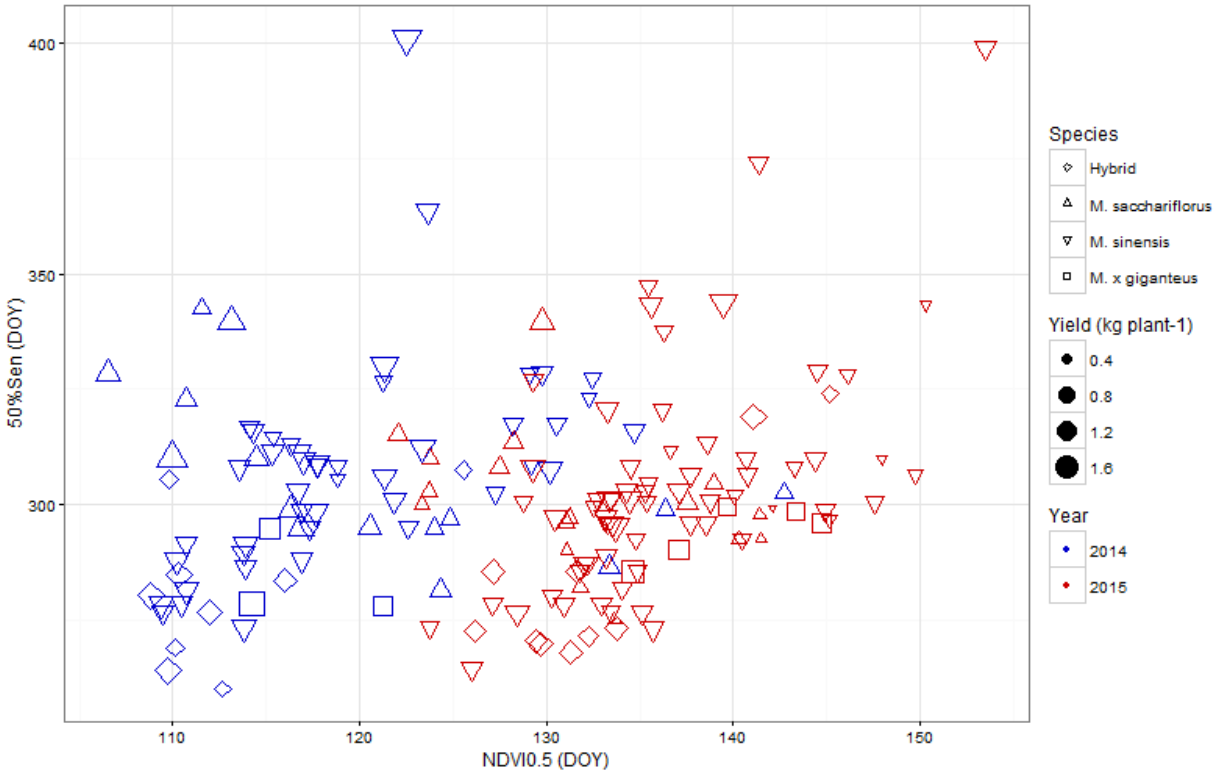


Figure 10.8: Interrelationship between early-season growth, the end of the growing season and final biomass yield in FT1 in 2014 and 2015. Colours show the two growing seasons, shapes distinguish the different species.

Discussion

Yield potential

The field trial demonstrated the availability of high yielding *M. sinensis* and *M. sacchariflorus* genotypes in the germplasm collections of European breeders. The collection, which was selected to be representative of breeder's germplasm, contains genotypes that are competitive with *M. x giganteus* in terms of biomass production in Belgium. These high yielding *M. sinensis* genotypes are

however not valuable candidate varieties to immediately replace *M. x giganteus* in the field, because vegetative propagation would be even more difficult because of their limited rhizomes (chapter three). They would however be interesting as breeding parents for high yielding seed based genotypes. Currently, *M. x giganteus* is generally propagated through rhizome cuttings or in vitro, making field establishment costly. The new varieties under development in breeding programs will be seed based, which is projected to significantly reduce propagation costs and increase propagation factors (Clifton-Brown et al., 2016; Xue et al., 2015). The observed yields ranged between 0.09 and 1.70 kg DM plant^{-1}. Expressed in ton per hectare this would amount, at the planting density of 20,000 plant ha^{-1} as the trial was planted, to yields ranging between 3 and 34 t DM ha^{-1}. These extrapolations from small plots to field level should of course be interpreted with caution due to the large variability between biomass plants in a field (Knörzer et al., 2013). Dry matter content of the biomass varied significantly between genotypes. The trial was harvested in both years at the moment that the dry matter content of *M. x giganteus* reached 80%. The large variation in dry matter content indicates that many genotypes had reached this harvestable dry matter content earlier in winter and could thus have been harvested earlier. Varieties with faster drying of the biomass in the field would be beneficial for farmers, as it allows more flexibility in the timing of harvest and reduces drying costs in years where *M. x giganteus* does not dry sufficiently and allows earlier emerging genotypes to benefit from the earlier emergence.

Variation in phenology

There was extensive variation in all phenological traits between the genotypes in the trial, which is consistent with the wide genetic variability in the germplasm and with earlier reports (Jensen et al., 2011; Robson et al., 2011; chapter six). Flowering was initiated, depending on the genotype, over a four-month period between June and September. Large variation has been reported in photoperiod and accumulated thermal time to initiate flowering (Jensen et al., 2011). The timing of flowering observed in our trial, between DOY 157 to 324, 232-1045 GDD_{10} or 16.5 to 8.5 h day length are very similar to the ranges of flowering diversity reported by (Jensen et al., 2011) who observed the onset of flowering to occur between DOY 160 to 329, 161 to 865 GDD_{10} or after photoperiods between 16.6 to 7.8 h in a trial with 244 miscanthus genotypes in Wales. *M. sacchariflorus* flowering is a quantitative short day response while *M. sinensis* is day neutral (Jensen et al., 2013). None of the *M. sacchariflorus* genotypes flowered in our trial indicating that the requirements for flower initiation for these genotypes were not met. This does not mean that no *M. sacchariflorus* genotype flowers under Belgian conditions as other *M. sacchariflorus* genotypes have been known to flower every year at our site (Van Hulle, unpublished data). Some genotypes had flowering periods stretching multiple months while other genotypes flowered shortly or did not flower at all. For breeding purposes, the

long flowering genotypes can be easily crossed with others, while the non-flowering genotypes will have to be forced to flower under controlled conditions if needed for breeding.

The 2014 and 2015 winters were exceptionally warm in Belgium, with little frost until late in winter. Senescence was thus not caused by frost but rather induced by low, above zero temperature or by changes in photoperiod. In genotypes for which senescence is temperature-driven or caused by frost, the senescence process was therefore likely delayed (Purdy et al., 2014). The fast senescence in some genotypes in early autumn is likely to be a result of the declining day length in that period or of an association with flowering, as a significant correlation between flowering and senescence was observed. While Robson et al. (2011) observed slower senescence in the *M. sinensis* genotypes compared to the *M. sacchariflorus* and *M. x giganteus* genotypes, this was not observed in our trial. The *M. sinensis* x *sacchariflorus* hybrid genotypes however senesced markedly earlier than the other species groups.

Relationship between phenology and biomass yield

Phenological traits were not strongly related with biomass yield in *M. sinensis* or in the *M. sinensis x sacchariflorus* hybrids, while in *M. sacchariflorus* an association between biomass yield and growing season duration was found. In *M. sacchariflorus* early emergence and late senescence was associated with higher biomass yields. Early emergence and early canopy formation allow plants to take maximum advantage of long days and large radiation sums in late spring and early summer (Monteith, 1977). The variation in emergence in our trial was limited however by the relatively late timing of the harvest, which was determined by the dry matter content of *M. x giganteus*. In FT2, as discussed in chapter nine, many of the genotypes that were also included in FT1 had already reached shoot lengths up to 40 cm by the time of harvest in FT1. The late harvest thus decreased the differences between the genotypes and might have masked the relationship between yield and early canopy formation in *M. sinensis* and hybrids. In *M. sacchariflorus* on the other hand the difference in emergence between the genotypes is especially large (chapter six) and a relationship between emergence and yield could still be observed. This shows that when new miscanthus varieties are introduced, harvest management will have to be optimized for these varieties, in order to maximize production. The low correlation between senescence and yield might be the result of the late senescence in both years. The autumns of 2014 and 2015 were exceptionally warm in Belgium, leading to delayed senescence. Most genotypes remained green until the end of October, differences in senescence between genotypes occurred mainly in November and December. After October days are short, temperatures and solar radiation are low and thus little extra biomass could be produced in genotypes that senesced later than others. Furthermore, green plants do not necessarily photosynthesize. There is not necessarily a strong link between senescence and photosynthesis, as

was recently shown in stay-green maize varieties (Swankaert et al., 2016). The stronger relationship between canopy duration and yield in *M. sacchariflorus* is thus likely a result of the large differences in early canopy formation in these genotypes, leading to large differences in canopy durations. Yield in these genotypes was only influenced by vegetative growth. In *M. sinensis* and hybrids on the other hand, flowering most likely had a confounding effect on the relationship between senescence and yield, although we did not observe an association between flowering and biomass yield.

Robson et al. (2013a) concluded from a field trial of 244 genotypes in Wales that long canopy durations are positively associated with biomass yield. They found both early emergence and late senescence to be associated with high yields. On the other hand, Zub et al. (2011) concluded from a trial with 21 genotypes in Northern France that high yielding genotypes emerge later and are mainly characterized by high growth rates in early summer and short durations of shoot growth. The results obtained in our study concur with those obtained by Zub et al. (2011). The different conclusions of the two studies could be due to morphological and phenological differences in the genotypes used, could be caused by the different methods to exactly determine growing season stages or could be a result of genotype by environment interactions. For example, in chapter seven we showed that emergence is relatively more variable in Wales compared to most other sites in the multilocation trials. It is possible that genotypes that are high yielding in the trials in Belgium or Northern France are less productive in the trial in Wales. A comparison of mean temperatures between the field trial site in Wales and Belgium is given in Table 3.3. For example, modelled yields for *M. x giganteus* in Wales are lower than those for Belgium/Northern France (Hastings et al., 2009). It is possible that in Belgium, genotypes adapted to warmer climates with high growth rates at high temperatures are able to outyield genotypes that are more adapted to cold regions which have an earlier emergence and later senescence. On the other hand, in Wales these cold adapted genotypes can take maximum advantage of the potential growing season while the warm adapted genotypes cannot reach their full potential. Later flowering is associated with higher biomass production under controlled conditions due to a longer growing period (Jensen et al., 2013), but this was not observed in our field trial, although the high yielding *M. x giganteus* genotypes do flower late and have a longer vegetative period than the *M. sinensis* genotypes, the longer vegetative period in *M. sacchariflorus* did not lead to higher yields compared to *M. sinensis*.

Implications for breeding

The large variation in yield and phenological traits indicates large potential for the improvement of miscanthus as a biomass crop. *M. sinensis, M. sacchariflorus* and hybrid genotypes that were as high yielding as *M. x giganteus* genotypes were observed in the trial and could serve as breeding material. Emergence, flowering and senescence were significantly correlated between years,

showing the genotypic determination of these phenological traits. This genotypic determination allows to optimize these traits through selective breeding. Similarly, Gifford et al. (2011) and Slavov et al. (2013) reported a high heritability of phenological traits such as heading date.

Morphological characteristics, such as tiller number, plant height, canopy height, stem diameter and plant diameter have been shown to be correlated with biomass yield in miscanthus (Jeżowski, 2008; Kaiser et al., 2015; Robson et al., 2013b; Zub et al., 2011). The studied germplasm in our trial is morphologically very diverse, but a strong correlation between height and yield was also observed. It is possible that this morphological diversity masked any contributions of phenology to yield. Larger gains in breeding for biomass are likely to be made by selecting for morphological traits rather than phenological traits. However, it is possible that when morphology has been optimized, phenological traits will become more important in breeding as was shown in this and the previous chapter.

While phenological traits did not necessarily have a large impact on biomass yield in our trial, optimization of these traits might be necessary for miscanthus agronomy and biomass quality. Early senescence was associated with lower moisture content and less leaves in the harvested biomass and was thus positively associated with biomass quality. Some genotypes senesced early, without low temperatures to induce senescence. The development of rapidly senescing, high quality genotypes should thus be possible. Potentially, genotypes that rapidly senesce in September could be harvested relatively dry in late autumn and be used as fuel in the same winter, which would decrease storage costs significantly. Although a number of high yielding genotypes were found in our trial, some of these genotypes also possessed agronomically undesirable traits for improved varieties, such as lodging, susceptibility to disease, vigorous spreading through rhizomes or delayed senescence. This shows that miscanthus is still a largely unimproved crop and that a number of undesirable traits will have to be removed by breeders. The large variation in flowering traits on the other hand could be beneficial for the public acceptance of miscanthus as a crop.

Chapter 11: General discussion and conclusions

As stated in the introduction chapter the general aims of this PhD thesis were:

1. To screen a broad miscanthus germplasm collection for frost tolerance.
2. To screen a broad miscanthus germplasm collection for chilling tolerance and early-season growth.
3. To determine the biochemical and physiological parameters underlying chilling tolerance.
4. To analyze the relationship between early-season growth and final biomass yield.

For each of these aspects we postulated several hypotheses and posed a number of research questions to guide the research.

Hypothesis 1: There exists a useful variation in frost tolerance in the genus *Miscanthus*, with genotypes with a lower LT_{50} than *M. x giganteus.*

RQ1: Does the variation in rhizome frost tolerance in miscanthus exceed −5°C?

We observed a substantial variation in rhizome frost tolerance among the tested genotypes. There was a difference of 4.5°C in LT_{50} between *M. sinensis* OPM44 the genotype with the lowest frost tolerance (LT_{50} = −0.4°C) and *M. sinensis* OPM64, the genotype most tolerant to frost (LT_{50} = −5.9°C) in our tests. The average LT_{50} for *M. x giganteus* was −2.6 ± 0.3°C. On average LT_{50} was −3.5 ± 0.1°C in *M. sinensis*, −2.6 ± 0.3 °C in *M. sacchariflorus* and −3.9 ± 0.2°C in the *M. sinensis x sacchariflorus* hybrids. While the *M. sinensis* and hybrid genotypes thus tended to have a better tolerance to frost, some *M. sacchariflorus* genotypes with good frost tolerance were also observed. Genotypes that can withstand lower temperatures than *M. x giganteus* are thus certainly available in the tested germplasm.

RQ2: How large is the variation in shoot frost tolerance in miscanthus?

