GrassMargins Final Report with Publishable Summaries

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List of Grass Margins Participants:

Participant no.	Participant Organisation Name	Abbreviation	Type of Organisation	Country
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2	The Provost Fellows & Scholars of the College of the Holy and Undivided Trinity of Queen Elizabeth Near Dublin (Trinity College Dublin)	TCD	RTD	Ireland
3	Sveriges Lantbruksuniversitet	SLU	RTD	Sweden
4	Aarhus Universitet	AU	RTD	Denmark
5	Tinplant Biotechnik und Pflanzenvermehrung GmbH	Tinplant	SME	Germany
6	Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences	CAS-MPG	RTD	China
7	The Establishment of the Russian Academy of Sciences, Institute of Cytology and Genetics; Siberian Branch of Russian Academy of Sciences	RAS	RTD	Russia
8	Knowledge Now Limited	K-NOW	SME	UK
9	Instytut Genetyki Roslin Polskiej Akademi Nauk (Polish Academy of Sciences)	PAS	RTD	Poland
10	DLF - Trifolium A/S	DLF	IMD	Denmark
11	The University of Sheffield	UFSD	RTD	UK
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Executive Summary

The objectives of GrassMargins were to develop and parameterise process-based crop models that can provide reliable predictions of perennial grass carbon assimilation, growth and yield under present and future climate scenarios and to use geographical mapping combined with ecological niche modelling to identify areas most suitable for the production of perennial rhizomatous grasses on marginal land. The process-based crop models were used to identify physiological traits to maximise biomass production of perennial grasses on marginal lands, reduce inputs and improve conversion of biomass feedstock to biofuels and bioenergy. The purpose was to provide the framework to evaluate potential traits for improvement of perennial grasses in advance of lengthy breeding programmes with the intention of identifying selection criteria that can be used in pre-breeding and selection of grasses.

Twenty nine different genotypes were selected from the gene pools from the partners and were successfully *in vitro* propagated for glasshouse experiments and field trials. Crosses of *Miscanthus* were made and novel hybrids were planted in field for further evaluations. Collection trips were undertaken to collect *Miscanthus* in Russia (Primorsky Krai) and China (Beijing, Hebei, Liaoning), and *Phalaris* and *Dactylis* in Europe to gain new genotypes not yet present in existing collections, and to examine the population genetic structure. The living *Miscanthus* collection at Teagasc (Ireland) has been genotyped by sequencing. To this dataset, genotypes from a well-documented collection in Japan, and more than 100 genotypes from the GrassMargins trip to the Far East have been added. Similarly, the newly collected European *Phalaris* and *Dactylis* accessions have been genotyped using a similar protocol.

The tolerance to a certain level of stress is of key importance for selection of the most promising grass species and varieties for production on marginal land and for production over a wide climatic gradient. The tolerance to salt, drought, flooding, cold and frost was analysed in the project grass species and varieties with a range of methodologies. Existing gene pools were screened in the field while specific tests were conducted under controlled conditions in lysimeter, greenhouse and climate chamber facilities. The analyses applied ranged from phenotypic development, over plant physiological measurements and compositional analysis, to expression libraries or DNA libraries for the determination of allele frequencies. There were significant species and interspecies variation in salt tolerance during growth with tall fescue being the most tolerant and reed canary grass the least tolerant species. Cocksfoot appeared from drought experiments under controlled conditions to be the most tolerant species – especially the variety Sevenup. However, when grasses were sown in a drought and salt affected field trial tall fescue and festulolium varieties

performed the best. The drought and salt tolerance during germination thus needs special attention in further work. Miscanthus and reed canary grass were the most flooding tolerant grasses while cocksfoot was very sensitive to flooding. Cold and frost tolerance was only tested within miscanthus and showed a promising variation to include in breeding for better performance in cool climates.

However, the results of the project have shown that grass yields obtained from marginal lands are not always lower than yields from good agricultural land. Moreover, in certain circumstances, yields from marginal land can exceed those from good agricultural land. This was particularly the case for Miscanthus grown on wet land. Additionally, grass yields on marginal land can be stimulated by the addition of high levels of nitrogen fertilizer while minimising nitrate leaching compared to annual crops but nitrogen levels can be reduced by the addition of legumes to grass mixtures. This result is also very significant as restrictions on the use of high nitrogen levels on annual crops due to failure to meet nitrate directive limits could turn land which was previously profitable for crop production into land which is marginal for crop production. However, our results have shown such lands can continue to be productive with low levels of nitrate losses if the land use is changed from crop production to the production of perennial grasses.

The Dissemination plan targeted the following audiences: Academic audience: this audience is largely represented within the project and the wider community involvement was guaranteed through either direct communication between researchers and groups (seminars, research visits etc) and via publication to external refereed sources, European Projects: this audience has been targeted via a systematic relationship with other projects such as OPTIMA and OPTMISC. In cooperation with these projects we have organised a series of workshops. Moreover strong links have been established with other EU FP7 projects such as WATBIO, or Nationally funded projects such as BFF, France; research networks (FIBRA) or research societies (miscanthus-society); General public and media: this audience was kept involved via our project website, the release of educational videos on social media channels and via interviews and TV appearances.

Summary Description of the Project Context and the Main Objectives

It is estimated that bioenergy currently contributes 12.9% (IEA Statistics, 2007) of the global primary energy use, but this is largely in the developing world where wood and dung are used in rural areas for heating and cooking. Furthermore, energy crops, at present, contribute relatively little to the overall energy supply from biomass. However, this is set to grow substantially in the next few decades in order to meet recent national and international commitments such as the European Union 2008 Directive on Renewable Energy that specifies a 20% share of energy from renewable sources by 2020 in all member states, and a 10% share of renewable energy specifically in the transport sector (DIRECTIVE 2009/28/EC). In order to meet these demands it is inevitable that biomass for energy will compete for land with food production and consequently it will be essential to maximise biomass production per unit of land area (Heaton et al., 2008a) and use marginal land which is of poor quality for agriculture and which yields low returns. Marginal land is, in broad terms, defined as, land at the margins of economic viability which is the last land to be brought into production and is the first land to be abandoned by farmers. This type of land generally has poor quality soils and experiences harsh climatic conditions. Although there is no clear agreement on the current amount of marginal land in Europe, some estimates put it at as much as one third of the total land area and there is no doubt that it will increase under predicted climate change (Olesen & Bindi 2004). It will also be essential to produce energy biomass with minimum resource inputs, in other words the ‘resource use efficiency’ of production must be maximised. In addition to growing biomass on marginal land there is likely to increasing interest in using current agricultural grasslands for biomass production because the Common Agricultural Policy (CAP) reforms coupled with the nitrates directive (91/676/EEC) have led to declining livestock numbers and a potential surplus of grassland biomass (Connolly et al., 2009).

The grand challenge for biomass production is to develop a new generation of energy crops which have a suite of desirable physical and chemical traits which offer the opportunity of a range of end uses while maximising biomass yields (Vermerris, 2008). Achieving this will depend on identifying the fundamental constraints on productivity and addressing these constraints with a combination of conventional breeding and modern genomic tools. It is now widely recognised that perennial rhizomatous grasses (PRGs) such as reed canary grass (*Phalaris arundinacea*) and *Miscanthus* spp. are a plant life form particularly well-suited to maximising outputs in terms of biomass yield while minimising inputs in terms of resources, and it is likely that these will become dedicated bioenergy crops in the relatively near future (Venendaal et al., 1997; Lewandowski et al., 2003b; El Bassam, 2008). Currently, these potential ‘second generation’ bioenergy crops are largely undomesticated and have not been subject to centuries of improvement as have our major food crops. At present most biofuels are derived from food crops such as maize and rape and are referred to as ‘first generation’ bioenergy crops. Breeding of appropriate species and genotypes to suit specific climates and soil conditions will be required. The objective of this proposal was to spur the development of perennial grass crops for bioenergy by addressing the factors hampering their employment on marginal land and achieving their full yield potential. We exploited their breeding potential and commercial relevance within an optimised production system to produce a stable source of biomass which will also include the opportunity to develop new plant-derived bioproducts. The delivery of optimised production of raw materials from agriculture as resources for added-value products will contribute to a more sustainable socio-economical and environmental performance of agriculture. This is because perennial grasses for energy can be grown on marginal lands or in environmentally sensitive areas, where conventional agricultural crops are either not economic to grow or where environmental demands restrict their production (Blanco-Canqui, 2010). Marginal soil may often vary and be low in fertility. Since the marginal soils may be present in environmentally sensitive areas intensive fertilization may not be possible and low-input systems with high use-efficiency of available resources are essential. To optimise the use of growth resources on marginal land, we will also investigated the performance of sown grasslands consisting of mixtures of cultivars or species and even legumes to supply nitrogen to grassland (Vandermeer, 1989; Tilman et al., 2001).

The selection of material by partners in the project focussed on four taxa of perennial grasses. These have been selected as a result of a preliminary analysis and known characteristics of these groups (El Bassam, 2008). The taxa were as follows:

Miscanthus - with ca. 12 species, is a genus native to eastern or south-eastern Asia (Hodkinson et al., 2001). Its natural geographic range extends from northeastern Siberia, 50°N in the temperate zone, to Polynesia 22°S, in the tropical zone, westwards to central India and eastwards to Polynesia. It is therefore found in a wide range of climatic zones and biomes. They are adapted to different habitats from agricultural grasslands, dry grassland and even wet, saline, and polluted land. Selection in these habitats has resulted in various ecotypes (Clifton-Brown et al., 2008). Some species such as *M. floridulus* generally grow best at sea level in tropical conditions but others such as *M. paniculatus* can tolerate temperate and/or high altitude conditions up to altitudes of up to 3,100 m on dry mountain slopes in China (Shouliang & Renvoize, 2006).

Phalaris - is a genus of ca. 20 species with a north temperate and circum-global distribution centred on the Mediterranean (Clayton & Renvoize, 1986; Cope & Gray, 2009). *Phalaris arundinacea* forms extensive monospecific stands and grows in wet places and shallow water that are unsuitable for most other agricultural purposes (Anderson et al., 2008). It grows throughout most of Europe except the extreme south (Tutin et al., 1980). However, related *Phalaris* spp. are found in the extreme south of Europe and would be a source of genes for the wider genepool.

Dactylis - is a monospecific genus. *Dactylis glomerata* is a large and highly variable mainly tetraploid complex. However, diploids exist in small areas around the Mediterranean, western and central Europe, north Italy, Ibiza, southern Spain, and Portugal. It grows in meadows, roadsides, open woodland, and stony hillsides (Tutin et al., 1980).

Festuca – is a genus of 450 largely perennial species. *Festuca arundinacea* is found in damp grassland, riverbanks and seashores. It is found in most of Europe but is absent from northern central and eastern Russia (Tutin et al., 1980). Large collections of *Festuca* exist and no new collections are planned as part of this proposal.

In the short term, the most likely forms of utilisation of grass biomass are combustion (Van Loo & Koppejan, 2008) and anaerobic digestion (Murphy & McCarthy, 2005). In terms of utilisation, *Miscanthus* is currently used as a co-firing feedstock in power stations in the UK

(DRAXPower, 2010) while reed canary grass (*Phalaris)* is used as a feedstock for power stations in Finland. While the combustion of grass in some cases appears to present technical problems compared to wood, there are now a number of commercial boilers suitable for the combustion of *Miscanthus* (Caslin et al., 2010). For anaerobic digestors, grasses in their vegetative stage are suitable as feedstocks (Dieterich, 2007, Smyth et al., 2009). Grass is currently used as a feedstock for anaerobic digestion in Germany (Weiland, 2010) where there are in excess of 4000 units in operation. It is also anticipated that within the foreseeable future feedstock for bio-ethanol production will utilise the cellulose and hemi-cellulose from perennial grasses (Milliken et al., 2007). Another alternative is to use grass as a feedstock for a green biorefinery which involves the application of technology to chemically and physically fractionate biomass into a number of products (Kromus, 2004; Grass, 2004). In simple form, this can involve the extraction of fibre and protein products from grass (Grass, 2004; O'Keefe 2010). However, high value chemicals such as lactic acid may also be extracted and used as a building block for plastic production (O'Keefe et al., 2010) and organic residues may end up in an anaerobic digestion facility to produce biogas and biofertilizer for recycling of the nutrient fractions and recalcitrant carbon. The selection of grasses will need to take these end uses into consideration.

This project had an international dimension by including invaluable research actors and experience from China and Russia and technical objectives were realized in the project consortium by including three integral SME companies and one large international seed company.

The S&T objectives of the project were:

1. Optimising perennial grasses for biomass production through modelling

The initial S&T objectives of this project are to develop parameterized models applicable to C_3 and C_4 perennial grasses including *Miscanthus*, *Dactylis glomerata* (cocksfoot), *Festuca arundinacea* (tall fescue) and *Phalaris arundinacea* (reed canary grass) and to accrue existing information on the project species which will be made available in an accessible format. This is necessary because although considerable knowledge on the distribution, taxonomy, agronomy and properties of C_3 and C_4 grasses exists, it is often difficult to access due to language barriers and presence in many different locations, as well as not being available in digitized form. Well-developed mechanistic models of crop and agro-ecosystem can play important roles in helping to assess crop growth and development through, for instance, designing proper irrigation and fertilization practices, and correspondingly in helping improve yields under different field conditions

The objectives of WP1 'Optimising perennial grasses for biomass production through modelling' are to:

1) Develop and parameterise process-based crop models that can provide reliable predictions of perennial grass carbon assimilation, growth and yield under present and future climate scenarios.

2) Use modelling to identify physiological traits to maximise biomass production of perennial grasses on marginal lands, reduce inputs and improve conversion of biomass feedstock to biofuels and bioenergy. Combine this with the use of geographical mapping and ecological niche modelling to identify areas most suitable for the production of the target species especially marginal land.
3) Provide the framework to evaluate potential traits for improvement of perennial grasses in advance of lengthy breeding programmes.
4) Selection criteria will incorporate outputs from WP3 – 'abiotic stress' and WP4 – 'improved drying under field conditions' and will be used in WP2 – 'pre-breeding and selection of grasses'.
5) Develop the grass portal database/webpage and publish the findings on the web and in journals.

2. Pre-breeding and characterization of C_3 and C_4 grass genetic materials

The candidate energy crops so far identified are largely undomesticated and have not undergone the centuries of improvement that characterise our current major food crops (Koonin, 2006; Vermerris, 2008). This even applies also to cocksfoot, tall fescue and reed canary grass where breeding started only in recent decades. The requirement will be to select appropriate species and genotypes that are adapted to the local soil and climatic conditions which exist in marginal areas. Selection criteria need to be based on the triple goals of maximising productivity, minimising inputs and maximising utilisation for energy production. Some of the traits of particular interest in breeding programmes are drought tolerance, frost tolerance, maintenance of growth at low temperature, chemical composition, resistance to pests and diseases, altering plant architectural features such as dwarf structure and erect leaves and differences in photosynthetic capacity.

The objectives of WP2 are to:

1) Investigate the question of whether current breeding selection is satisfactory for species included in this study and to identify whether many of the required characteristics can be manipulated in breeding programmes.
2) Make further crosses in *Miscanthus* to produce potentially novel varieties with high biomass yield and adaptation to marginal land.
3) Propagate interesting *Miscanthus* genotypes and new hybrids to grow in marginal environments.
4) Collect accessions, in Europe, China and Russia (in all partner countries in the proposal), of *Phalaris, Miscanthus* and *Dactylis* from natural populations including native salt exposed stands.
5) Investigate the genetic diversity of the novel grass species accessions collected during the project and *Miscanthus* species from collections belonging to the different partner groups.

3. Adaptation to abiotic stress conditions influencing biomass yield

In order to limit the competition between bioenergy and food production and to create resilient production systems that can tolerate the increased climatic variation foreseen with climate change, it is important to increase the stress tolerance of the target perennial grasses. Important stress factors that we have selected to address are drought, salt, flooding and cold.

The objectives of WP3 ‘Abiotic stress’ were to:

1) Extend the potential area for non-food grass production into marginal lands that are saline, drought or flooding prone, or experience low winter and spring temperatures in order to reduce competition with food production.
2) Enlarge the growing season and climatic production zones for highly productive C_4 species by improving their cold tolerance.

This will be achieved by screening grass gene-pools for stress tolerance in multi-locations, by improving our understanding of the adaptive mechanisms, by investigating allelic variation of the genes controlling the adaptive traits, and by testing yields, quality and management of selected stress tolerant genotypes under field conditions.

4. Optimising biomass production of perennial grasses under different climatic conditions and improvement of the drying of standing biomass in the field

If grasslands are to be used as a feedstock for industrial and energy purposes, it is imperative that grassland productivity is maximised both from the point of view of maximising returns to the farmer but also from the point of view of minimising competition between food production, while contributing additional services to society such as environmental protection (grassland and biodiversity preservation). The overall objectives of WP4 are to:

1) Use growth trials to optimise the production of perennial grasses as feedstocks for anaerobic digestion, combustion, second generation biofuel production and biorefining.
2) Identify high yielding grass genotypes which exhibit enhanced moisture loss after cutting grass species accessions.
3) Identify high yielding *Miscanthus* genotypes which also display enhanced moisture loss in standing crops.