Although shoot frost tolerance could only be investigated once, on March 28th 2014, it was clear that for this trait a large variation exists in the germplasm. After the cold spell a large range of frost stress sensitivity was observed. While some genotypes did not suffer any visible damage due to the frost event, other genotypes showed changes in leaf coloration, indicating a reduction in chlorophyll content, anthocyanin build-up and even photobleaching. The leaves of the most sensitive genotypes had been effectively killed off by the frost event. However, since the frost event took place early in the growing season, the shoot apices were most likely still below ground when the stress event occurred and consequently all plants were able to recover quickly. This was the first study to report shoot frost damage in a large miscanthus collection. Further research will be necessary to validate the results since we only observed one frost event, for example the effects of more severe or repeated frost events are likely to be more damaging than the one reported.

RQ3: Which phenological characteristics relate to frost tolerance in miscanthus?

The relationship between phenology and frost tolerance of rhizomes observed in our trial was not very strong. Heading date (r = 0.32 in *M. sinensis*) and the moment of 50% senescence (r = 0.33 and 0.72 in *M. sinensis* and *M. sacchariflorus* respectively) were significantly correlated with rhizome LT_{50} indicating that an earlier flowering and earlier senescence were linked to a higher frost tolerance. We did not observe a relationship between rhizome moisture content and LT_{50}.

Conclusion: Hypothesis 1 is supported. A large variation in frost tolerance was observed in the collection, both in the above ground and below ground parts of the plants. Many genotypes were found to be more tolerant than the tested *M. x giganteus* genotypes, especially among the *M. sinensis* and hybrids genotypes with higher frost tolerance were common. This wide variation in frost tolerance in the germplasm will offer breeders the possibility to develop new varieties with improved frost tolerance, given that frost tolerance is a sufficiently heritable trait and can be combined with high biomass yield. The observed lower limits of frost tolerance in miscanthus are similar to the lowest LT_{50} reported in Clifton-Brown and Lewandowski (2000) and would allow to plant miscanthus on a commercial scale in significantly colder areas than where *M. x giganteus* can be currently cultivated (Hastings et al., 2009), provided that the season characteristics are appropriate for cultivation, as discussed below.

Hypothesis 2: There exists a useful variation in chilling tolerance and early-season growth in the genus *Miscanthus*, with genotypes allowing an earlier growing season than *M. x giganteus*.

RQ4: How large is the variation in chilling tolerance and early-season growth in miscanthus?

We observed a substantial variation in chilling tolerance and early-season growth in miscanthus. We evaluated this in several experiments, both under controlled and under field conditions. We derived several parameters from these measurements in order to quantify and compare early-season growth. For example, early-season growth rates ranged between 0.6 and 2.8 mm GDD^{-1}, and plants reached a length of 50 cm between 690 and 1243 GDD in 2014 and 593 and 1300 GDD in 2015. A number of genotypes, from all species groups, could be identified which had stronger earlier growth than *M. x giganteus*. Compared to *M. x giganteus*, these genotypes emerged earlier and formed a canopy faster. Especially in the colder 2015 spring these differences were apparent.

RQ5: What is the most efficient method to measure chilling tolerance and early-season growth?

We have set up several experiments to measure chilling tolerance and early-season growth and applied various methods to quantify these traits, such as shoot growth measurements and chlorophyll fluorescence. A first comparison of methods indicated that shoot growth, leaf growth, measurement of photosynthesis and analysis of water soluble carbohydrates might be useful for screening (chapter four). Shoot growth was strongly correlated with plant biomass, as described in chapter five and allowed to derive efficient parameters for comparison of the genotypes (chapter six). Growth under controlled

conditions did not correlate well to growth in the field. Growth in the field is dependent on more factors than chilling tolerance alone, for example, rhizome reserves or photosynthetic capacity. Furthermore, the chosen growth chamber conditions might not have been representative enough to accurately reflect field conditions in spring. Overall, the most efficient way to screen large numbers of genotypes for early-season growth appears to be the measurement of shoot growth in field trials in the early growing season over multiple years.

RQ6: Can chilling tolerance and early-season growth be screened in growth chamber experiments?

Detailed measurements of photosynthesis and leaf growth allowed to distinguish the chilling tolerance of *M. x giganteus* IL10 and *M. sinensis* Goliath IL11 (chapter four). A comparison of leaf growth of a larger set of genotypes at 20°C and at 14°C under controlled conditions revealed a substantial reduction in growth at 14°C but did not reveal large differences in growth reduction among the genotypes. The growth under controlled conditions was not strongly correlated to growth in the field. Chlorophyll fluorescence measurements on the plants grown at 14°C showed that the photosynthetic capacity of none of the genotypes tested was markedly stressed at 14°C. This information was used in the setup of new experiments, outside of the research presented in this thesis, in which new genotypes, produced from crosses of *M. sinensis, M. sacchariflorus* and *M. lutarioriparius* accessions, were subjected to chilling stress at 8 and 12°C, where after chlorophyll fluorescence was measured. In this new study, groups of genotypes differing in cold tolerance could be distinguished clearly, however the relationship with field performance is still to be determined.

RQ7: How large is the genotype x environment effect on early-season growth?

The comparison of early-season growth in the OPTIMISC multilocation trials revealed strong genotype by environment interactions. Even when the observations from Adana, Turkey, where early-season growth was not determined by temperature but rather by water availability, were excluded from the dataset, these effects were still apparent. No genotype was the earliest or latest in all locations, showing the need for locally adapted varieties to optimally take advantage of local growing conditions. Several genotypes, such as OPM06, OPM11 and OPM14, did show a more consistent behaviour across locations, so it might also be possible to breed varieties that perform well consistently across locations.

Conclusion: Hypothesis 2 is supported. We observed a large variation in chilling tolerance and early-season growth in our germplasm collection. Our results indicate that chilling tolerance is just one of the parameters influencing early-season growth in the field, therefore screening of chilling tolerance alone is not sufficient to predict early-season growth under field conditions. The observed variation can potentially allow miscanthus breeders to breed for longer growing seasons and earlier canopy formation, which could theoretically increase biomass yields. As early-season growth traits were shown to have a relatively high heritability, breeding for these traits should be feasible.

Hypothesis 3: Variation in chilling tolerance in *Miscanthus* is linked to variation in biochemical and physiological traits.

RQ8: Which biochemical traits, such as ROS, PPDK or soluble sugars relate to chilling stress tolerance in miscanthus?

We analyzed a number of biochemical traits related to ROS, PPDK and soluble sugars in five extreme genotypes, representative of *M. sinensis, M. x giganteus*, and *M. sinensis x sacchariflorus* hybrids. The genotypes chosen for their assumed chilling tolerance were distinct in a number of these traits from the genotypes chosen as chilling sensitive. PCA analyses showed that chilling tolerant genotypes were characterized by higher levels of malondialdehyde, raffinose, sucrose and higher catalase activity while the chilling sensitive genotypes were characterized by higher concentrations of glucose, fructose and higher pyruvate-Pi-dikinase activity later in the growing season. *M. x giganteus* was intermediate between both groups. We have therefore identified certain biochemical traits underlying chilling tolerance which can be used to distinguish extreme genotypes. Of these traits, WSC appear to be most promising as marker trait for the identification of chilling tolerant genotypes.

Conclusion: Hypothesis 3 is supported. We observed distinct differences in biochemical traits between the genotypes chosen as chilling tolerant and those chosen as chilling sensitive. If these differences can be proven to be also related to chilling tolerance in a larger germplasm collection, they could potentially be used to screen for chilling tolerant genotypes in the early stages of breeding and selection.

Hypothesis 4: Increased cold tolerance and early-season growth are linked with increased biomass yield.

RQ9: What is the relationship between growing season duration and final yield?

We found that long growing season durations were generally associated with lower biomass yields, since genotypes with late senescence were somewhat lower yielding than earlier senescing genotypes. Heading date, which marks the end of vegetative growth, was not markedly associated with biomass yield. The duration of shoot growth and the end of shoot growth were significantly correlated with biomass yield, the genotypes that reached their maximum height earlier tended to be higher yielding. Most genotypes reached their maximum heights by August. After that date it is unlikely that substantial amounts of extra biomass were produced. The end of the growing season is thus not likely important for yield in Belgium. The end of the growing season is likely only important in terms of nutrient remobilization to the rhizomes and biomass quality at harvest.

RQ10: What is the relationship between early-season growth and final yield?

We found that genotypes with strong early-season growth were generally higher yielding. The genotypes that reached higher shoot lengths early in the season and developed their canopy faster had higher final biomass yields. The relationship was not very strong however and explained only a relatively small part

of the variation in yield. Morphological traits, such as shoot number or shoot length, are more direct targets for biomass yield optimization in breeding. Early-season growth is also related to the amount of biomass plants can produce and so also to morphological traits such as shoot length and shoot number.

Conclusion: Hypothesis 4 is partially supported. Strong early-season growth appeared to be linked with high final biomass yields in both field trials, however we did not demonstrate a link between cold tolerance in se and final biomass yield.

General conclusion

A large and useful variation in cold tolerance traits was observed in the miscanthus germplasm studied. Several genotypes were identified with higher frost tolerance than the currently used *M. x giganteus*. Several large scale screening methods were tested to identify these genotypes. To distinguish frost tolerant genotypes, regrowth assays proved most useful. To detect genotypes with high early-season growth, measuring shoot growth in field trials in spring proved the best method. Chilling tolerant genotypes could be distinguished from chilling sensitive genotypes on a biochemical level when growing under field conditions. *M. x giganteus* was intermediate between chilling tolerant and chilling sensitive genotypes. Genotypes with early-season growth might be higher yielding, in our field trial there were significant correlations between strong early-season growth and high biomass yield.

Further research

In this thesis a broad overview of the variation in chilling and frost tolerance was obtained, but there are still numerous questions to be answered about miscanthus cold tolerance. Below, a few suggestions for further research are discussed.

There was a high variation in morphology in our germplasm, which may have had a strong influence on our results and masked some effects of early-season growth. A study with a full-sib population derived from a cross between morphologically similar genotypes differing in cold tolerance and early-season growth might be advisable in the future to overcome this confounding effect. The recent progress made in miscanthus seed production makes this now a likely option in future studies. This would allow to better determine the relationship between early-season growth, cold tolerance and final biomass yield. Additionally, this population could also be used for a QTL analysis which could assist and accelerate breeding if DNA polymorphisms correlated with early-season growth traits and cold tolerance can be found.

No secondary traits that would be efficient marker traits for frost tolerance were found in our study, but a detailed metabolical analysis of the rhizomes of genotypes with high and low frost tolerance looking at specific cold tolerance related traits, such as proline, raffinose or membrane saturation could still reveal useful traits for fast screening. Acclimation should also be taken into account in such research as this is likely to have an impact on these metabolites. Substantial variation in frost

tolerance was found in the germplasm, but recent winters were mild and very little winter mortality was observed in our field trials and in those of the OPTIMISC multi-location trials. Further research is necessary to determine the effect of different harvest dates on rhizome frost tolerance. The genotypes determined as frost tolerant by our screening should be trialed in a location where frost stress is likely to occur in the first winter in order to determine the value of the screening test developed here.

Although we did not find large differences in chlorophyll fluorescence in our experiments under the conditions used in the growth chambers, a screening at lower temperatures (8 and 12°C) of the miscanthus germplasm based on our experiences did reveal significant differences (Muenich et al., 2016). In future research chlorophyll fluorescence might thus still be a useful option for screening chilling tolerant genotypes. However, as the plants could not be grown in the growth chambers at 12°C, it would thus be best to grow plants at higher temperatures (20°C), let them acclimate to low temperature (15°C) and then expose them to chilling stress (10°C).

The biochemical analysis revealed interesting traits that distinguish chilling tolerant and chilling sensitive genotypes. A larger scale screening of these traits would be of interest to test if these traits can be used as early markers for chilling tolerance. Similarly as Purdy et al. (2015) who proposed the use of WSC as marker traits for biomass yield. The variation between seedlings, early established and mature plants will be important to take into account. If carbohydrate composition can be proven to be similar between early and later growth phases, these traits might be useful markers for rapid early selection of novel genotypes. Further research will be also necessary to unravel the mechanisms that lead to these observed differences in order to better understand which traits can best be used as markers for chilling tolerance.

As this PhD project was part of a project involving the leading European miscanthus breeders, the results obtained in our field trials are readily applicable for the development of new varieties. Crosses of the best *M. sinensis* genotypes or highest yielding *M. sinensis* genotypes or between the best *M. sinensis* and *M. sacchariflorus* genotypes might yield seed based genotypes that are competitive with *M. x giganteus*. Although most *M. sinensis* genotypes were in general lower yielding than *M. x giganteus*, the lower costs of seed propagation would make cultivation of the new varieties competitive with *M. x giganteus* (Clifton-Brown et al., 2016).

Defining an ideotype

The research presented in this thesis allow us to think about a miscanthus ideotype that could guide further miscanthus breeding efforts. As a biomass crop, the most important trait of this ideotype would be high biomass yield, which generally means a tall plant with a high shoot number (chapter nine and ten). Additionally, the biomass should be of high quality, i.e. low moisture, ash and leaf content. Our research indicates that high yields are associated with strong growth early in the year, high shoot growth rates and a short duration of shoot growth (chapters five, six, seven,

nine and ten). The ideotype should have a high carbon assimilation rate (chapters five and eight). Since no strong relationship between heading date, senescence and biomass yield was found (chapter ten), the ideotype should flower and senesce relatively early. This would improve biomass quality (chapter ten) and would improve frost tolerance (chapter four). The ideotype should be free of the undesirable traits that were observed in a number of our genotypes, such as lodging, disease susceptibility, spreading through rhizomes or stay green (chapter ten). Furthermore, depending on the zone it is intended for, the ideotype should be highly tolerant to local abiotic stress conditions. In cold areas the ideotype should have a high rhizome and shoot frost tolerance (chapter four) in order to avoid winter mortality and shoot damage by a late frost event that would likely be associated with early emergence. Since water availability is the main limit on biomass production in miscanthus (Heaton et al., 2004), the ideotype should have a high water use efficiency. The ideotype should be propagated by seeds, to keep establishment costs down. We identified high yielding genotypes in all species tested. The ideotype should thus not necessarily be a *M. sinensis x sacchariflorus* hybrid. Almost all *M. sacchariflorus* genotypes in our trials had creeping rhizomes, which might create problems at the field borders. While spreading at the borders is undesirable, this might be overcome with a border of grass mown several times through the year. The spreading habit of *M. sacchariflorus* also allows it to fill in gaps in the field that might be caused by bad establishment or winter mortality. The spreading habit also makes the rhizomes easier to harvest and vegetative propagation of *M. sacchariflorus* is likely to be cheaper and easier compared to *M. x giganteus*. The *M. sinensis* genotypes on the other hand did not spread and are more promising breeding material for further improvement. *M. sinensis* does not have a spreading habit and would thus be more suitable for cultivation. It forms few rhizomes however, so commercial *M. sinensis* varieties would only be seed propagated.