Project S&T Outcomes

WP1: Optimising perennial grasses for biomass production

Our objectives were to develop and parameterise process-based crop models that can provide reliable predictions of perennial grass carbon assimilation, growth and yield under present and future climate scenarios and to use geographical mapping combined with ecological niche modelling to identify areas most suitable for the production of perennial rhizomatous grasses on marginal land. The process-based crop models were used to identify physiological traits to maximise biomass production of perennial grasses on marginal lands, reduce inputs and improve conversion of biomass feedstock to biofuels and bioenergy. The purpose was to provide the framework to evaluate potential traits for improvement of perennial grasses in advance of lengthy breeding programmes with the intention of identifying selection criteria that can be used in pre-breeding and selection of grasses.

Development of a generic plant growth and development model

We developed a generic plant production model based on WIMOVAC (Windows Intuitive Model of Vegetation response to Atmosphere and Climate), which is based on key physiological and micrometeorological processes underlying plant production (Humphries and Long, 1995). The modules included: leaf photosynthesis, photosynthate translocation, light environment inside the canopy, canopy architecture reconstruction and phenology. The product is a user-friendly interface to enable users to combine the desktop model with the particular climate and soil conditions to identify optimal physiological parameters (including those related to leaf angle, photosynthesis, thermal degree day for reaching particular stage) and optimal management practices (such as water or nitrogen application schedule). The model can be parameterised for C4 (Miscanthus) and C3 (reed canary grass, cocksfoot and tall fescue) grasses.

We have published the C4 metabolism model, which incorporates the basic metabolic processes of C4 photosynthesis and have used this model to explore the major biochemical and anatomical features required to gain an efficient NADP-ME type C4 photosynthesis In addition to this new development, we also have a model of C3 photosynthesis developed from earlier projects. These two models form the basis to link to the canopy photosynthesis models. We have further developed the framework which integrates the canopy architecture information with the steady state photosynthesis models to explore the physiological parameters which can increase the total canopy CO_2 uptake. To do this, we collected architectural parameters for a single plant, and then developed software to recreate the 3D canopy architecture. This 3D canopy architecture model is further combined with a custom-built ray-tracing algorithm to predict the light environments inside a canopy (Song et al., 2013). The basic photosynthetic parameters can be incorporated into this model. We have therefore developed a new method of predicting canopy photosynthesis, which in principle has higher accuracy than the currently used sunlit-shade model.

To test the accuracy of the canopy photosynthesis model, we have used a canopy photosynthesis measurement system to measure the canopy photosynthetic CO_2 uptake rate for both a tobacco and also a rice canopy. We also use this canopy photosynthesis model to predict the canopy photosynthetic CO_2 uptake rates. Our comparison showed that the correlation coefficient (R^2) between the measured and calculated canopy photosynthesis rates are higher than 0.8 (Fig 1.1). This suggests that the new canopy photosynthesis model that we developed can be used to accurately predict canopy photosynthetic efficiency.

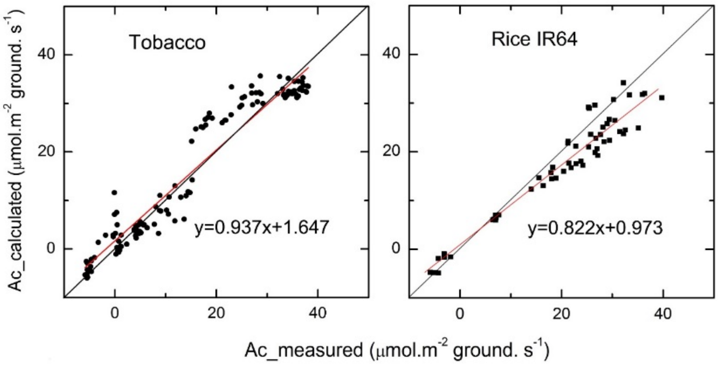


Figure 1.1. The correlation between measured can predicted canopy photosynthesis. These results show that our canopy photosynthesis model can accurately predict canopy photosynthetic CO_2 uptake rates.

To enable a direct prediction of the crop performance for any crop with defined architecture and leaf physiological parameters, we developed an algorithm to link the architecture information, metabolic information with the generic model of WIMVOAC. Basically, the leaf metabolism model is used to build an A-Q (light response) curve and A-Ci (CO_2 response) curve, which are used to derive the key physiological parameters required as input for WIMOVAC. This integration now enables WIMOVAC to predict performance of plants from molecular level and architectural level manipulations.

We have further developed a Java version WIMOVAC. This new Java WIMOVAC can be easily parameterized for different crops. This software has a user-friendly user interface and can be used to explore the potential impacts of manipulating physiological and agronomic parameters on primary productivity of bioenergy crops. The basic physiological processes incorporated into this new version are shown in Fig. 1.2. It has a database for all the essential physiological parameters, architectural parameters. The users can define their simulation scenarios both from the form and also from the database.

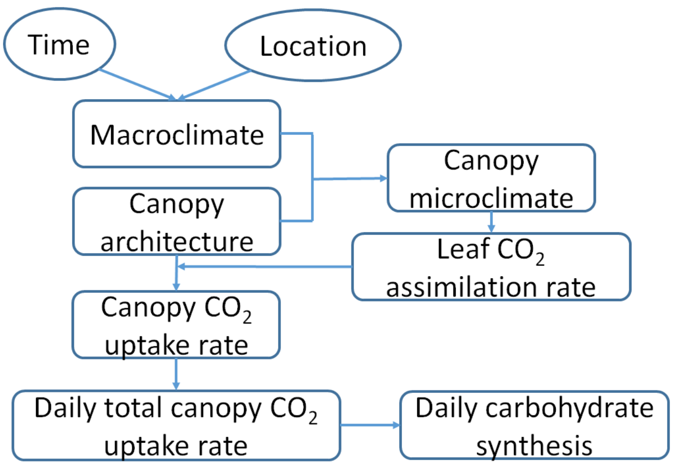


Figure 1.2. The major processes incorporated into this new Java version WIMOVAC.

We collaborated with partners within Grassmargins to parameterize WIMOVAC for both Miscanthus and Festulolium. In particular, we used parameters for photosynthesis under different temperature ranges, and derived new temperature response functions for both species. These new functions are used in WIMOVAC to predict the primary productivity of these crops. Because the basic partitioning table for these species have not been systematically collected in the project we have used parameters from literature on related species to represent the photosynthate partitioning at different developmental stages. As a result, the current model can predict the canopy photosynthesis more accurately, as compared to the prediction for the final biomass predictions. Fig. 1.3 shows the predicted comparison between measured and predicted changes in biomass over time for Festulolium.

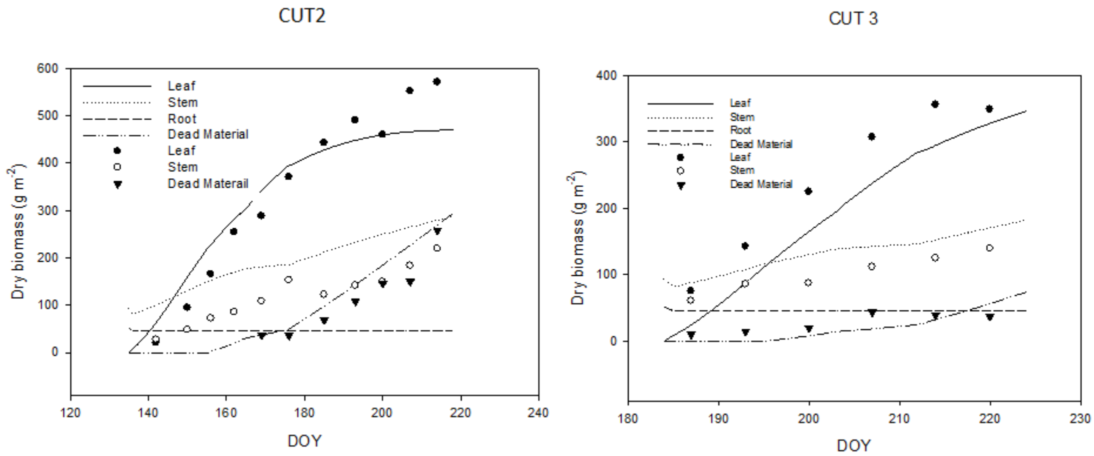


Figure 1.3. The predicted and measured biomass of different organs in Festulolium after the second and third cut. The lines represent the simulation results. The dotted symbols represents the measured values.

With the model available, we are able to systematically evaluate the potential impacts of manipulating different architectural and physiological parameters on canopy photosynthesis. Our analysis shows that altering the leaf chlorophyll content has major impacts on primary productivity. Our analysis showed that the shaded leaves contribute to about 50% of the total canopy photosynthetic efficiency. For crops with lower leaf area index, leaf angle is an important parameter controlling canopy photosynthesis. We have further used the derived temperature response functions for both Miscanthus and Festulolium to predict the canopy photosynthesis at different latitudes. We have 4 accessions of Miscanthus and one Festulolium species in the comparison. The analysis identifies the accession that will show higher canopy photosynthetic CO_2 uptake rate at different latitudes. Because the partitioning of photosynthate into different organs are not well parameterized, we view this prediction will be potentially altered after detailed information on this partitioning is incorporated into the model.

High resolution mapping and ecological niche modelling of perennial grasses for biomass production.

High resolution mapping and ecological niche modelling of perennial rhizomatous grasses was undertaken to establish their distribution and factors limiting production. To investigate the limiting ecological factors for the growth of the target bioenergy grasses in Europe we collated existing geographical and ecological data on the species and their close relatives. We then constructed ecological niche models and model biogeography to help predict the areas with highest potential for production of each of the grasses and to identify the abiotic (climatic, edaphic) factors that determine their current distribution. Once these factors had been identified breeders are able to better select germplasm for development of new varieties especially for growth in marginal habitats. Collation of data has been undertaken via the ‘GrassPortal’ database which collates information from Floras and herbaria searches and national biodiversity databases. We have used high resolution mapping and climatic envelope modelling (CEM) to identify regions in Europe including marginal habitats most suitable for the growth of each bioenergy crop. Data has been collected from existing sources and has been supplemented with newly digitized occurrence data from herbarium specimens in Royal Botanic Gardens Kew, England; British Museum, London, England; Leiden, Netherlands; Copenhagen Denmark; and Trinity College Dublin Ireland. Georeferenced distribution records of the target species (i.e. with information on latitude/longitude) was obtained from GrassPortal. Data on current climate from all over the world is already linked to these georeferenced records within the GrassPortal system, using the global climatology reconstructed for the CRU CL 2.0 dataset (http://www.cru.uea.ac.uk). Computation of the CEMs used BIOCLIM and was implemented in DIVA-GIS. More sophisticated algorithms were also tested, including Genetic Algorithm for Rule-set Production (GARP) and Maxent. Model prediction performance was tested with the area under the curve statistics. Another way to test model output is Cohen's kappa statistic of similarity (k), applicable when presence and absence data are available.

Data from a variety of national, regional and global species databases (e.g. Global Biodiversity Information Facility and digitised Kew Gardens herbarium records) were combined with data collected by the GrassMargins project team. Baseline climate and climate change datasets, developed by the Ecochange Project (www.ecochange-project.eu), were used to undertake the modelling at a 10 km resolution. Nineteen principal bioclimatic variables were derived from the monthly temperature and rainfall values in order to generate more biologically meaningful variables.

The bioclimatic variables represent annual trends (e.g., mean annual temperature, annual precipitation) seasonality (e.g., annual range in temperature and precipitation) and extreme or limiting environmental factors (e.g., temperature of the coldest and warmest month, and precipitation of the wet and dry quarters). Six variables were used to undertake the modelling, including three temperature variables, two precipitation variables, and potential evapotranspiration. The distribution (current and future projections) of the various perennial grass species/subspecies were modelled using an ensemble of species distribution modelling techniques available in the BIOMOD2 library (http://cran.r-project.org/web/packages/biomod2/biomod2.pdf), run and calibrated within the R statistical programming environment (R Development Core Team, 2013): Generalised Linear Models, Random Forests, Generalised Boosting Models, Artificial Neural Networks, Classification Tree Analysis, Multiple Adaptive Regression Splines, Flexible Discriminant Analysis, and Generalised Additive Models, Maxent, and Surface Range Envelope. An ensemble approach to the modelling was used to account for the uncertainties associated with the different regression and machine learning techniques outlined above using a consensus approach rather than comparing single modelling outcomes. A split-sample cross-validation procedure was used to evaluate the models using the area under the curve (AUC) of the receiver operating characteristic (ROC) (Swets, 1988), Cohen's Kappa statistic and the True Skill Statistic (TSS) (Allouche *et al.*, 2006). Two time slices (2021 to 2050 and 2050 to 2080) were used to investigate the impacts of climate change on the future distribution of the IAS. The chosen climate change scenarios included both a range of RCMs (CCSM3, HadCM3 and ECHAM5) and SRES (B1, A2, A1b) providing a minimum of 12 different climate change datasets to account for the RCM and SRES uncertainty in the modelling

In summary, significant additional occurrence records have been added to the GrassPortal database and species occurrence data are now spatially relatable to a range of environmental and land use datasets. Species distribution models (SDM) have been built for each of the species, predicting with their distributions and identifying the suitable climate conditions required by each species. Key climatic drivers of the species distributions have been quantified comparing variable importance in the SDM and potential range change responses of the species under climate change have been quantified.

WP 2: Pre-breeding and selection of grasses

To extend the growing area of miscanthus it is necessary to find genotypes better adapted to different climatic and soil conditions. The high biomass cultivar *M.* × *giganteus* as an interspecific hybrid has a high degree of heterzygosity. A large contribution to biomass yield can be expected from heterosis in hybrids made from crossings of different genetic backgrounds. Collection of new genotypes can broaden the genetic basis of agronomically valuable cultivars, and provides new genotypes for direct testing and breeding of future crops. Investigating the genetic diversity of existing gene pools and novel collected accessions is a prerequisite to identify breeding potential, or possibly gaps in the present collections, and provides valuable information for the planning of future breeding work.

Propagation of *Miscanthus* genotypes for experiments

Twenty nine different genotypes were propagated for experiments and field trials. They were selected from collections held in Ireland, Denmark, Sweden, Poland, Germany and Russia and include different *Miscanthus* species (Table 2.1). Genetically uniform clones were obtained by micropropagation starting with buds. Approximately 25.000 in vitro propagated plants were shipped to the project partners for their different trials. Micropropagated shoots of all *Miscanthus* genotypes were kept in long term storage throughout the project duration.

Table 2.1: Micropropagated clones of *Miscanthus*

***Miscanthus* species**	**Number of genotypes**
M. sinensis	9
M. sacchariflorus	4
M. lutarioriparia	1
M. × *giganteus*	12
M. × *giganteus* related hybrids	3

Creation of novel hybrids of Miscanthus

Depending on the target region for hybrids, various genome combinations are conceivable for Northern Europe (*M. sinensis* with *M. sinensis* or *M. sacchariflorus* day-neutral), Middle Europe (*M. sinensis* with *M. sacchariflorus* large-stemmed) or Southern Europe (*M. sacchariflorus* large-stemmed with *M. sinensis*, *M. sacchariflorus* or *M. floridulus*). For these crossings Teagasc (Ireland) supplied genotypes from a number of European collections while Aarhus University (Denmark) provided genotypes collected at various locations in Japan.

Forty two *M. sinensis* x *M. sinensis* crossings were mainly made between genotypes from gene pool collected in Japan and held by Aarhus University (Denmark) and the gene pool held by Teagasc (Ireland) origin from European collections. It is highly likely that there is a genetic diversity between these pools. The crossings were done in isolated pairs in the greenhouse. The strongest seedlings were selected twice in the greenhouse. Novel hybrids (736 hybrids in Ireland and 658 hybrids in Denmark) were transferred in the field either for further evaluations. The survival rate after the first winter varied within each crossing group from 33 to 100%. One cross seems to be promising (by the high survival and fast growing rate) for intensive further breeding work as a base of a probably novel variety. Additional 30 crosses were made in the same way, and the resulting seeds are stored at -20°C for further experiments.

Three *M. sinensis* x *M. sacchariflorus* (dayneutral) crossings were made as described above. The Siberian branch of Russian Academy of Science selected one *M. sacchariflorus* genotype which had similarity to *M. sacchariflorus* Robustus. Novel hybrids (80 hybrids in Ireland and 168 hybrids in Denmark) were transferred in the field. Their survival of the first winter varied within each crossing group between 60 and 100%. Additional 903 novel hybrids were used for experiments under controlled environmental conditions in growth chambers at Aarhus University (Denmark) for the selection of cold tolerant genotypes. Additional two crosses were made and the resulting seeds were stored at -20°C.