Bibliography

Aebi, H., 1984. Catalase *in vitro*. Methods in Enzymology. Methods Enzymol. 105, 121 –126.

Agüera, F., Villalobos, F.., Orgaz, F., 1997. Evaluation of sunflower (*Helianthus annuus*, L.) genotypes differing in early vigour using a simulation model. Eur. J. Agron. 7, 109–118. doi:10.1016/S1161-0301(97)00023-3

An, G.-H., Miyakawa, S., Kawahara, A., Osaki, M., Ezawa, T., 2008. Community structure of arbuscular mycorrhizal fungi associated with pioneer grass species *Miscanthus sinensis* in acid sulfate soils: Habitat segregation along pH gradients. Soil Sci. Plant Nutr. 54, 517–528. doi:10.1111/j.1747-0765.2008.00267.x

Anderson, E., Arundale, R., Maughan, M., Oladeinde, A., Wycislo, A., Voigt, T., 2011. Growth and agronomy of *Miscanthus x giganteus* for biomass production. Biofuels 2, 167–183.

Angelini, L.G., Ceccarini, L., Nassi o Di Nasso, N., Bonari, E., 2009. Comparison of *Arundo donax* L. and *Miscanthus x giganteus* in a long-term field experiment in Central Italy: Analysis of productive characteristics and energy balance. Biomass and Bioenergy 33, 635–643. doi:10.1016/j.biombioe.2008.10.005

Anzoua, K.G., Suzuki, K., Fujita, S., Toma, Y., Yamada, T., 2015. Evaluation of morphological traits, winter survival and biomass potential in wild Japanese *Miscanthus sinensis* Anderss. populations in northern Japan. Grassl. Sci. 61, 83–91. doi:10.1111/grs.12085

Arnoult, S., Obeuf, A., Béthencourt, L., Mansard, M.-C., Brancourt-Hulmel, M., 2015. Miscanthus clones for cellulosic bioethanol production: Relationships between biomass production, biomass production components, and biomass chemical composition. Ind. Crops Prod. 63, 316–328. doi:10.1016/j.indcrop.2014.10.011

Aroca, R., Irigoyen, J., Sánchez-Díaz, M., 2001. Photosynthetic characteristics and protective mechanisms against oxidative stress during chilling and subsequent recovery in two maize varieties differing in chilling sensitivity. Plant Sci. 161, 719–726.

Arredondo, J.T., Schnyder, H., 2003. Components of leaf elongation rate and their relationship to specific leaf area in contrasting grasses. New Phytol. 158, 305–314. doi:10.1046/j.1469-8137.2003.00745.x

Arundale, R.A., Dohleman, F.G., Heaton, E.A., Mcgrath, J.M., Voigt, T.B., Long, S.P., 2014. Yields of *Miscanthus x giganteus* and Panicum virgatum decline with stand age in the Midwestern USA. GCB Bioenergy 6, 1–13. doi:10.1111/gcbb.12077

Barre, P., Turner, L., Escobar-Gutiérrez, A., 2015. Leaf length variation in perennial forage grasses. Agriculture 5, 682–696. doi:10.3390/agriculture5030682

Baxter, X.C., Darvell, L.I., Jones, J.M., Barraclough, T., Yates, N.E., Shield, I., 2014. Miscanthus combustion properties and variations with *Miscanthus* agronomy. Fuel 117, 851–869. doi:10.1016/j.fuel.2013.09.003

Beale, C.V., Bint, D.A., Long, S.P., 1996. Leaf photosynthesis in the C4-grass *Miscanthus x giganteus*, growing in the cool temperate climate of southern England. J. Exp. Bot. 47, 267–273.

Belintani, N.G., Guerzoni, J.T.S., Moreira, R.M.P., Vieira, L.G.E., 2012. Improving low-temperature tolerance in sugarcane by expressing the ipt gene under a cold inducible promoter. Biol. Plant. 56, 71–77. doi:10.1007/s10535-012-0018-1

Bhosale, S.U., Rymen, B., Beemster, G.T.S., Melchinger, a E., Reif, J.C., 2007. Chilling tolerance of Central European maize lines and their factorial crosses. Ann. Bot. 100, 1315–21. doi:10.1093/aob/mcm215

Bradford, M.M., 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Qantities of Protein Utilizing the Priciple of Protein-Dye Binding. Anal. Biochem. 72, 248–254.

Bultynck, L., Ter Steege, M.W., Schortemeyer, M., Poot, P., Lambers, H., 2004. From individual leaf elongation to whole shoot leaf area expansion: a comparison of three *Aegilops* and two *Triticum* species. Ann. Bot. 94, 99–108. doi:10.1093/aob/mch110

Cannell, M.G.R., Milne, R., Sheppard, L.J., Unsworth, M.H., 1987. Radiation interception and productivity in willow. J. Appl. Ecol. 24, 261–278. doi:10.2307/2403803

Carter, P.R., 1995. Late spring frost and postfrost clipping effect on corn growth and yield. J. Prod. Agric. 8, 203–209.

Chenu, K., Chapman, S.C., Hammer, G.L., McLean, G., Salah, H.B.H., Tardieu, F., 2008. Short-term responses of leaf growth rate to water deficit scale up to whole-plant and crop levels: an integrated modelling approach in maize. Plant, Cell Environ. 31, 378–91. doi:10.1111/j.1365-3040.2007.01772.x

Chou, C., Chang, F., 1988. Population study of *Miscanthus floridulus* II. Ecotypic variation of *M. floridulus* and *M. transmorrisonensis* as affected by altitude in Nantou, Taiwan. Bot. Bull. Acad. Sin. 29, 301–314.

Chou, C.-H., Lee, Y.-F., Chiu, C.Y., Wang, Y.C., Hsu, F.H., 1991. Growth performance of *M. floridulus* and *M. transmorrisonensis* and their acclimation to temperatures and water stresses. Bot. Bull. Acad. Sin. 32, 87–96.

Christian, D.G., Haase, E., 2001. Agronomy of Miscanthus, in: Jones, M.B., Walsh, M. (Eds.), Miscanthus for Energy and Fibre. Earthscan, London, pp. 21–45.

Clevering, O.A., Brix, H., Lukavská, J., 2001. Geographic variation in growth responses in *Phragmites australis*. Aquat. Bot. 69, 89–108.

Clifton-Brown, J.C., Jones, M.B., 1997. The thermal response of leaf extension rate in genotypes of the C4-grass *Miscanthu*s: an important factor in determining the potential productivity of different genotypes. J. Exp. Bot. 48, 1573–1581.

Clifton-Brown, J.C., Lewandowski, I., 2000. Overwintering problems of newly established *Miscanthus* plantations can be overcome by identifying genotypes with improved rhizome cold tolerance. New Phytol. 148, 287–294. doi:10.1046/j.1469-8137.2000.00764.x

Clifton-Brown, J.C., Neilson, B., Lewandowski, I., Jones, M.B., 2000. The modelled productivity of *Miscanthus x giganteus* (GREEF et DEU) in Ireland. Ind. Crops Prod. 12, 97–109.

Clifton-Brown, J.C., Lewandowski, I., Andersson, B., Basch, G., Christian, D.G., Kjeldsen, J.B., Jørgensen, U., Mortensen, J. V., Riche, A.B., Schwarz, K.-U., Tayebi, K., Teixeira, F., 2001a. Performance of 15 *Miscanthus* genotypes at five sites in Europe. Agron. J. 93, 1013–1019.

Clifton-Brown, J.C., Long, S.P., Jørgensen, U., 2001b. *Miscanthus* productivity, in: Jones, M.B., Walsh, M. (Eds.), Miscanthus for Energy and Fibre. Earthscan, London, pp. 46–67.

Clifton-Brown, J.C., Lewandowski, I., 2002. Screening *Miscanthus* genotypes in field trials to optimise biomass yield and quality in Southern Germany. Eur. J. Agron. 16, 97–110. doi:10.1016/S1161-0301(01)00120-4

Clifton-Brown, J.C., Stampfl, P.F., Jones, M.B., 2004. *Miscanthus* biomass production for energy in Europe and its potential contribution to decreasing fossil fuel carbon emissions. Glob. Chang. Biol. 10, 509–518. doi:10.1111/j.1529-8817.2003.00749.x

Clifton-Brown, J., Schwarz, K.-U., Hastings, A., 2015. History of the development of Miscanthus as a bioenergy crop: from small beginnings to potential realisation. Biol. Environ. Proc. R. Irish Acad. 115B, 1–13. doi:10.3318/BIOE.2015.05

Clifton-Brown, J., Hastings, A., Mos, M., McCalmont, J. P., Ashman, C., Awty-Carroll, D., Cerazy, J., Chiang, Y.-C., Cosentino, S., Cracroft-Eley, W., Scurlock, J., Donnison, I. S., Glover, C., Gołąb, I., Greef, J. M., Gwyn, J., Harding, G., Hayes, C., Helios, W., Hsu, T.-W., Huang, L. S., Jeżowski, S., Kim, D.-S., Kiesel, A., Kotecki, A., Krzyzak, J., Lewandowski, I., Lim, S. H., Liu, J., Loosely, M., Meyer, H., Murphy-Bokern, D., Nelson, W., Pogrzeba, M., Robinson, G., Robson, P., Rogers, C., Scalici, G., Schuele, H., Shafiei, R., Shevchuk, O., Schwarz, K.-U., Squance, M., Swaller, T., Thornton, J., Truckses, T., Botnari, V., Vizir, I., Wagner, M., Warren, R., Webster, R., Yamada, T., Youell, S., Xi, Q., Zong, J. and Flavell, R., 2016, Progress in upscaling Miscanthus biomass production for the European bio-economy with seed-based hybrids. GCB Bioenergy. doi:10.1111/gcbb.12357

Davey, C.L., Jones, L.E., Squance, M., Purdy, S.J., Maddison, A.L., Cunniff, J., Donnison, I., Clifton-Brown, J., 2015. Radiation capture and conversion efficiencies of *Miscanthus sacchariflorus, M. sinensis* and their naturally occurring hybrid M. x giganteus. GCB Bioenergy n/a-n/a. doi:10.1111/gcbb.12331

de Souza, A.P., Arundale, R. a., Dohleman, F.G., Long, S.P., Buckeridge, M.S., 2013. Will the exceptional productivity of *Miscanthus x giganteus* increase further under rising atmospheric CO2? Agric. For. Meteorol. 171–172, 82–92. doi:10.1016/j.agrformet.2012.11.006

Demmig-Adams, B., Adams, W.W., 2006. Photoprotection in an ecological context: The remarkable complexity of thermal energy dissipation. New Phytol. 172, 11–21. doi:10.1111/j.1469-8137.2006.01835.x

Doerffling, K., Doerffling, H., Lesselich, G., Luck, E., Melz, G., 1997. Improvement of frost tolerance in winter wheat by *in vitro* selection of proline-overproducing mutants. Acta Agron. Hungarica 45, 295–299.

Dohleman, F.G., Long, S.P., 2009. More productive than maize in the Midwest: How does Miscanthus do it? Plant Physiol. 150, 2104–15. doi:10.1104/pp.109.139162

Domon, J.-M., Baldwin, L., Acket, S., Caudeville, E., Arnoult, S., Zub, H., Gillet, F., Lejeune-Hénaut, I., Brancourt-Hulmel, M., Pelloux, J., Rayon, C., 2013. Cell wall compositional modifications of *Miscanthus* ecotypes in response to cold acclimation. Phytochemistry 85, 51–61. doi:10.1016/j.phytochem.2012.09.001

Du, Y., Nose, A., Wasano, K., 1999. Effects of chilling temperature on photosynthetic rates, photosynthetic enzyme activities and metabolite levels in leaves of three sugarcane species. Plant, Cell Environ. 22, 317–324.

Du, Y., Nose, A., 2002. Effects of chilling temperature on the activity of enzymes of sucrose synthesis and the accumulation of saccharides in leaves of three sugarcane cultivars differing in cold sensitivity. Photosynthetica 40, 389–395.

Dwiyanti, M.S., Stewart, J.R., Nishiwaki, A., Yamada, T., 2014. Natural variation in *Miscanthus sinensis* seed germination under low temperatures. Grassl. Sci. 60, 194–198. doi:10.1111/grs.12051

Eitzinger, J., Kössler, C., 2002. Microclimatological characteristics of a miscanthus (*Miscanthus cv. giganteus*) stand during stable conditionsat night in the nonvegetative winter period. Theor. Appl. Climatol. 72, 245–257. doi:10.1007/s00704-002-0681-9

Ensminger, I., Busch, F., Huner, N., 2006. Photostasis and cold acclimation: sensing low temperature through photosynthesis. Physiol. Plant. 126, 28–44. doi:10.1111/j.1399-3054.2005.00627.x

Equiza, M., Mirave, J., Tognetti, J., 1997. Differential inhibition of shoot vs. root growth at low temperature and its relationship with carbohydrate accumulation in different wheat cultivars. Ann. Bot. 80, 657–663.

Ezaki, B., Nagao, E., Yamamoto, Y., Nakashima, S., Enomoto, T., 2008. Wild plants, *Andropogon virginicus* L. and *Miscanthus sinensis* Anders, are tolerant to multiple stresses including aluminum, heavy metals and oxidative stresses. Plant Cell Rep. 27, 951–61. doi:10.1007/s00299-007-0503-8

Farage, P.K., Blowers, D., Long, S.P., Baker, N.R., 2006. Low growth temperatures modify the efficiency of light use by photosystem II for CO_2 assimilation in leaves of two chilling-tolerant C4 species, *Cyperus longus* L. and *Miscanthus x giganteus*. Plant, Cell Environ. 29, 720–728. doi:10.1111/j.1365-3040.2005.01460.x

Farrell, A.D., Clifton-Brown, J.C., Lewandowski, I., Jones, M.B., 2006. Genotypic variation in cold tolerance influences the yield of *Miscanthus*. Ann. Appl. Biol. 149, 337–345. doi:10.1111/j.1744-7348.2006.00099.x

Fonteyne, S., Lootens, P., Muylle, H., Van den Ende, W., De Swaef, T., Reheul, D., Roldán-Ruiz, I., 2016a. Chilling tolerance and early vigor related characteristics evaluated in two *Miscanthus* genotypes. Photosynthetica 54, 295–306. doi:10.1007/s11099-016-0193-y

Fonteyne, S., Muylle, H., De Swaef, T., Reheul, D., Roldán-Ruiz, I., Lootens, P., 2016b. How low can you go?—Rhizome and shoot frost tolerance in miscanthus germplasm. Ind. Crops Prod. 89, 323–331. doi:10.1016/j.indcrop.2016.05.031

Fonteyne, S., Roldan-Ruiz, I., H., M., T., D.S., D., R., P., L., 2016c. A review of frost and chilling stress in *Miscanthu*s and its importance to biomass yield, in: Barth, S., Murphy-Bokern, D., Kalinina, O., Taylor, G., Jones, M. (Eds.), Perennial Biomass Crops for a Resource Constrained World. Springer New York, New York.