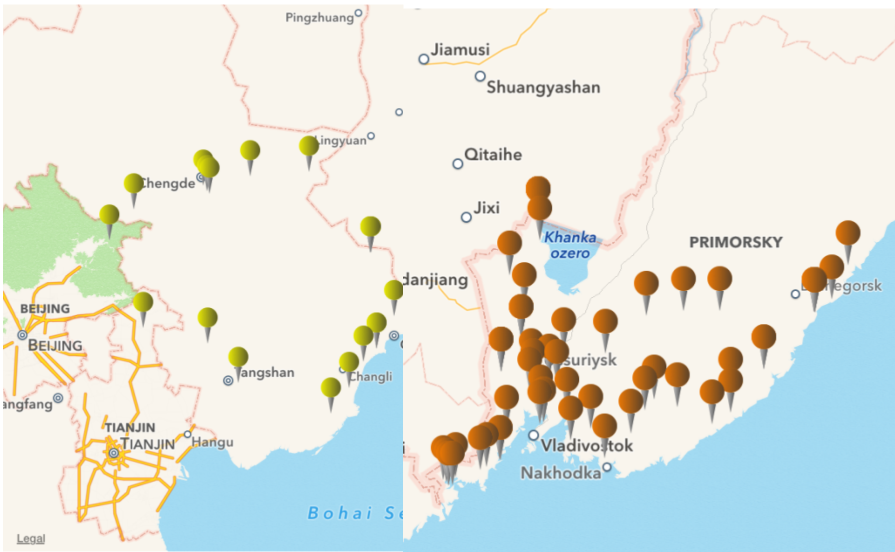
Inclusion of *M. lutarioriparius* in crossing programs is considered promising because of its high biomass accumulation, frost survival and flooding tolerance. The *M. lutarioriparius* genotype was provided from Kai-Uwe Schwarz (JKI Braunschweig, Germany) and had been collected at 29°19N; 112°57O in China. A short day treatment was undertaken and only the crossings seeds from *M. lutarioriparius* were harvested. Novel hybrids (186 hybrids) were planted in the field in Denmark for further evaluations.

One aim for *Miscanthus* improvement is the resynthesis of new triploid hybrids similar to the widely cultivated *M. × giganteus* genotype. Potential crossings are *M. sinensis* x *M. sacchariflorus* large-stemmed. A field experiment was started in Spain (N 36° 43´) to induce flowering at *M. sacchariflorus* large-stemmed. Only the seeds from *M. sacchariflorus* were harvested and are stored at -20°C for further experiments.

Polyploidisation in *Miscanthus* can directly lead to gigantism, and is also the basis to construct new genomic combinations. Of interest are *M. sacchariflorus* (day neutral) and late flowering genotypes of *M. sinensis*. Both are diploid, and tetraploid forms would be desirable as breeding material. The callus from 12 genotypes out of 20 was able to multiply and was treated with colchicine 313 µM. The treated callus from five genotypes was able to form shoots. Between 18-100% of the regenerated shoots were tetraploid. 149 tetrapolid plants were transferred to soil and sent to partners for further evaluations.

Collection of new genotypes of *Phalaris*, *Dactylis* and *Miscanthus*

To give an overview on genetic variability within *Phalaris*, *Dactylis* and *Miscanthus* reviews were provided. To enlarge the genetic variability in particular with respect to cold resistant genotypes, and to examine *Miscanthus* population genetic structure a collecting trip was undertaken during September 2014 to collect *Miscanthus* in Russia (Primorsky Krai). Rhizome, leaf, seed and herbarium samples were taken from over 20 populations. 10 populations were sampled intensively and the other populations sampled for two individuals. Rhizome material was planted in pots. *Miscanthus sacchariflorus* and *M. sinensis* were collected. Some material was identified as potentially hybrid and this will require genetic analysis to confirm. Additional a *Miscanthus* collecting trip was also undertaken in China (Beijing, Hebei, Liaoning) during mid September. Rhizome, leaf, seed and herbarium samples were taken from over 20 populations. Rhizome material was planted in pots. Only *Miscanthus sacchariflorus* was collected as no *M. sinensis* was found. One specimen showed characteristics of hybridization and will be investigated further by DNA analysis. Summary maps of collecting localities are shown below (Figure 2.1).



China Russia

Figure 2.1: Collecting localities for Miscanthus germplasm in China and Russia.

Germplasm collections of *Phalaris* and *Dactylis* were done in Europe (Poland, Germany, Denmark, Sweden, UK and Ireland). Ten Populations of *Dactylis glomerata* and of *Phalaris arundinacea* have been sampled in each country for seed, rhizome and leaf. Seed was collected from 30 individuals per population, leaf from 15 individuals per population and rhizomes from 2 individuals per population. Voucher specimens were collected during the collection expedition of *Phalaris* and *Dactylis* in Europe. These are herbarium specimens that serve as reference material for species identification and verify the status of the germplasm collected. These are kept at in the herbarium at Trinity College Dublin. Voucher specimens for *Miscanthus* are housed in Vladivostok Botanic Gardens and Trinity College Dublin herbaria. Ecological, edaphic and geographic information has been collected for all of the collections described above for *Phalaris, Dactylis* and *Miscanthus*. The information overlaps with the ecological niche modelling of WP1. This has been submitted for safe archival and uploading on to GrassPortal.

Phalaris and _Dactylis_ Genetic diversity assessments by plastid markers

DNA has been extracted from all plant material collected from *Phalaris* and *Dacytlis* in Europe and plastid DNA amplified and sequenced using long read PCR. Genotyping of the newly collected *Dactylis* and *Phalaris* accessions from Poland, Germany, Denmark, Sweden, UK and Ireland was undertaken using plastid DNA SNPs generated via long read PCR and MiSeq sequencing. An example of the genetic analysis for *Phalaris* is given in Figure 2.2. The datasets are of high quality. The aim for *Phalaris* and *Dactylis* genotyping was to cover at least half of the plastid genome and to test the markers on a large number of samples collected across north-west Europe to assess genetic diversity and examine population genetic structure. These data would complement the nuclear genome SNPs for these two species. Plastid markers are particularly well suited for the examination of geographic population genetic structure in grasses as they are maternally inherited and therefore distributed by seed dispersal.

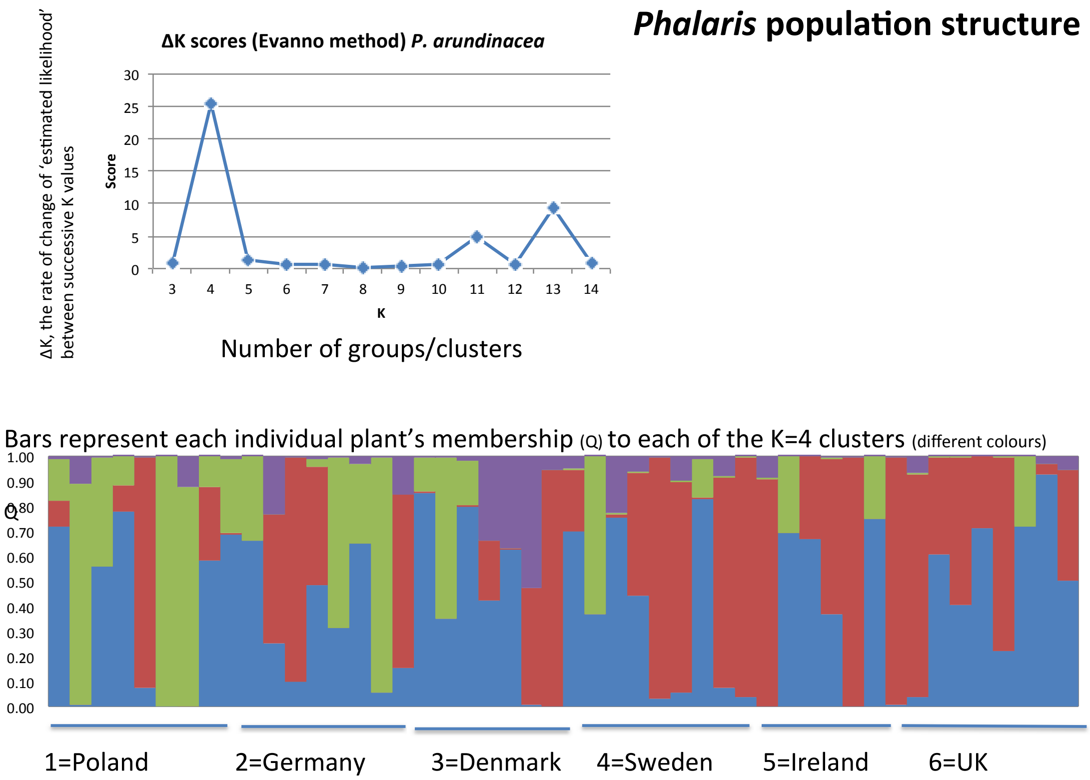


Figure 2.2: Population genetic structure in European Phalaris samples genotyped using plastid SNPs of near complete genomes.

Genotyping by sequencing of *Dactylis* and *Phalaris*

Dactylis and *Phalaris* accessions have been collected from the wild in 2012 (Ireland, UK) and 2013 (Denmark, Sweden, Poland, Germany). DNA of 96 *Phalaris* and 88 *Dactylis* DNAs has been extracted for gbs at a provider. The sequencing had to be repeated because of a low read quality, and in agreement with the provider samples with low read counts were re-sequenced. The results are in, and are at present analysed.

Microsatellite marker in *Phalaris*

A normalized cDNA library from *Phalaris* RNA from different tissues has been sequenced on the FLX-454 Roche next generation sequencing platform. The reads were assembled into putative transcripts, and this sequence information was used to design Microsatellite markers. 60 PCR primer pairs for microsatellite amplification have been synthesized and partially tested. The markers were tested for polymorphisms between various accessions from across the collection range Ireland, UK, Denmark, Sweden, Germany, Poland) of *Phalaris*. PCR reactions of ten primer pairs have been prepared. To identify polymorphisms, seven samples have been sent away for cloning, and Sanger-sequencing of 10 clones for each primer pair. These data are presently analysed. For the remaining PCR products the concentration obtained has been too low for the provider, so we cloned them, and sent isolated plasmid samples away for sequencing.

Genotyping by sequencing of Miscanthus

A gbs (genotyping by sequencing) experiment has been conducted to analyse the living collection of *Miscanthus* held at Teagasc Oak Park, Carlow. This consists mainly of the high biomass (3n) sterile hybrid *M.* × *giganteus*, its parent species *M. sinensis* and *M. sacchariflorus*, and spontaneus and artificial hybrids. This is an important task for the assessment of pre-breeding material as many of these plants were duplicated from existing European collections, and information about the geographic origins and genetic constitution are in many cases sketchy or unreliable. This set of samples is enlarged by samples from other collections such as Trinty College Dublin, and in particular plants obtained from well documented collection trips to Japan (obtained via Aarhus University, Denmark) and China (obtained by Chinese Academy of Science). In Sept 2014 a collection trip to the *Miscanthus* northern range has been organized, and material from this trip to Far East Russia and China (Province Hebei) have been included in the gbs data set as well. In total, 146 genotypes from the Oak Park field (Teagasc, Ireland) collection were analysed, 116 samples from the GrassMargins collection travel to Far East Russia and the Chinese Hebei province, 14 sample from the collection of Chinese Academy of Science in Shanghai, and 20 samples from the Japan collection, held at Aarhus University in Denmark.

Our gbs follows the protocol from Elshire et al. 2011 (PLoS ONE 6(5): e19379. doi:10.1371), with modifications. Next generation sequencing is applied to obtain molecular markers that are the basis of the final genotyping analysis, without reliance on pre-existing genomic information. From restriction enzyme (RE) digested genomic DNA, reads are generated starting at the RE sites, in our experiment of the enzyme PstI. Only fragments of up to a few hundred base pairs are converted into sequencing libraries by a PCR process, further reducing the number of read starting points. Reads from homologue sites are aligned, and searched for positions with some variability in the base composition (SNPs, single nucleotide polymorphism). Because of the high read count of ~150M reads per Illumina HiSeq lane, the investigated samples are highly multiplexed, and are de-multiplexed after the sequencing based on sample specific in-line barcode sequences. The optimal combination of RE, taxon, and multiplexing can only be determined experimentally: the more frequently a RE cuts, the higher is the potential number of SNPs. At the same time the required number of reads per sample increases as each RE sites needs to be covered adequately, and the extent of possible multiplexing decreases.

The *Miscanthus* samples have been sequenced in four Illumina sequencing cell lanes, each multiplexed between 96x and 56x. The data set has been analysed with help from Dr. Paul Cormican with whom we have developed a software pipeline for *Miscanthus* gbs. The initial quality check was done with FastQC, the Stacks package has been used for de-multiplexing, alignment of reads into stacks, and identification of SNPs. At present a dendrogram has been constructed comprising 130 *Miscanthus* accessions (see Figure 2.3). The graph is based on ~ 11000 SNP markers, and summarizes the results of the first two gbs experiments. A large number of rather uniform accessions at the bottom of the figure (3n accessions, green dots) indicate that most *M.* × *giganteus* accessions are very close, and presumably clonal copies that have spread through various collections. The variability among the 2n accessions is much higher (red and yellow dots, mostly *M. sinensis*). The remaining genotypes have been sequenced in additional two gbs experiments, and the data need to be incorporated into the existing dendrogram. The latest data contain in particular results from the GrassMargins collection to Far East Russia (Primorsky Krai region) and China (province Hebei), additional genotypes obtained from Chinese Academy of Science (Shanghai), and more samples from the Japan collection obtained via Aarhus University (Denmark). The parameters for genetic distance analysis and the criteria for the dendrogram construction are going to be optimized over the next weeks.

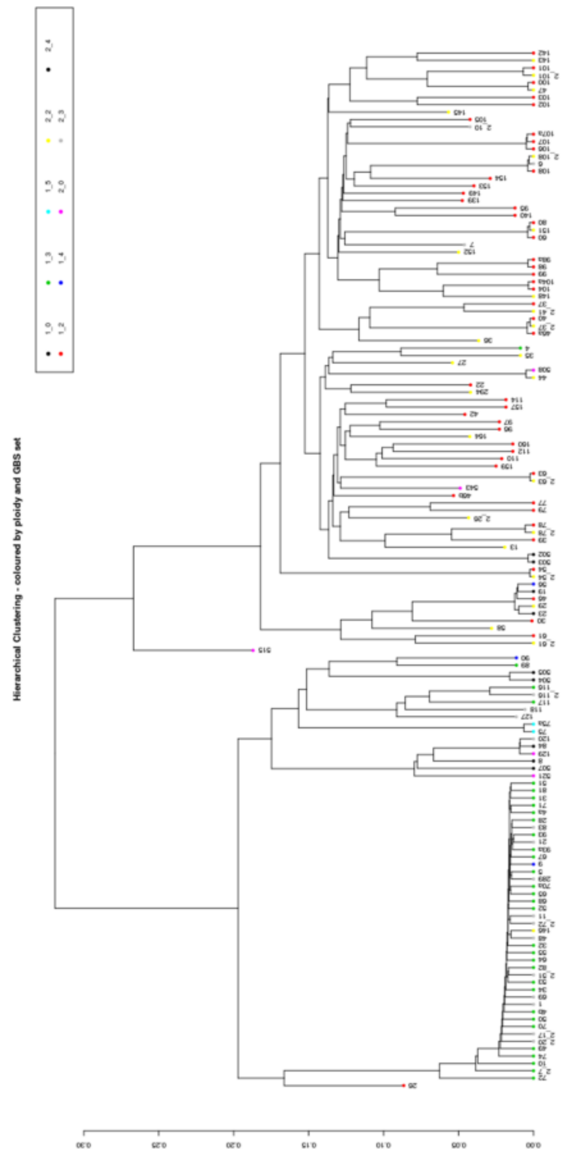


Figure 2.3: Hierarchical clustering of 130 Miscanthus accessions based on 11000 gbs derived SNPs; the graph represents the results from two out of the total of four Illumina sequencing lanes

WP3: Tolerance to abiotic stress

The tolerance to a certain level of stress is of key importance for selection of the most promising grass species and varieties for production on marginal land and for production over a wide climatic gradient. The tolerance to salt, drought, flooding, cold and frost was analysed in the project grass species and varieties with a range of methodologies. Existing gene pools were screened in the field while specific tests were conducted under controlled conditions in lysimeter, greenhouse and climate chamber facilities. The analyses applied ranged from phenotypic development, over plant physiological measurements and compositional analysis, to expression libraries or DNA libraries for the determination of allele frequencies.