Foyer, C.H., Vanacker, H., Gomez, L.D., Harbinson, J., Gom, L.D., 2002. Regulation of photosynthesis and antioxidant metabolism in maize leaves at optimal and chilling temperatures: Review. Plant Physiol. Biochem. 40, 659–668.

Fracheboud, Y., Haldimann, P., Leipner, J., Stamp, P., 1999. Chlorophyll fluorescence as a selection tool for cold tolerance of photosynthesis in maize (*Zea mays* L.). J. Exp. Bot. 50, 1533–1540. doi:10.1093/jxb/50.338.1533

Franks, C.D., Burow, G.B., Burke, J.J., 2006. A comparison of U.S. and Chinese sorghum germplasm for early-season cold tolerance. Crop Sci. 46, 1371. doi:10.2135/cropsci2005.08-0279

Friesen, P.C., Peixoto, M.M., Busch, F.A., Johnson, D.C., Sage, R.F., 2014. Chilling and frost tolerance in *Miscanthus* and *Saccharum* genotypes bred for cool temperate climates. J. Exp. Bot. 63, 3749–3758. doi:10.1093/jxb/eru105

Friesen, P.C., Peixoto, M.M., Lee, D.K., Sage, R.F., 2015. Sub-zero cold tolerance of *Spartina pectinata* (prairie cordgrass) and *Miscanthus x giganteus*: candidate bioenergy crops for cool temperate climates. J. Exp. Bot. 66, 4403–4413. doi:10.1093/jxb/erv085

Fritz, M., Formowitz, B., Jodl, S., Eppel-Hotz, A., Kuhn, W., 2009. Miscanthus als Nachwachsender Rohstoff - Ergebnisse aus bayerischen Forschungsarbeiten.

Fryer, M., Andrews, J., Oxborough, K., Blowers, D., Baker, N., 1998. Relationship between CO_2 Assimilation, Photosynthetic Electron Transport, and Active O_2 Metabolism in Leaves of Maize in the Field during Periods of Low Temperature. Plant Physiol. 116, 571–80.

Gaddum, J.H., n.d. Probit Analysis. doi:10.1038/161417a0

Gauder, M., Graeff-Hönninger, S., Lewandowski, I., Claupein, W., 2012. Long-term yield and performance of 15 different *Miscanthus* genotypes in southwest Germany. Ann. Appl. Biol. 160, 126–136. doi:10.1111/j.1744-7348.2011.00526.x

Genty, B., Briantais, J.-M., Baker, N.R., 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim. Biophys. Acta 990, 87–92. doi:10.1016/S0304-4165(89)80016-9

Gifford, J.M., Chae, W.B., Swaminathan, K., Moose, S.P., Juvik, J.A., 2015. Mapping the genome of *Miscanthus sinensis* for QTL associated with biomass productivity. GCB Bioenergy 7, 797–810. doi:10.1111/gcbb.12201

Głowacka, K., Adhikari, S., Peng, J., Gifford, J., Juvik, J.A., Long, S.P., Sacks, E.J., 2014a. Variation in chilling tolerance for photosynthesis and leaf extension growth among genotypes related to the C4 grass *Miscanthus x giganteus*. J. Exp. Bot. 65, 5267–78.

Głowacka, K., Clark, L. V., Adhikari, S., Peng, J., Ryan Stewart, J., Nishiwaki, A., Yamada, T., Jørgensen, U., Hodkinson, T.R., Gifford, J., Juvik, J.A., Sacks, E.J., 2014b. Genetic variation in *Miscanthus x giganteus* and the importance of estimating genetic distance thresholds for differentiating clones. GCB Bioenergy 7, 386–404. doi:10.1111/gcbb.12166

doi:10.1093/jxb/eru287

Głowacka, K., Ahmed, A., Sharma, S., Abbott, T., Comstock, J.C., Comstock, J.C., Long, S.P., Sacks, E.J., 2015a. Can chilling tolerance of C4 photosynthesis in *Miscanthus* be transferred to sugarcane? GCB Bioenergy n/a-n/a. doi:10.1111/gcbb.12283

Głowacka, K., Jorgensen, U., Kjeldsen, J.B., Korup, K., Spitz, I., Sacks, E.J., Long, S.P., 2015b. Can the exceptional chilling tolerance of C4 photosynthesis found in *Miscanthus x giganteus* be exceeded? Screening of a novel Miscanthus Japanese germplasm collection. Ann. Bot. 115, 981–990. doi:10.1093/aob/mcv035

Gray, G.R., Heath, D., 2005. A global reorganization of the metabolome in *Arabidopsis* during cold acclimation is revealed by metabolic fingerprinting. Physiol. Plant. 124, 236–248. doi:10.1111/j.1399-3054.2005.00507.x

Greef, J., Deuter, M., 1993. Syntaxonomy of *Miscanthus x giganteus* GREEF et DEU. Angew. Bot. 67, 87–90.

Hastings, A., Clifton-Brown, J., Wattenbach, M., Mitchell, C.P., Smith, P., 2009a. The development of MISCANFOR, a new *Miscanthus* crop growth model: towards more robust yield predictions under different climatic and soil conditions. GCB Bioenergy 1, 154–170. doi:10.1111/j.1757-1707.2009.01007.x

Hastings, A., Clifton-Brown, J., Wattenbach, M., Mitchell, C.P., Stampfl, P., Smith, P., 2009b. Future energy potential of *Miscanthus* in Europe. GCB Bioenergy 1, 180–196. doi:10.1111/j.1757-1707.2009.01012.x

Heaton, E., Voigt, T., Long, S.P., 2004. A quantitative review comparing the yields of two candidate C4 perennial biomass crops in relation to nitrogen, temperature and water. Biomass and Bioenergy 27, 21–30. doi:10.1016/j.biombioe.2003.10.005

Heaton, E.A., Dohleman, F.G., Miguez, A.F., Juvik, J.A., Lozovaya, V., Widholm, J., Zabotina, O.A., Mcisaac, G.F., David, M.B., Voigt, T.B., Boersma, N.N., Long, S.P., 2010. Miscanthus: A promising biomass crop. Adv. Bot. Res. 56, 76–124. doi:10.1016/B978-0-12-381518-7.00003-0

Himken, M., Lammel, J., Neukirchen, D., Czypionka-Krause, U., Olfs, H.W., 1997. Cultivation of Miscanthus under West European conditions: Seasonal changes in dry matter production, nutrient uptake and remobilization. Plant Soil 189, 117–126. doi:10.1023/A:1004244614537

Hodges, D., Andrews, C., 1997. Antioxidant enzyme responses to chilling stress in differentially sensitive inbred maize lines. J. Exp. Bot. 48, 1105–1113.

Hodges, D.M., DeLong, J.M., Forney, C.F., Prange, R.K., 1999. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta 207, 604–611. doi:10.1007/s004250050524

Hodkinson, T.R., Chase, M.W., Renvoize, S.A., 2002. Characterization of a Genetic Resource Collection for *Miscanthus* (*Saccharinae, Andropogoneae, Poaceae*) using AFLP and ISSR PCR. Ann. Bot. 89, 627–636. doi:10.1093/aob/mcf091

Hope, H., McElroy, A., 1990. Low-temperature tolerance of switchgrass (*Panicum virgatum* L.). Can. J. Plant Sci. 70, 1091–1096.

Janská, A., Marsík, P., Zelenková, S., Ovesná, J., 2010. Cold stress and acclimation - what is important for metabolic adjustment? Plant Biol. 12, 395–405. doi:10.1111/j.1438-8677.2009.00299.x

Jensen, E., Farrar, K., Thomas-Jones, S., Hastings, A., Donnison, I., Clifton-Brown, J., 2011. Characterization of flowering time diversity in *Miscanthus* species. GCB Bioenergy 3, 387–400. doi:10.1111/j.1757-1707.2011.01097.x

Jensen, E.F., Robson, P.R.H., Norris, J., Cookson, A., Farrar, K., Donnison, I.S., Clifton-Brown, J.C., 2013. Flowering induction in the bioenergy grass *Miscanthus sacchariflorus* is a quantitative short-day response, whilst delayed flowering under long days increases biomass accumulation. J. Exp. Bot. 64, 541–552. doi:10.1093/jxb/err313

Jiao, X., Kørup, K., Andersen, M.N., Petersen, K.K., Prade, T., Jezowski, S., Ornatowski, S., Górynowicz, B., Spitz, I., Lærke, P.E., Jørgensen, U., 2016. Low-temperature leaf photosynthesis of a *Miscanthus* germplasm collection correlates positively to shoot growth rate and specific leaf area. Ann. Bot. 117, 1229–1239. doi:10.1093/aob/mcw042

Jiao, X., Kørup, K., Andersen, M.N., Sacks, E.J., Zhu, X.-G., Laerke, P.E., Jørgensen, U., 2016. Can miscanthus C4 photosynthesis compete with festulolium C3 photosynthesis in a temperate climate? GCB Bioenergy n/a-n/a. doi:10.1111/gcbb.12342

Jones, M.B., Finnan, J., Hodkinson, T.R., 2015. Morphological and physiological traits for higher biomass production in perennial rhizomatous grasses grown on marginal land. GCB Bioenergy 7, 375–385. doi:10.1111/gcbb.12203

Jørgensen, U., Mortensen, J., Ohlsson, C., 2003. Light interception and dry matter conversion efficiency of miscanthus genotypes estimated from spectral reflectance measurements. New Phytol. 157, 263–270. doi:10.1046/j.1469-8137.2003.00661.x

Jørgensen, U., Muhs, H.-J., 2001. Miscanthus breeding and improvement, in: Jones, M.B., Walsh, M. (Eds.), Miscanthus for Energy and Fibre. Earthscan, London, pp. 68–85.

Jørgensen, U., Schwarz, K.-U., 2000. Why do basic research? A lesson from commercial exploitation of miscanthus. New Phytol. 148, 190–193.

Kaiser, C.M., Clark, L. V., Juvik, J.A., Voigt, T.B., Sacks, E.J., 2015. Characterizing a germplasm collection for yield, yield components, and genotype × environment interactions. Crop Sci. 55, 1978–1994. doi:10.2135/cropsci2014.12.0808

Kaiser, C.M., Sacks, E.J., 2015. Cold-tolerance of *Miscanthus* seedlings and effects of spring and autumn frosts on mature clonally replicated cultivars. Crop Sci. 55, 2401–2416. doi:10.2135/cropsci2014.10.0679

Kandel, T.P., Hastings, A., Jørgensen, U., Olesen, J.E., 2016. Simulation of biomass yield of regular and chilling tolerant *Miscanthus* cultivars and reed canary grass in different climates of Europe. Ind. Crops Prod. 86, 329–333. doi:10.1016/j.indcrop.2016.04.007

Kao, W., Tsai, T., Chen, W., 1998. A comparative study of *Miscanthus floridulus* (Labill) Warb and *M. transmorrisonensis* Hayata: photosynthetic gas exchange, leaf characteristics and growth in controlled environments. Ann. Bot. 81, 295–299.

Kao, W.-Y., Chang, K.-W., 2001. Altitudinal trends in photosynthetic rate and leaf characteristics of *Miscanthus* populations from central Taiwan. Aust. J. Bot. 49, 509–514.

Kaplan, F., Guy, C.L., 2005. RNA interference of *Arabidopsis* beta-amylase8 prevents maltose accumulation upon cold shock and increases sensitivity of PSII photochemical efficiency to freezing stress. Plant J. 44, 730–743. doi:10.1111/j.1365-313X.2005.02565.x

Keunen, E., Peshev, D., Vangronsveld, J., Van Den Ende, W., Cuypers, A., 2013. Plant sugars are crucial players in the oxidative challenge during abiotic stress: Extending the traditional concept. Plant, Cell Environ. 36, 1242–1255. doi:10.1111/pce.12061

Klotke, J., Kopka, J., Gatzke, N., Heyer, A.G., 2004. Impact of soluble sugar concentrations on the acquisition of freezing tolerance in accessions of *Arabidopsis thaliana* with contrasting cold adaptation - Evidence for a role of raffinose in cold acclimation. Plant, Cell Environ. 27, 1395–1404. doi:10.1111/j.1365-3040.2004.01242.x

Knaupp, M., Mishra, K.B., Nedbal, L., Heyer, A.G., 2011. Evidence for a role of raffinose in stabilizing photosystem II during freeze-thaw cycles. Planta 234, 477–486. doi:10.1007/s00425-011-1413-0

Knörzer, H., Hartung, K., Piepho, H.-P., Lewandowski, I., 2013. Assessment of variability in biomass yield and quality: what is an adequate size of sampling area for miscanthus? GCB Bioenergy 5, 572–579. doi:10.1111/gcbb.12027

Koster, K.L., Lynch, D. V., 1992. Solute Accumulation and compartmentation during the cold acclimation of puma rye. Plant Physiol. 98, 108–113. doi:10.1104/pp.98.1.108

Kucharik, C.J., VanLoocke, A., Lenters, J.D., Motew, M.M., 2013. Miscanthus establishment and overwintering in the midwest usa: a regional modeling study of crop residue management on critical minimum soil temperatures. PLoS One 8, e68847. doi:10.1371/journal.pone.0068847

Larsen, S.U., Jørgensen, U., Kjeldsen, J.B., Lærke, P.E., 2013. Long-term miscanthus yields influenced by location, genotype, row distance, fertilization and harvest season. BioEnergy Res. 7, 620–635. doi:10.1007/s12155-013-9389-1

Lê, S., Josse, J., Husson, F., 2008. FactoMineR: An R Package for multivariate analysis. J. Stat. Software. 25, 1–18.

Leipner, J., Fracheboud, Y., Stamp, P., 1999. Effect of growing season on the photosynthetic apparatus and leaf antioxidative defenses in two maize genotypes of different chilling tolerance. Environ. Exp. Bot. 42, 129–139. doi:10.1016/S0098-8472(99)00026-X

Lesur, C., Jeuffroy, M.-H., Makowski, D., Riche, A.B., Shield, I., Yates, N., Fritz, M., Formowitz, B., Grunert, M., Jorgensen, U., Laerke, P.E., Loyce, C., 2013. Modeling long-term yield trends of *Miscanthus x giganteus* using experimental data from across Europe. F. Crop. Res. 149, 252–260. doi:10.1016/j.fcr.2013.05.004

Lewandowski, I., Clifton-Brown, Murphy-Bokern, D. 2015. Developing perennial biomass crops for expanding European bio-based feedstock supply in a resource constrained world. Summary of the Perennial biomass crops for a resource constrained world conference, Hohenheim, Germany.

Lewandowski, I., Clifton-Brown, J.C.J., Scurlock, J.M.O., Huisman, W., 2000. Miscanthus: European experience with a novel energy crop. Biomass and Bioenergy 19, 209–227. doi:10.1016/S0961-9534(00)00032-5

Lewandowski, I., Clifton-Brown, J.C.J., Scurlock, J.M.O., Huisman, W., 2000. Miscanthus: European experience with a novel energy crop. Biomass and Bioenergy 19, 209–227. doi:10.1016/S0961-9534(00)00032-5

Lewandowski, I., Kalinina, O., Kiesel, A., Clifton-Brown, J., Farrar, K., I., D., Huxley, L., Nunn, C., Trindade, L., Dolstra, O., van der Weijde, T., van der Linden, G., Chen, C.-L., Roland-Ruiz, I., Muylle, H., Lootens, P., Fonteyne, S., Harding, G., Mos, M., Schwarz, K.U., Meyer, H., Muller-Samann, K., Xi, Q., Özgüven, M., Khokhlov, N., Tarakanov, I., Schüle, H., 2015. OPTIMISC - Developing Miscanthus Production Systems for Marginal Lands, in: Proceedings of the 23rd European Biomass Conference and Exhibition. Vienna, Austria, pp. 6–8. doi:10.5071/23rdEUBCE2015-1CP.2.2

Lewandowski, I., Kalinina, O., Kiesel, A., Iqbal, Y., Wagner, M., Clifton-Brown, J.C., Hastings, A., Farrar, K., Donnison, I.S., Huxley, L.M., Nunn, C., Trindade, L.M., Dolstra, O., van der Weijde, T., Chen, C.-L., van der Linden, G., Roldán-ruiz, I., Muylle, H., Lootens, P., Fonteyne, S., Harding, G., Mos, M., Schwarz, K.-U., Meyer, H., Muller-Samann, K., Xi, Q., Özgüven, M., Khokhlov, N., Tarakanov, I., Schuele, H., (2016). Progress on Optimizing Miscanthus Biomass Production for the European Bioeconomy: Results of the EU FP7 Project OPTIMISC. Front. Plant Sci. 7, 1620. doi:10.3389/fpls.2016.01620

Lewandowski, I., Scurlock, J., Lindvall, E., Christou, M., 2003. The development and current status of perennial rhizomatous grasses as energy crops in the US and Europe. Biomass and Bioenergy 25, 335–361. doi:10.1016/S0961-9534(03)00030-8

Li, X.M., Chen, X.M., Xiao, Y.G., Xia, X.C., Wang, D. Sen, He, Z.H., Wang, H.J., 2014. Identification of QTLs for seedling vigor in winter wheat. Euphytica 198, 199–209. doi:10.1007/s10681-014-1092-6

Lichtenthaler, H.K., Buschmann, C., 2001. Chlorophylls and Carotenoids: Measurement and characterization by UV-VIS. Curr. Protoc. Food Anal. Chem. F4.3.1-F4., 1–8. doi:10.1002/0471142913

Livingston, D.P., Premakumar, R., Tallury, S.P., 2006. Carbohydrate partitioning between upper and lower regions of the crown in oat and rye during cold acclimation and freezing. Cryobiology 52, 200–8. doi:10.1016/j.cryobiol.2005.11.001

Loehle, C., 1998. Height growth rate tradeoffs northern determine and southern range limits for trees. J. Biogeogr. 25, 735–742. doi:10.1046/j.1365-2699.1998.2540735.x

Long, S.P., Spence, A.K., 2013. Toward cool C(4) crops. Annu. Rev. Plant Biol. 64, 701–22. doi:10.1146/annurev-arplant-050312-120033

Longin, C.F.H., Sieber, A.-N., Reif, J.C., 2013. Combining frost tolerance, high grain yield and good pasta quality in durum wheat. Plant Breed. 132, 353–358. doi:10.1111/pbr.12064

Lootens, P., Van Waes, J., Carlier, L., 2004. Effect of a short photoinhibition stress on photosynthesis, chlorophyll a fluorescence, and pigment contents of different maize cultivars. can a rapid and objective stress indicator be found? Photosynthetica 42, 187–192. doi:10.1023/B:PHOT.0000040589.09614.a0

Marocco, A., Lorenzoni, C., Fracheboud, Y., 2005. Chilling stress in maize. Maydica 50, 571–580. doi:10.1007/978-0-387-79418-1

Matros, A., Peshev, D., Peukert, M., Mock, H., Van den Ende, W., 2015. Sugars as hydroxyl radical scavengers. Proof-of-concept by studying the fate of sucralose in *Arabidopsis*. Plant J. 82, 822–839.

Matsuoka, M., Furbank, R.T., Fukayama, H., Miyao, M., 2001. Molecular engineering of C4 photosynthesis. Annu. Rev. Plant Physiol. Plant Mol. Biol. 52, 297–314. doi:10.1146/annurev.arplant.52.1.297

Maughan, M., Bollero, G., Lee, D.K., Darmody, R., Bonos, S., Cortese, L., Murphy, J., Gaussoin, R., Sousek, M., Williams, D., Williams, L., Miguez, F., Voigt, T., 2012. *Miscanthus x giganteus* productivity: the effects of management in different environments. GCB Bioenergy 4, 253–265. doi:10.1111/j.1757-1707.2011.01144.x

Menkir, A., Larter, E.N., 1985. Growth responses of early maturing inbred lines of corn to suboptimal temperatures. Can. J. Plant Sci. 65, 79–86. doi:10.4141/cjps85-011

Miguez, F.E., Villamil, M.B., Long, S.P., Bollero, G.A., 2008. Meta-analysis of the effects of management factors on *Miscanthus x giganteus* growth and biomass production. Agric. For. Meteorol. 148, 1280–1292. doi:10.1016/j.agrformet.2008.03.010

Mittler, R., 2006. Abiotic stress, the field environment and stress combination. Trends Plant Sci. 11, 15–19. doi:10.1016/j.tplants.2005.11.002

Morsy, M.R., Jouve, L., Hausman, J.-F., Hoffmann, L., Stewart, J.M., 2007. Alteration of oxidative and carbohydrate metabolism under abiotic stress in two rice (*Oryza sativa* L.) genotypes contrasting in chilling tolerance. J. Plant Physiol. 164, 157–67. doi:10.1016/j.jplph.2005.12.004

Mortaignie, E., 2014. Evaluatie van het effect van koudestress op de jeugdgroei van Miscanthus. Master Thesis. Ghent University. Belgium

Muennich, C., Kørup, K., Klaas, M., Barth, S., Kjeldsen, J.B., Finnan, J., Fonteyne, S., Jankowska, M., Jørgensen, U., 2016. Creation and evaluation of novel cold tolerant Miscanthus hybrids, in: Barth, S., Murphy-Bokern, D., Kalinina, O., Taylor, G., Jones, M. (Eds.), Perennial Biomass Crops for a Resource Constrained World. Springer New York, New York.

Murchie, E.H., Lawson, T., 2013. Chlorophyll fluorescence analysis: A guide to good practice and understanding some new applications. J. Exp. Bot. 64, 3983–3998. doi:10.1093/jxb/ert208

Muylle, H., Hulle, S. Van, Vliegher, A. De, Baert, J., Bockstaele, E. Van, Roldán-Ruiz, I., 2015. Dry matter yield and energy balance of annual and perennial lignocellulosic crops for bio-refinery use: a four-year field experiment in Belgium. Eur. J. Agron. 63, 62–70. doi:10.1016/j.eja.2014.11.001

Nägele, T., Heyer, A.G., 2013. Approximating subcellular organisation of carbohydrate metabolism during cold acclimation in different natural accessions of *Arabidopsis thaliana*. New Phytol. 198, 777–787. doi:10.1111/nph.12201

Naidu, S.L., Long, S.P., 2004. Potential mechanisms of low-temperature tolerance of C4 photosynthesis in *Miscanthus x giganteus*: an *in vivo* analysis. Planta 220, 145–55. doi:10.1007/s00425-004-1322-6

Naidu, S.L., Moose, S.P., Al-shoaibi, A.K., Raines, C.A., Long, S.P., 2003. Cold tolerance of C4 photosynthesis in *Miscanthus x giganteus*: adaptation in amounts and sequence of C4 photosynthetic enzymes. Plant Physiol. 132, 1688–1697. doi:10.1104/pp.103.021790.photosynthetic

Nishizawa, A., Yabuta, Y., Shigeoka, S., 2008. Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. Plant Physiol. 147, 1251–63. doi:10.1104/pp.108.122465

Nogueira, F.T.S., De Rosa Jr, V.E., Menossi, M., Ulian, U.C., Arruda, P., 2003. rna expression profiles and data mining of sugarcane response to low temperature. Plant Physiol. 132, 1811–1824. doi:10.1104/pp.102.017483.to

Pastori, G., Foyer, C.H., Mullineaux, P., 2000. Low temperature-induced changes in the distribution of H_2O_2 and antioxidants between the bundle sheath and mesophyll cells of maize leaves. J. Exp. Bot. 51, 107–113.

Peixoto, M.D.M., Friesen, P.C., Sage, R.F., 2015. Winter cold-tolerance thresholds in field-grown *Miscanthus* hybrid rhizomes. J. Exp. Bot. 66, 4415–4425. doi:10.1093/jxb/erv093

Peixoto, M.D.M., Sage, R.F., 2016. Improved experimental protocols to evaluate cold tolerance thresholds in *Miscanthus* and switchgrass rhizomes. GCB Bioenergy 8, 257–268. doi:10.1111/gcbb.12301

Peshev, D., Vergauwen, R., Moglia, A., Hideg, E., Van den Ende, W., 2013. Towards understanding vacuolar antioxidant mechanisms: a role for fructans? J. Exp. Bot. 64, 1025–38. doi:10.1093/jxb/ers377

Peter, R., Eschholz, T.W., Stamp, P., Liedgens, M., 2009. Early Growth of flint maize landraces under cool conditions. Crop Sci. 49, 169. doi:10.2135/cropsci2007.10.0538

Peter, R., Eschholz, T.W., Stamp, P., Liedgens, M., 2009. Swiss Flint maize landraces—A rich pool of variability for early vigour in cool environments. F. Crop. Res. 110, 157–166. doi:10.1016/j.fcr.2008.07.015

Płażek, A., Dubert, F., Janowiak, F., Krępski, T., Tatrzańska, M., 2011. Plant age and *in vitro* or *in vivo* propagation considerably affect cold tolerance of Miscanthus x giganteus. Eur. J. Agron. 34, 163–171. doi:10.1016/j.eja.2011.01.002

Presterl, T., Ouzunova, M., Schmidt, W., Möller, E.M., Röber, F.K., Knaak, C., Ernst, K., Westhoff, P., Geiger, H.H., 2007. Quantitative trait loci for early plant vigour of maize grown in chilly environments. Theor. Appl. Genet. 114, 1059–1070. doi:10.1007/s00122-006-0499-4

Purdy, S.J., Maddison, A.L., Jones, L.E., Webster, R.J., Andralojc, J., Donnison, I., Clifton-Brown, J., 2013. Characterization of chilling-shock responses in four genotypes of *Miscanthus* reveals the superior tolerance of *M. x giganteus* compared with *M. sinensis* and *M. sacchariflorus*. Ann. Bot. 111, 999–1013. doi:10.1093/aob/mct059

Purdy, S.J., Cunniff, J., Maddison, A.L., Jones, L.E., Barraclough, T., Castle, M., Davey, C.L., Jones, C.M., Shield, I., Gallagher, J., Donnison, I., Clifton-Brown, J., 2014. Seasonal carbohydrate dynamics and climatic regulation of senescence in the perennial grass, *Miscanthus.* BioEnergy Res. 8, 7–9. doi:10.1007/s12155-014-9500-2

Purdy, S.J., Maddison, A.L., Cunniff, J., Donnison, I., Clifton-Brown, J., 2015. Non-structural carbohydrate profiles and ratios between soluble sugars and starch serve as indicators of productivity for a bioenergy grass. AoB Plants 7, 1–12. doi:10.1093/aobpla/plv032

Queval, G., Issakidis-Bourguet, E., Hoeberichts, F.A., Vandorpe, M., Gakière, B., Vanacker, H., Miginiac-Maslow, M., Van Breusegem, F., Noctor, G., 2007. Conditional oxidative stress responses in the *Arabidopsis* photorespiratory mutant cat2 demonstrate that redox state is a key modulator of daylength-dependent gene expression, and define photoperiod as a crucial factor in the regulation of H_2O_2-induced cel. Plant J. 52, 640–657. doi:10.1111/j.1365-313X.2007.03263.x

Quinn, L.D., Stewart, J.R., Yamada, T., Toma, Y., Saito, M., Shimoda, K., Fernández, F.G., 2011. Environmental tolerances of *Miscanthus sinensis* in invasive and native populations. BioEnergy Res. 5, 139–148. doi:10.1007/s12155-011-9163-1

Raineri, J., Ribichich, K.F., Chan, R.L., 2015. The sunflower transcription factor HaWRKY76 confers drought and flood tolerance to *Arabidopsis thaliana* plants without yield penalty. Plant Cell Rep. 34, 2065–2080. doi:10.1007/s00299-015-1852-3

Revilla, P., Malvar, R., Cartea, M., 2000. Inheritance of cold tolerance at emergence and during early-season growth in maize. Crop Sci. 40, 1579–1585.