Salt tolerance

A greenhouse screening of cocksfoot, tall fescue, Festulolium and reed canary grass on Rockwool blocks was performed. The plants were established without salt stress during 60 days and were later challenged with stepwise increasing salt concentrations from 0.5% to 2.5% salt (w/v). We found variation within and between all the tested species. A ranking of the tested species starting with the most tolerant looks like the following:

Tall fescue > Festulolium > Cocksfoot > Reed canary

Early in the screen, under low to moderate salt concentrations, the species and varieties mainly responded in different growth rates and not so much in discoloration (except for reed canary) or development of dead leaf material. We took leaf samples for RNA sequencing of accessions coping well with the stress and from those severely affected. With increasing salt concentrations, especially those accessions with high biomass production under low to moderate salt conditions were the ones to die fastest, as if the high growth rate was the cause for the salt poisoning at higher concentrations.

Even though tall fescue was the most salt tolerant species in general, different accessions showed quite variable in their growth and appearance especially after 122 days of salt-stress (shown as % green shoots in Fig.3.1).

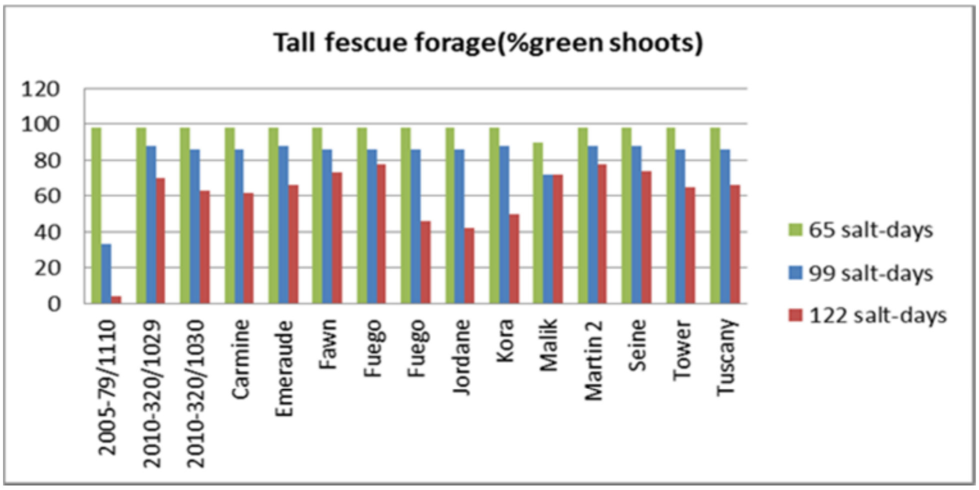


Figure 3.1. Salt stress effects on tall fescue varieties in a greenhouse experiment

Reed canary grass was the most sensitive species which showed discolorations and growth reduction already at low salt concentrations (Fig. 3.2).

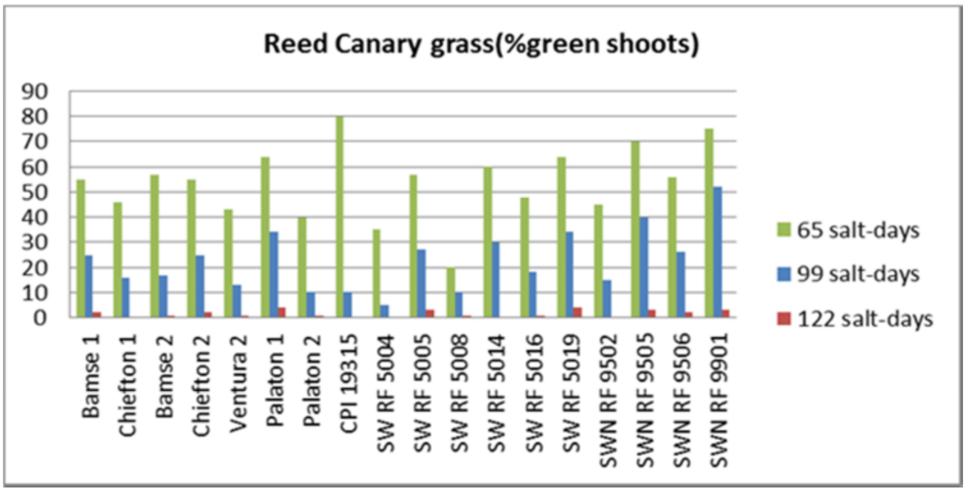


Figure 3.2. Salt stress effects on reed canary grass varieties in a greenhouse experiment

The growth of miscanthus failed in the Rockwool blocks and was repeated in larger soil pots. In general a clear growth retardation of all tested clones could be seen under increasing salt stress. The highest salt tolerance was observed in the varieties Strictus and H7 which were still green at a salt concentration of 1.5% NaCl (Fig. 3.3).

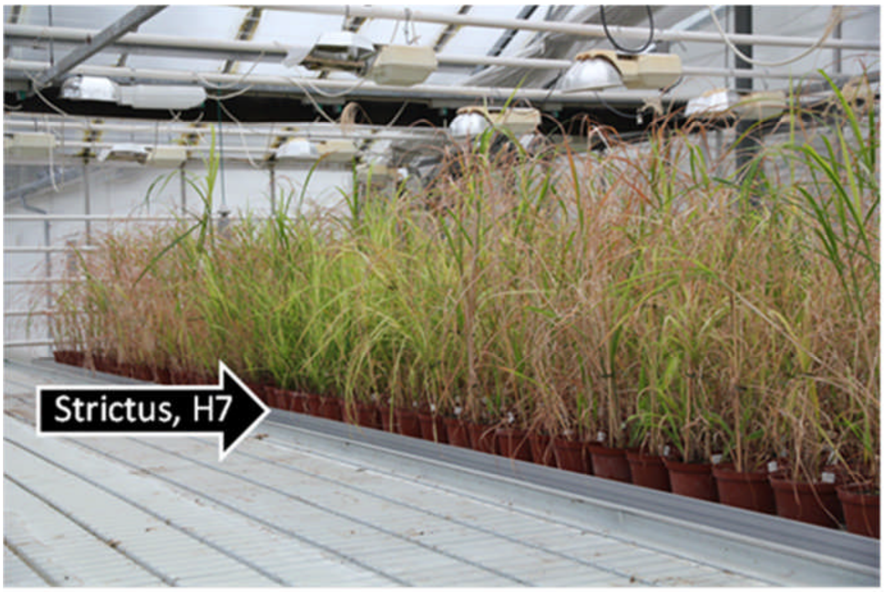


Figure 3.3. Salt stress effects on miscanthus varieties in a greenhouse experiment

The screening results were followed by a field trial with cocksfoot, tall fescue, reed canary grass and festulolium on an area in France subjected to both salt and drought stress. However, in general the grass establishment was poor due to harsh climatic conditions. The field was not managed at all, except by grazing of cattle for meat production. Tall fescue and festulolium varieties performed best, followed by cocksfoot varieties. Reed canary grass did not establish at all on the field. The best performance (ground cover, weed competition and disease resistance) was rated for the varieties Tower and Hykor in the tall fescue/festulolium group. Furthermore, festuca-like varieties performed better than lolium-types in the festulolium group.

Cocksfoot performed clearly not as well as tall fescue/festulolium, but the variety performing best was Sevenup. These difficulties encountered in the establishment of a proper field trial in a heavily salt contaminated soil pinpointed that it is important also to test for germination efficiency under salt stress. Salt tolerance during germination is not related to salt tolerance in an established sward. Furthermore, a breeding effort has to be initiated to select for the highest yielding grass varieties under marginal conditions (low or no fertilizer, high salt or drought stress). All the tested varieties were originally developed for high yield with appropriate fertilization and no abiotic stress, and they had a hard time to compete with the natural grasses already established at the trial site.

Drought tolerance

The use of stable isotope ratios as an indicator of water use efficiency was investigated in a greenhouse experiment on the three C3 species. We investigated if $\Delta^{13}C$ is correlated to season-long WUE (WUE_{SL}).All investigated species and varieties showed lower $\Delta^{13}C$ when exposed to drought, and there were significant correlations between $\Delta^{13}C$ and WUE_{SL} (Fig. 3.4).