Robson, P., Mos, M., Clifton-Brown, J., Donnison, I., 2011. Phenotypic variation in senescence in miscanthus: towards optimising biomass quality and quantity. BioEnergy Res. 5, 95–105. doi:10.1007/s12155-011-9118-6

Robson, P., Farrar, K., Gay, A.P., Jensen, E.F., Clifton-Brown, J.C., Donnison, I.S., 2013a. Variation in canopy duration in the perennial biofuel crop *Miscanthu*s reveals complex associations with yield. J. Exp. Bot. 64, 2373–83. doi:10.1093/jxb/ert104

Robson, P., Jensen, E., Hawkins, S., White, S.R., Kenobi, K., Clifton-Brown, J., Donnison, I., Farrar, K., 2013b. Accelerating the domestication of a bioenergy crop: identifying and modelling morphological targets for sustainable yield increase in *Miscanth*us. J. Exp. Bot. 64, 4143–55. doi:10.1093/jxb/ert225

Rodríguez, V.M., Butrón, A., Sandoya, G., Ordás, A., Revilla, P., 2007. Combining maize base germplasm for cold tolerance breeding. Crop Sci. 47, 1467–1474. doi:10.2135/cropsci2006.10.0648

Rodríguez, V.M., Romay, M.C., Ordás, A., Revilla, P., 2010. Evaluation of European maize (*Zea mays* L.) germplasm under cold conditions. Genet. Resour. Crop Evol. 57, 329–335. doi:10.1007/s10722-009-9529-9

Rosser, B., 2012. Evaluation of *Miscanthus* winter hardiness and yield potential in Ontario. Master Thesis. University of Guelph. Canada

Roy, G.R., 2016. The role of lake effect snow cover in reducing the susceptibility of *Miscanthus x giganteus* to extreme cold soil temperatures in Michigan. Cold Reg. Sci. Technol. 127, 37–48. doi:10.1016/j.coldregions.2016.04.007

Ruelland, E., Vaultier, M.N., Zachowski, A., Hurry, V., 2009. Cold signalling and cold acclimation in plants. Adv. Bot. Res. 49, 36–126. doi:10.1016/S0065-2296(08)00602-2

Rymen, B., Fiorani, F., Kartal, F., Vandepoele, K., Inzé, D., Beemster, G.T.S., 2007. Cold nights impair leaf growth and cell cycle progression in maize through transcriptional changes of cell cycle genes. Plant Physiol. 143, 1429–38. doi:10.1104/pp.106.093948

Sadok, W., Naudin, P., Boussuge, B., Muller, B., Welcker, C., Tardieu, F., 2007. Leaf growth rate per unit thermal time follows QTL-dependent daily patterns in hundreds of maize lines under naturally fluctuating conditions. Plant, Cell Environ. 30, 135–146. doi:10.1111/j.1365-3040.2006.01611.x

Sage, R.F., de Melo Peixoto, M., Friesen, P., Deen, B., 2015. C4 bioenergy crops for cool climates, with special emphasis on perennial C4 grasses. J. Exp. Bot. 66, 4195–4212. doi:10.1093/jxb/erv123

Sage, R.F., Kubien, D.S., 2007. The temperature response of C3 and C4 photosynthesis. Plant, Cell Environ. doi:10.1111/j.1365-3040.2007.01682.x

Saint Pierre, C., 2012. Phenotyping in controlled environments vs. field conditions, in: Reynolds, M., Pask, A., Mullan, D. (Eds.), WHEAT Physiological Breeding I: Interdisciplinary Approaches to Improve Crop Adaptation. CIMMYT, Mexico, D.F., pp. 124–131.

Sandve, S.R., Kosmala, A., Rudi, H., Fjellheim, S., Rapacz, M., Yamada, T., Rognli, O.A., 2011. Molecular mechanisms underlying frost tolerance in perennial grasses adapted to cold climates. Plant Sci. 180, 69–77. doi:10.1016/j.plantsci.2010.07.011

Scebba, F., Arduini, I., Ercoli, L., Sebastiani, L., 2006. Cadmium effects on growth and antioxidant enzymes activities in *Miscanthus sinensis*. Biol. Plant. 50, 688–692. doi:10.1007/s10535-006-0107-0

Schneider, T., Keller, F., 2009. Raffinose in chloroplasts is synthesized in the cytosol and transported across the chloroplast envelope. Plant Cell Physiol. 50, 2174–2182. doi:10.1093/pcp/pcp151

Slavov, G., Robson, P., Jensen, E., Hodgson, E., Farrar, K., Allison, G., Hawkins, S., Thomas-Jones, S., Ma, X.-F., Huang, L., Swaller, T., Flavell, R., Clifton-Brown, J., Donnison, I., 2013. Contrasting geographic patterns of genetic variation for molecular markers vs. phenotypic traits in the energy grass *Miscanthus sinensis*. GCB Bioenergy 5, 562–571. doi:10.1111/gcbb.12025

Sobkowiak, A., Jończyk, M., Adamczyk, J., Szczepanik, J., Solecka, D., Kuciara, I., Hetmańczyk, K., Trzcinska-Danielewicz, J., Grzybowski, M., Skoneczny, M., Fronk, J., Sowiński, P., 2016. Molecular foundations of chilling-tolerance of modern maize. BMC Genomics 17, 125. doi:10.1186/s12864-016-2453-4

Song, Y., Jain, A.K., Landuyt, W., Kheshgi, H.S., Khanna, M., 2015. Estimates of biomass yield for perennial bioenergy grasses in the USA. BioEnergy Res. 8, 688–715. doi:10.1007/s12155-014-9546-1

Spence, A.K., 2012. Understanding the physiological and molecular basis of chilling tolerance across species of the C4 genera *Miscanthu*s and *Sparti*na. PhD. Thesis. University of Illinois. USA.

Spence, A.K., Boddu, J., Wang, D., James, B., Swaminathan, K., Moose, S.P., Long, S.P., 2014. Transcriptional responses indicate maintenance of photosynthetic proteins as key to the exceptional chilling tolerance of C4 photosynthesis in *Miscanthus x giganteus*. J. Exp. Bot. 65, 3737–47. doi:10.1093/jxb/eru209

Stewart, J.R., Toma, Y., Fernández, F.G., Nishiwaki, A., Yamada, T., Bollero, G., 2009. The ecology and agronomy of *Miscanthus sinensis*, a species important to bioenergy crop development, in its native range in Japan: a review. GCB Bioenergy 1, 126–153. doi:10.1111/j.1757-1707.2009.01010.x

Stoller, E.W., 1977. Differential cold tolerance of quackgrass and johnsongrass rhizomes. Weed Sci. 25, 348–351.

Strigens, A., Freitag, N.M., Gilbert, X., Grieder, C., Riedelsheimer, C., Schrag, T.A., Messmer, R., Melchinger, A.E., 2013. Association mapping for chilling tolerance in elite flint and dent maize inbred lines evaluated in growth chamber and field experiments. Plant, Cell Environ. 36, 1871–1887. doi:10.1111/pce.12096

Swanckaert, J., Pannecoucque, J., Van Waes, J., Steppe, K., Van Labeke, M.-C. Reheul, D. (2016) 'Stay-green characterization in Belgian forage maize', The Journal of Agricultural Science, pp. 1–11. doi: 10.1017/S002185961600085X.

Tarkowski, Ł.P., Van den Ende, W., Tarkowski, L.P., Van den Ende, W., 2015. Cold tolerance triggered by soluble sugars: a multifaceted countermeasure. Front. Plant Sci. 6, 203. doi:10.3389/fpls.2015.00203

Tiryaki, I., Andrews, D., 2001. Sorghum-germination and seedling cold tolerance in sorghum: ii. parental lines and hybrids. Agron. J. 93, 1391–1397.

Trudgill, D.L., Honek, A., Li, D., Straalen, N.M., Van Straalen, N.M., 2005. Thermal time - concepts and utility. Ann. Appl. Biol. 146, 1–14. doi:10.1111/j.1744-7348.2005.04088.x

Usuda, H., Ku, M.S.B., Edwards, G.E., 1984. Rates of photosynthesis relative to activity of photosynthetic enzymes, chlorophyll and soluble protein content among ten C4 species. Aust. J. Plant Physiol. 11, 509–17.

Valluru, R., Van den Ende, W., 2008. Plant fructans in stress environments: emerging concepts and future prospects. J. Exp. Bot. 59, 2905–16. doi:10.1093/jxb/ern164

Van den Ende, W., El-Esawe, S.K., 2014. Sucrose signaling pathways leading to fructan and anthocyanin accumulation: A dual function in abiotic and biotic stress responses? Environ. Exp. Bot. 108, 4–13. doi:10.1016/j.envexpbot.2013.09.017

van der Weijde, T., Alvim Kamei, C.L., Torres, A.F., Vermerris, W., Dolstra, O., Visser, R.G.F., Trindade, L.M., 2013. The potential of C4 grasses for cellulosic biofuel production. Front. Plant Sci. 4, 107. doi:10.3389/fpls.2013.00107

Van Hulle, S., Van Waes, C., De Vliegher, A., Baert, J., Muylle, H., Hulle, S., Waes, C., Vliegher, A., 2012. Comparison of dry matter yield of lignocellulosic perennial energy crops in a long-term Belgian field experiment., in: Grassland-a European Resource. pp. 499–501.

Vargas, L.A., Andersen, M.N., Jensen, C.R., Jorgensen, U., 2002. Estimation of leaf area index, light interception and biomass accumulation of *Miscanthus sinensis* “Goliath” from radiation measurements. Biomass and Bioenergy 22, 1–14.

Vergauwen, L., 2016. Bepaling van de genetische diversiteit in een Miscanthus collectie. Report. ILVO, Belgium.

Verhulst, N., Govaerts, B., Nelissen, V., Sayre, K.D., Crossa, J., Raes, D., Deckers, J., 2011. The effect of tillage, crop rotation and residue management on maize and wheat growth and development evaluated with an optical sensor. F. Crop. Res. 120, 58–67. doi:10.1016/j.fcr.2010.08.012

Verhulst, P.-F., 1838. Notice sur la loi que la population poursuit dans son accroissement. Corresp. mathématique Phys. 10, 113–121.

Voorend, W., Lootens, P., Nelissen, H., Roldán-Ruiz, I., Inzé, D., Muylle, H., 2014. LEAF-E: a tool to analyze grass leaf growth using function fitting. Plant Methods 10, 37. doi:10.1186/1746-4811-10-37

Wang, D., Naidu, S.L., Portis, A.R., Moose, S.P., Long, S.P., 2008a. Can the cold tolerance of C4 photosynthesis in *Miscanthus x giganteus* relative to *Zea mays* be explained by differences in activities and thermal properties of Rubisco? J. Exp. Bot. 59, 1779–87. doi:10.1093/jxb/ern074

Wang, D., Portis, A.R., Moose, S.P., Long, S.P., 2008. Cool C4 photosynthesis: pyruvate Pi dikinase expression and activity corresponds to the exceptional cold tolerance of carbon assimilation in Miscanthus x giganteus. Plant Physiol. 148, 557–67. doi:10.1104/pp.108.120709

Warmund, M.R., Fuller, R., Dunn, J.H., 1998. Survival and recovery of “Meyer” zoysiagrass rhizomes after extracellular freezing. J. Am. Soc. Hortic. Sci. 123, 821–825.

Weih, M., 2009. Genetic and environmental variation in spring and autumn phenology of biomass willows (*Salix* spp.): Effects on shoot growth and nitrogen economy. Tree Physiol. 29, 1479–1490. doi:10.1093/treephys/tpp081

Weng, J.H., Ueng, R.G., 1997. Effect of temperature on photosynthesis of Miscanthus clones collected from different elevations. Photosynthetica 34, 307–311.

Weng, J.H.J., Hsu, F.H.F., 2001. Gas exchange and epidermal characteristics of Miscanthus populations in Taiwan varying with habitats and nitrogen application. Photosynthetica 39, 35–41.

Withers, K.K., 2015. Morphological Adaptations and Membrane Stabilizing Mechanisms of Overwintering Miscanthus (Poaceae). PhD. Thesis. University of Guelph.

Wos, G., Willi, Y., 2015. Temperature-stress resistance and tolerance along a latitudinal cline in North American *Arabidopsis lyrata*. PLoS One 10, e0131808. doi:10.1371/journal.pone.0131808

Xue, S., Kalinina, O., Lewandowski, I., 2015. Present and future options for *Miscanthus* propagation and establishment. Renew. Sustain. Energy Rev. 49, 1233–1246. doi:10.1016/j.rser.2015.04.168

Yadav, S.K., 2010. Cold stress tolerance mechanisms in plants. A review. Agron. Sustain. Dev. 30, 515–527. doi:10.1051/agro/2009050

Yan, J., Chen, W., Luo, F., Ma, H., Meng, A., Li, X., Zhu, M., Li, S., Zhou, H., Zhu, W., Han, B., Ge, S., Li, J., Sang, T., 2011. Variability and adaptability of *Miscanthus* species evaluated for energy crop domestication. GCB Bioenergy 4, 49–60. doi:10.1111/j.1757-1707.2011.01108.x

Yan, W., Hunt, L., 1999. An equation for modelling the temperature response of plants using only the cardinal temperatures. Ann. Bot. 84, 607–614.

Yin, X., Goudriaan, J., Lantinga, E.A., Vos, J., Spiertz, H.J., 2002. A flexible sigmoid function of determinate growth. Ann. Bot. 91, 361–371. doi:10.1093/aob/mcg029

Yu, L., Chen, X., Wang, Z., Wang, S., Wang, Y., Zhu, Q., Li, S., Xiang, C., 2013. Arabidopsis enhanced drought tolerance1/homeodomain glabrous11 confers drought tolerance in transgenic rice without yield penalty. Plant Physiol. 162, 1378–1391. doi:10.1104/pp.113.217596

Yu, L., Ding, G., Huai, Z., Zhao, H., 2013. Natural variation of biomass yield and nutrient dynamics in *Miscanthus*. F. Crop. Res. 151, 1–8. doi:10.1016/j.fcr.2013.07.001

Zhang, J., Xu, Y., Chen, W., Dell, B., Vergauwen, R., Biddulph, B., Khan, N., Luo, H., Appels, R., Van den Ende, W., 2015. A wheat 1-FEH w3 variant underlies enzyme activity for stem WSC remobilization to grain under drought. New Phytol. 205, 293–305.

Zhao, H., Wang, B., He, J., Yang, J., Pan, L., Sun, D., Peng, J., 2013. Genetic diversity and population structure of *Miscanthus sinensis* germplasm in China. PLoS One 8, e75672. doi:10.1371/journal.pone.0075672

Zhu, X.G., Long, S.P., Ort, D.R., 2010. Improving photosynthetic efficiency for greater yield. Annu Rev Plant Biol 61, 235–261. doi:10.1146/annurev-arplant-042809-112206

Zub, H.W., Arnoult, S., Brancourt-Hulmel, M., 2011. Key traits for biomass production identified in different Miscanthus species at two harvest dates. Biomass and Bioenergy 35, 637–651. doi:10.1016/j.biombioe.2010.10.020

Zub, H.W., Arnoult, S., Younous, J., Lejeune-Hénaut, I., Brancourt-Hulmel, M., 2012a. The frost tolerance of *Miscanthus* at the juvenile stage: Differences between clones are influenced by leaf-stage and acclimation. Eur. J. Agron. 36, 32–40. doi:10.1016/j.eja.2011.08.001

Zub, H.W., Brancourt-Hulmel, M., 2010. Agronomic and physiological performances of different species of Miscanthus, a major energy crop. A review. Agron. Sustain. Dev. 30, 201–214. doi:10.1051/agro/2009034

Zub, H.W., Rambaud, C., Bethencourt, L., Brancourt-Hulmel, M., 2012b. Late emergence and rapid growth maximize the plant development of Miscanthus clones. BioEnergy Res. 5, 841–854. doi:10.1007/s12155-012-9194-2

Curriculum vitae

Personal data

Address: Pieter Lachaertstraat 44, 9000, Ghent, Belgium

Mobile phone: 0476/685928

E-mail: fonteynesimon@gmail.com

Date of birth: 31/03/1988

Place of birth: Ghent

Education

2012 - present PhD. Candidate – Doctor in applied biological sciences: agriculture
Ghent University, Department of plant breeding and sustainable crop production (Ghent, Belgium).
Thesis subject: “Cold stress tolerance in miscanthus: genotypic variation, physiological background and the relation with biomass yield”

2006 - 2011 Master in bioscience engineering - agriculture
Ghent University (Ghent, Belgium)
Thesis subject: “The role of ethylene, auxin and abscisic acid in the interaction between rice and the brown spot pathogen *Cochliobolus miyabeanus*”.

2010 Erasmus semester at Universität fur Bodenkultur (Vienna, Austria)

2004-2006 Latijn-wetenschappen at Heilig Hart College Waregem

2000-2004 Latijn at Heilig Hart College Waregem

Work experience

2012 – present PhD. Candidate at the Plant Sciences Unit, ILVO (Institute for Agricultural and Fisheries Research), Melle, Belgium

2012 Intern at the Wheat Agronomy Department, CIMMYT, (International Maize and Wheat Improvement Center), Ciudad Obregon/Texcoco, Mexico

Publications

Peer reviewed articles

Fonteyne, S., Muylle, H., De Swaef, T., Reheul, D., Roldán-Ruiz, I., Lootens, P. (2016). How low can you go? – Rhizome and shoot frost tolerance in *Miscanthus* germplasm. Ind. Crop Prod. 89, 323-331.

Fonteyne, S., Lootens, P., Muylle, H., van Den Ende, W., de Swaef, T., Reheul, D., Roldan-Ruiz, I., (2016). Chilling tolerance and early vigour related characteristics evaluated in two *Miscanthus* genotypes. Photosynthetica. 54, 295–306.

Grahmann, K., Govaerts, B., Fonteyne, S., Guzmán C., Galaviz Soto, A.P., Buerkert, A., Verhulst, N. (2016). Nitrogen fertilizer placement and timing affects bread wheat (*Triticum aestivum*) quality and yield in an irrigated bed planting system. Nutr. Cycl. Agroecosys. 106: 185-199

Lewandowski, I., Kalinina, O., Kiesel, A., Iqbal, Y., Wagner, M., Clifton-Brown, J.C., Hastings, A., Farrar, K., Donnison, I.S., Huxley, L.M., Nunn, C., Trindade, L.M., Dolstra, O., van der Weijde, T., Chen, C.-L., van der Linden, G., Roldán-ruiz, I., Muylle, H., Lootens, P., Fonteyne, S., Harding, G., Mos, M., Schwarz, K.-U., Meyer, H., Muller-Samann, K., Xi, Q., Özgüven, M., Khokhlov, N., Tarakanov, I., Schuele, H., (2016). Progress on optimizing miscanthus biomass production for the European bioeconomy: Results of the EU FP7 project OPTIMISC. Front. Plant Sci. doi: 10.3389/fpls.2016.01620

Fonteyne, S., Muylle, H., Lootens, P., Kerchev, P., van den Ende, W., Staelens, A., Reheul, D., Roldán-Ruiz, I. (submitted). Physiological basis of chilling tolerance and early-season growth in *Miscanthus.* Ann. Bot.

Book chapters

Fonteyne, S., Roldan-Ruiz, I., Muylle, H., De Swaef, T., Reheul, D., Lootens, P. (2016). A review of frost and chilling stress in *Miscanthus* and its importance to biomass yield. In: Barth, S., Murphy-Bokern, D., Kalinina, O., Taylor, G. and Jones, M. (eds): Perennial Biomass Crops for a Resource Constrained World. Springer, New York.

Muennich, C., Kørup, K., Klaas, M., Barth, S., Kjeldsen, J.B., Finnan, J., Fonteyne, S., Jankowska, M., Jørgensen, U. (2016). Creation and evaluation of novel cold tolerant *Miscanthus* hybrids. In: Barth, S., Murphy-Bokern, D., Kalinina, O., Taylor, G. and Jones, M. (eds): Perennial Biomass Crops for a Resource Constrained World. Springer, New York.

Abstracts of presentations at scientific conferences

Fonteyne, S., Lootens, P., Muylle, H., Reheul, D., Roldan-Ruiz, I., (2013). OPTIMISC: optimizing miscanthus biomass production - chilling stress tolerance. 9th International Conference on Renewable Resources and Biorefineries.

Clifton-Brown, J., Kalinina, O., Dolstra, O., Donnison, I., Farrar, K., Fonteyne, S., Harding, G, Hastings, A, Huxley, L, Khokhlov, N, Kiesel, A, Lootens, P., Meyer, H, Mos, M, Müller-Sämann, K, Muylle, H., Nunn, C, Ozgüven, M, Roldán-Ruiz, I., Schüle, H, Schwarz, K, Tarakanov, I, Trindade, L, Truckses, T, Anisimov, A, van der Linden, G, van der Weijde,

T, Xi, Q, Lewandowski, I. (2014). Current advances in breeding and testing miscanthus. Plant Biology Europe FESPB/EPSO 2014 Congress.

Lootens, P., Fonteyne, S., De Swaef, T., Muylle, H., Reheul, D., Roldán-Ruiz, I. (2014). Effect of short-term chilling stress on the photosynthetic light reactions of Miscanthus sacchariflorus genotypes. Plant Biology Europe FESPB/EPSO 2014 Congress.

Fonteyne, S., Lootens, P., De Swaef, T., Muylle, H., Reheul, D., Roldán-Ruiz, I. (2014). Developing a screening protocol for chilling tolerance in miscanthus. Plant Biology Europe FESPB/EPSO 2014 Congress.

Fonteyne, S., Lootens, P., De Swaef, T., Muylle, H., Reheul, D., Roldán-Ruiz, I. (2014). Evaluating growth of miscanthus at low temperatures using LEAF-E. PhD. students Meeting of EU consortia: OPTIMISC, OPTIMA, GrassMargins, WatBio and BFF.

Fonteyne, S., Lootens, P., De Swaef, T., Muylle, H., Reheul, D., Roldán-Ruiz, I. (2014). Growth and photosynthesis of miscanthus under chilling stress. PePG Ecophysiology workshop.

Robson, P., Cope S., Naomi, Donnison, I., Fonteyne, S., Lootens, P., Malinowska, M., Purdy, S., Scalici, G., Stavridou, E., Taylor, G., Webster, R. (2014). Abiotic stress tolerance: the impacts of variation in water, salinity and temperature on growth and yield. Miscanthus Syposium and Safari.

Muylle, H., Fonteyne, S., Lootens, P., Roldán-Ruiz, I. (2015). Evaluation of a collection of about hundred miscanthus genotypes for use in fiber board production in Belgium. Internationale Miscanthus Tagung - "Quo Vadis Miscanthus".

Lootens, P., Fonteyne, S., De Swaef, T., Muylle, H., Reheul, D., Roldán-Ruiz, I. (2015) Phenotyping of early vigor in relation to chilling tolerance under field conditions using "differential base temperature": a case study with > 100 miscanthus genotypes. Plant Organ Growth Symposium

Fonteyne, S., Lootens, P., De Swaef, T., Muylle, H., Reheul, D., Roldán-Ruiz, I., (2015). Determining genotypic variation in chilling stress tolerance through growth and photosynthesis measurements in miscanthus. Plant Organ Growth Symposium.

van der Weijde, T., Kiesel, A., Iqbal, Y., Fonteyne, S., Muylle, H., Mos, M., Dolstra, O., Roldán-Ruiz, I., Lewandowski, I., Trindade, L.. (2015). OPTIMISC: Optimization of miscanthus biomass quality for various biobased-products. Perennial biomass crops for a resource constrained world.

Nunn, C., Kalinina, O., Ozgüven, M., Tarakanov, I., Schule, H., van der Weijde, T., Anisimov, A., Fonteyne, S., Kokhlov, N., Kot, V., Lewandowski, I., Lootens, P., Trindade, L., Lewandowski, I., Iqbal, Y., Hastings, A., Clifton-Brown, J., (2015). End of growing season

and ripening in miscanthus germplasm grown in six countries. Perennial biomass crops for a resource constrained world.

Lootens, P., Fonteyne, S., De Swaef, T., Muylle, H., Roldán-Ruiz, I., Reheul, D., Genotypic variation in the frost tolerance of miscanthus rhizomes. (2015). Perennial biomass crops for a resource constrained world.

Hastings, A., Nunn, C., Kalinina, O., Ozgüven, M., Tarakanov, I., Schüle, H., van der Weijde, T., Anisimov, A., Dolstra, O., Fonteyne, S., Khokhlov, N., Kot, V., Lewandowski, I., McAlmont, J., Meyer, H., Mos, M., Lootens, P., Schwarz, KU., Trindade, L., Iqbal, Y., Xi, Q., Clifton-Brown, J., (2015). Understanding miscanthus genotype and environment interactions using the model MiscanFor. Perennial biomass crops for a resource constrained world.

Fonteyne, S., Lootens, P., Roldán-Ruiz, I., Muylle, H., (2015). Yield and quality characteristics of miscanthus genotypes in different value chains. Perennial biomass crops for a resource constrained world

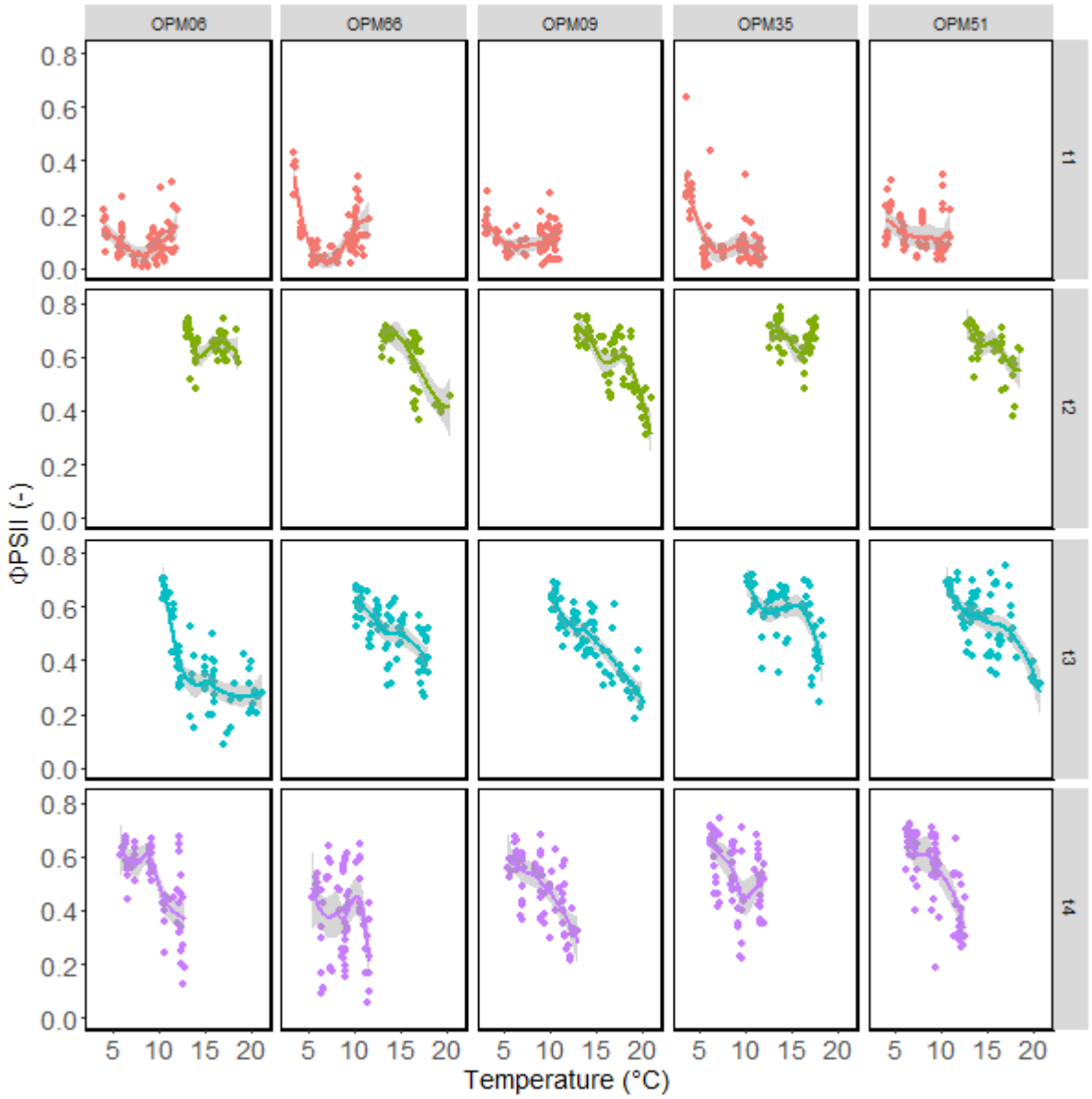
Fonteyne, S., Muylle, H., De Swaef, T., Kalinina, O., Nunn, C., Ozgüven, M., Schüle, H., Tarakanov, I., van der Weijde, T., Anisimov, A., Clifton-Brown, J., Lewandowski, I., Trindade, L., Rehuel, D., Roldán-Ruiz, I., Lootens, P. (2015). Genotypic variation in chilling tolerance in miscanthus germplasm. Perennial biomass crops for a resource constrained world.

Appendix I: Supplementary figures

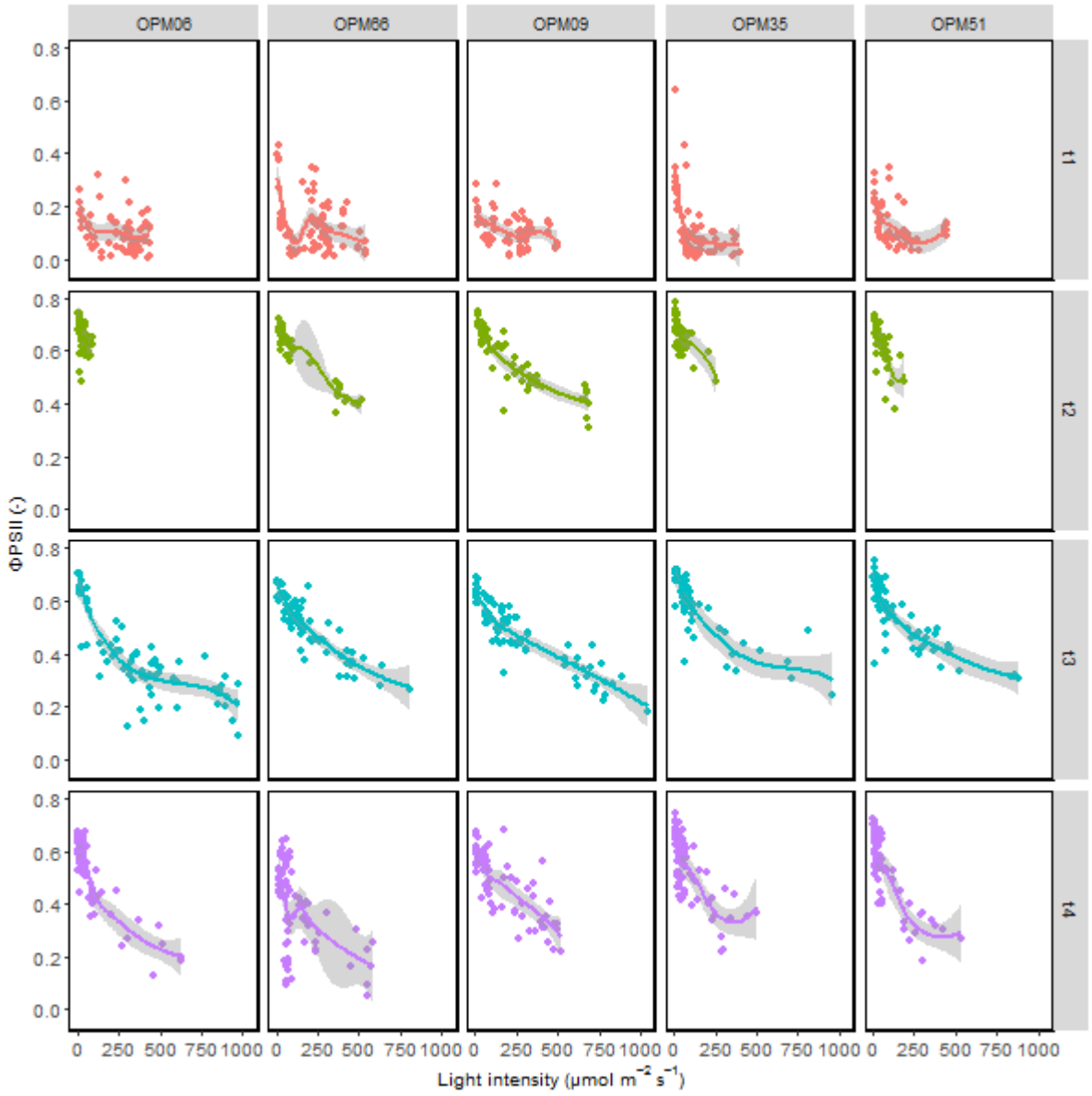
Suplementary table 7.1: Analysis of variance of traits measured in the common garden experiment in 2014 and 2015. AGR: absolute growth rate, L50: thermal time to 50 cm shoot length, L30: thermal time to 30 cm shoot length; LFR: leaf formation rate; Leaf: thermal time to formation of leaf4; S50 thermal time to formation of 50% of shoots.

Year	Trait	Effect	Degrees of freedom	Sum of Squares	Mean square error	F-value	p
2014	AGR	Intercept	1	372.0683	372.0683	925.5386	0*
		Species	3	2.4191	0.8064	3.882	0.011244*
		Genotype(Species)	98	22.5529	0.2301	4.7068	0*
		Block	5	1.966	0.3932	8.0422	0*
		Error	381	18.6284	0.0489		
	L50	Intercept	1	205753988	205753988	4537.691	0.000000*
		Species	3	1034603	344868	10.117	0.000007*
		Genotype(Species)	98	3745562	38220	7.948	0.000000*
		Block	5	129650	25930	5.392	0.000083*
		Error	381	1832101	4809		
	L30	Intercept	1	128110574	128110574	5822.589	0.000000*
		Species	3	606368	202123	9.860	0.000009*
		Genotype(Species)	98	2257009	23031	8.975	0.000000*
		Block	5	31029	6206	2.418	0.035465*
		Error	381	977667	2566		
	LFR	Intercept	1	0.018613	0.018613	1560.608	0.000000*
		Species	3	0.000463	0.000154	12.650	0.000000*
		Genotype(Species)	98	0.001321	0.000013	4.216	0.000000*
		Block	5	0.000016	0.000003	1.011	0.410565
		Error	381	0.001219	0.000003		
	Leaf4	Intercept	1	142457130	142457130	9202.948	0.000000*
		Species	3	182115	60705	4.608	0.004541*
		Genotype(Species)	98	1426806	14559	4.345	0.000000*
		Block	5	39512	7902	2.358	0.039772*
		Error	381	1276618	3351		
	S50	Intercept	1	129968326	129968326	6213.489	0.000000*
		Species	3	102588	34196	1.542	0.207875
		Genotype(Species)	98	2353467	24015	2.613	0.000000*
		Block	5	38853	7771	0.845	0.518161
		Error	381	3502005	9192		
2015	AGR	Intercept	1	390.7729	390.7729	1530.252	0.000000*
		Species	2	1.3085	0.6543	2.468	0.090859
		Genotype(Species)	82	24.1833	0.2949	7.670	0.000000*
		Block	5	0.1905	0.0381	0.991	0.423479
		Error	304	11.6886	0.0384		
	L50	Intercept	1	117411551	117411551	4380.255	0.000000*
		Species	2	20369	10185	0.400	0.671537
		Genotype(Species)	82	2332804	28449	10.648	0.000000*
		Block	5	35479	7096	2.656	0.022813*
		Error	304	812222	2672		
	L30	Intercept	1	76596740	76596740	5010.575	0.000000*
		Species	2	78148	39074	2.823	0.065043
		Genotype(Species)	82	1267861	15462	10.423	0.000000*

	Block	5	26269	5254	3.542	0.003972*
	Error	304	450969	1483		
LFR	Intercept	1	0.006763	0.006763	1300.061	0.000000*
	Species	3	0.000031	0.000010	2.326	0.080097
	Genotype(Species)	82	0.000394	0.000005	3.296	0.000000*
	Block	5	0.000023	0.000005	3.097	0.009626*
	Error	307	0.000447	0.000001		
Leaf4	Intercept	1	61907667	61907667	5397.798	0.000000*
	Species	2	40731	20365	1.811	0.169489
	Genotype(Species)	82	1007517	12287	3.724	0.000000*
	Block	5	21871	4374	1.326	0.252995
	Error	304	1003048	3300		
S50	Intercept	1	54597732	54597732	2667.703	0.000000*
	Species	2	27844	13922	0.636	0.531803
	Genotype(Species)	82	1996247	24344	7.758	0.000000*
	Block	5	9729	1946	0.620	0.684557
	Error	304	953920	3138		



Supplementairy figure 8.1: Φ_{PSII} in function of temperature plotted per genotype and measuring date.



Supplementairy figure 8.2: Φ_{PSII} in function of light intensity plotted per genotype and measuring date.

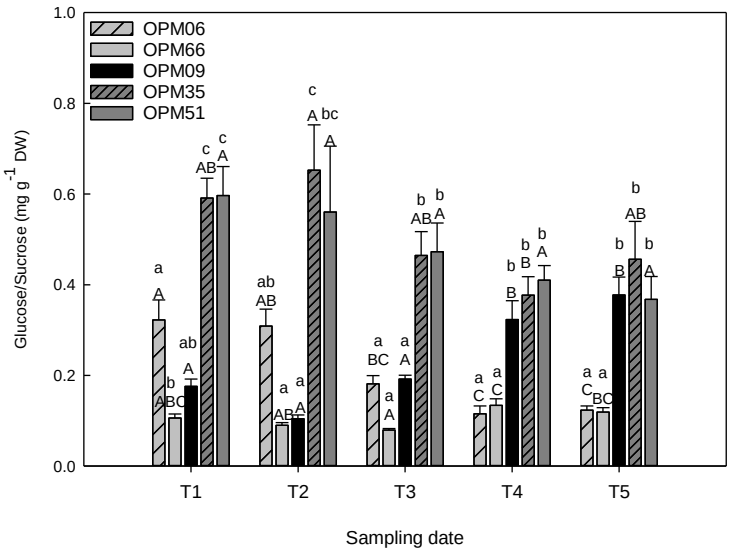
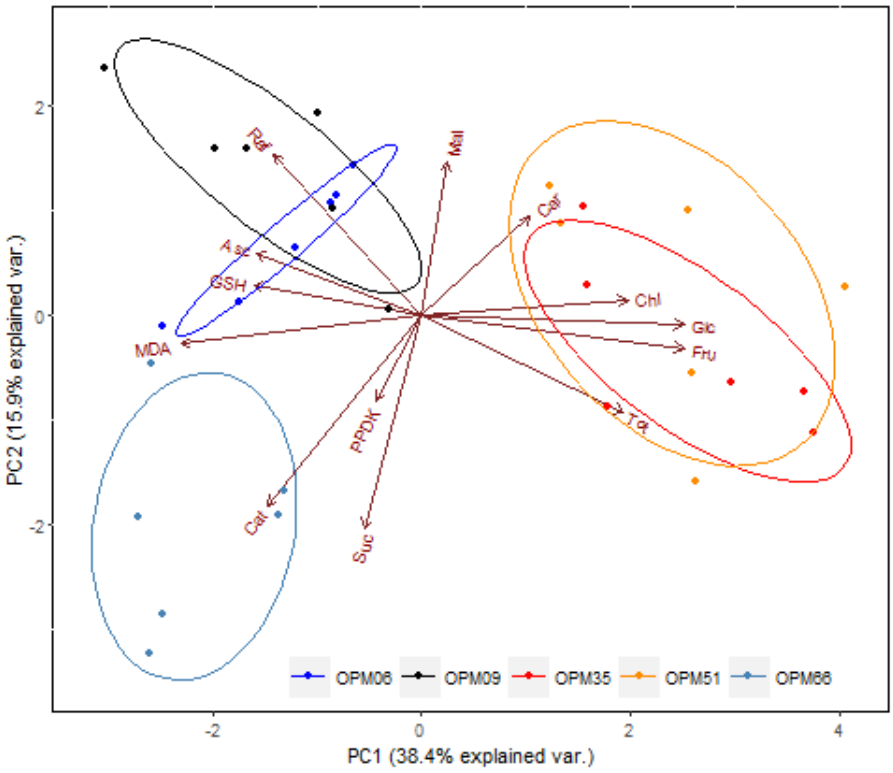
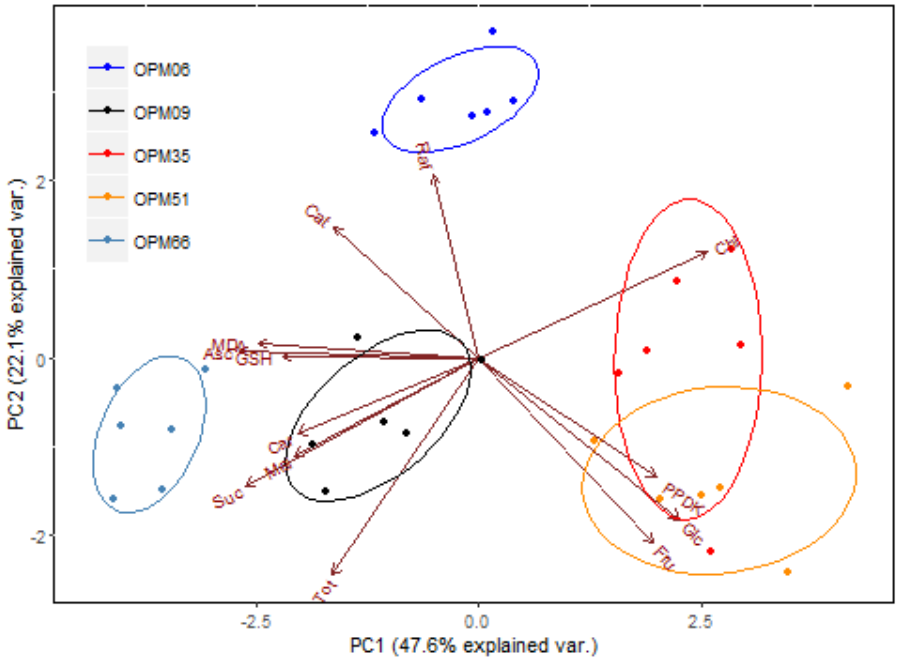


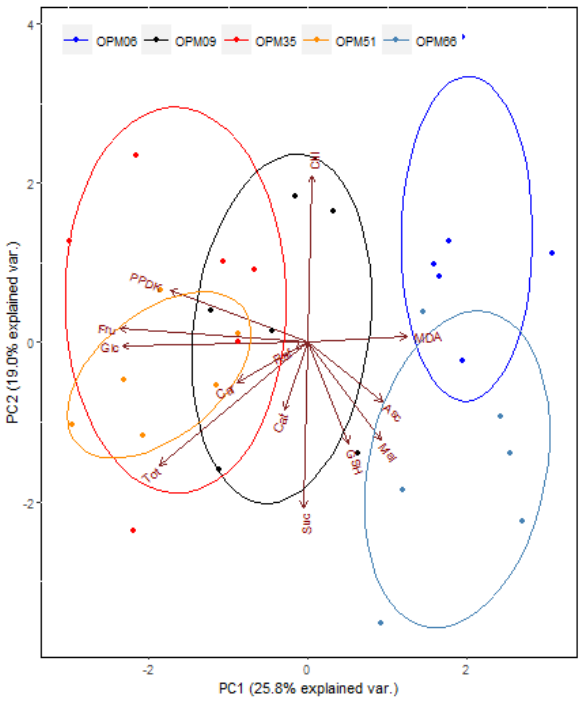
Figure 8.3: Mean ratio of glucose/sucrose content in the leaves per genotype and per sampling date. Symbols show mean values per genotype and sampling date; error bars show standard error (n=6). Different patterns of the bars indicate different genotypes. Uppercase letters refer to homogenous groups of sampling dates for a specific genotype, lowercase letters refer to homogenous groups of genotypes for a specific sampling date ($p < 0.05$).



Supplementairy figure 4: PCA of biochemcial components on T1. Dots show values of the first and second component for individual plants. Different colors indicate different genotypes.



Supplementairy figure 5: PCA of biochemcial components on T3. Dots show values of the first and second component for individual plants. Different colors indicate different genotypes.



Supplementairy figure 6: PCA of biochemcial components on T5. Dots show values of the first and second component for individual plants. Different colors indicate different genotypes.