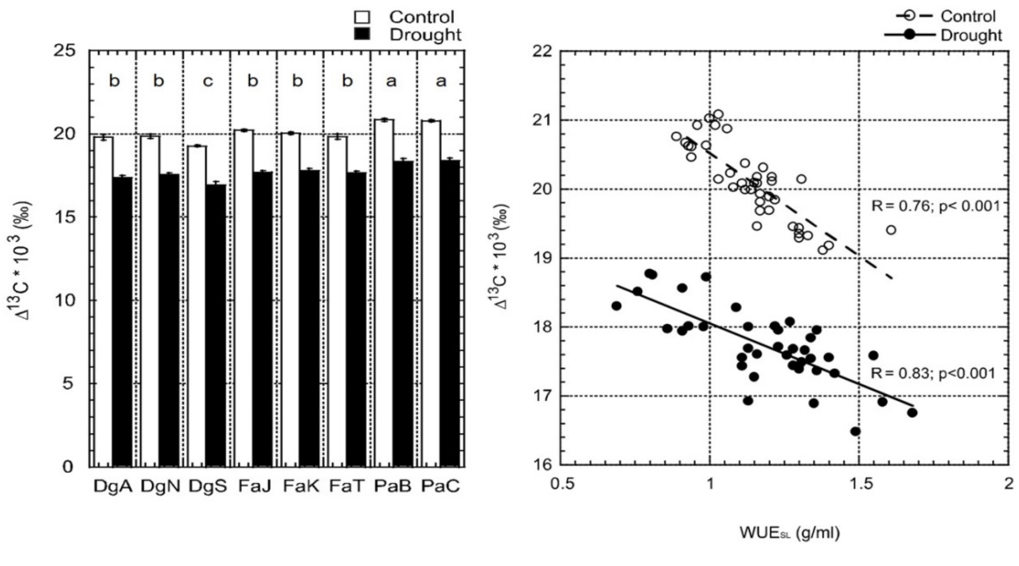


Figure 3.4. Discrimination of ^{13}C (‰) in shoot material of species and varieties under control (white bars) and drought treatment (black bars) – LEFT. Relationship between $\Delta^{13}C$ discrimination (‰) and WUE_{SL} (g/ml) - RIGHT.

Cocksfoot obtained highest shoot dry weight under both control and drought conditions while reed canary grass showed lowest shoot dry weight. Similar patterns, with tall fescue being intermediate between the high-performing cocksfoot and poor-performing reed canary, were found also for shoot:root ratio and WUE_{SL}. Within-species genotypic variations were most pronounced in cocksfoot, where the variety Sevenup performed best in terms of both shoot dry weight and WUE_{SL}.

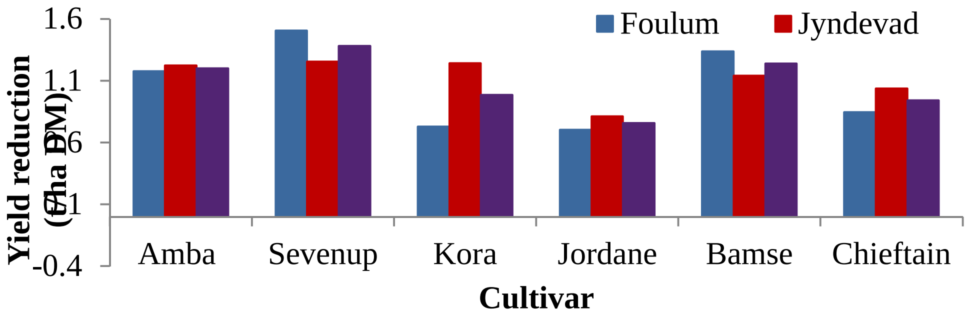


Figure 3.5. Yield reduction (difference in DM yield between control and drought stressed plots) in one cut under semi-field conditions at two soil types (Jyndevad - coarse sand, Foulum - loamy sand).

Drought stress was then tested under semi-field conditions where the effect on productivity, stomatal conductance (g_s), leaf water potential (Ψ_l), ratio vegetation index (RVI) was followed. Across soil types and treatments, the cocksfoot variety Sevenup had the highest DM yield. Both across soil types and treatments as well as after drought stress, the DM yield was significantly higher in the varieties Sevenup, Kora and Jordane compared to Amba, Bamse and Chieftain. Even though not the highest yielding variety, Jordane had the lowest yield reduction as a result of drought stress (Figure 3.5).

Flooding tolerance

Up to ten genotypes of each five grass species were screened under semi-field conditions. After Flooding Treatment Initiation (FTI) the water table was gradually increased (2 cm/day) over a 13-days period until it reached the soil surface. Mean daily shoot Growth Rate (GR) was calculated, Leaf Chlorophyll Fluorescence (LCF) measured, and root porosity (percentage air space within roots) was quantified.

Before flooding cocksfoot had significantly higher GRs (2.1 cm/day) than the other four species, but shortly after FTI, GR quickly decreased and cocksfoot ended up as the most flooding affected species. GRs of tall fescue and festulolium also decreased throughout the period, but at a slower rate than cocksfoot (Fig. 3.6). In contrast, GRs of RCG and miscanthus increased after FTI. Some differences between genotypes within each species were also detected, but they were less significant.

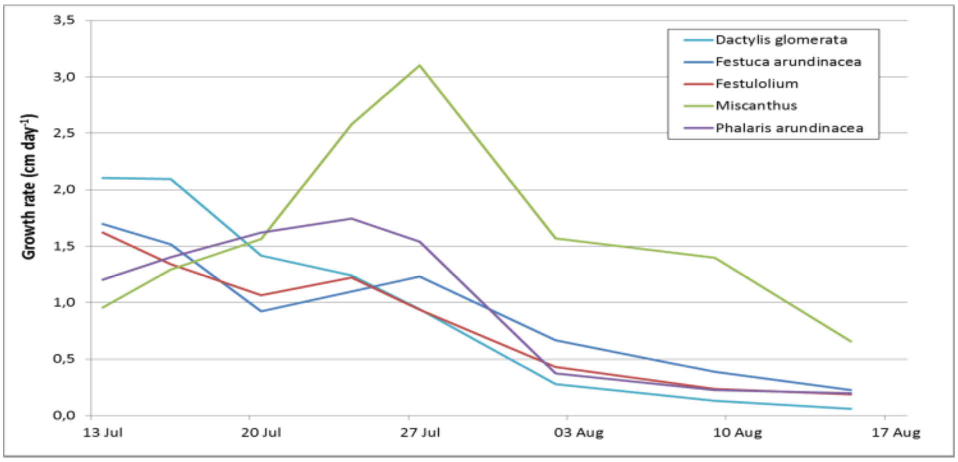


Figure 3.6. Daily shoot growth rates before onset of increased water table (from July 15th) until full flooding (from July 28th).

Surprisingly, miscanthus seemed to be the most flooding tolerant of the five species which was supported by measurements of root porosity. Four weeks after FTI, 47 % air space in miscanthus roots, 18 % in RCG, 12 % in tall fescue and 10% in festulolium were detected across varieties.

Naturally flooded sites have been established in Ireland and Denmark. Reed canary grass, tall fescue, festulolium and miscanthus have all thrived well at the flooded sites, while cocksfoot established well but ended up yielding poorer than the other species.

Cold tolerance

The heredity of cold tolerance was investigated on 881 offspring from 13 *M. sinensis* x *M. sacchariflorus* crossings. They were grown in the greenhouse and measured for chlorophyll *a* fluorescence (F_v/F_m) during cold treatment in growth chambers. A high F_v/F_m value is an indication of high cold tolerance. F_v/F_m varied between 0.825 and 0.439 when measured at 12°C and between 0.741 and 0.315 at 8°C. Higher mean F_v/F_m was measured on seedlings from seeds harvested from *M. sacchariflorus* than on seedlings from *M. sinensis* mothers, indicating participation of the maternal cytoplasm in the hereditability of this trait. A total of 33 hybrids with high F_v/F_m values were selected and have been planted in the field in Denmark in order to test their phenotypic performance.

Winter survival was registered on offspring from crosses between *M. sinensis* x *M. sinensis* and *M. sinensis* x *M. sacchariflorus.* The winter survival was 79 and 91% in Ireland and Denmark, respectively. The offspring were planted according to the origin of parents. The survival rate within each group varied from 33 to 100%. For seven crossings all offspring survived the winter in both Ireland and Denmark. In Denmark the plants started flowering in the beginning of August. On August 24th 2015 the shoot length of the offspring varied between 28 and 203 cm. An offspring from crossing of *M. sinensis* S 94 with *M. sinensis* D-60 had the longest shoot. The average shoot length of offspring from this crossing was the highest of all crossings with 167 cm. As all offspring from this crossing also survived the winter in both countries they might be interesting for future investigation and cultivation.

Miscanthus genotypes were followed under field conditions in Poland during variable temperatures in spring. A few genotypes had rather high Fv/Fm values even at the two lowest temperatures (Fig. 3.7). It seemed from shoot growth rates that there was most often a better growth of the genotypes with high Fv/Fm but the correlation was not unique.

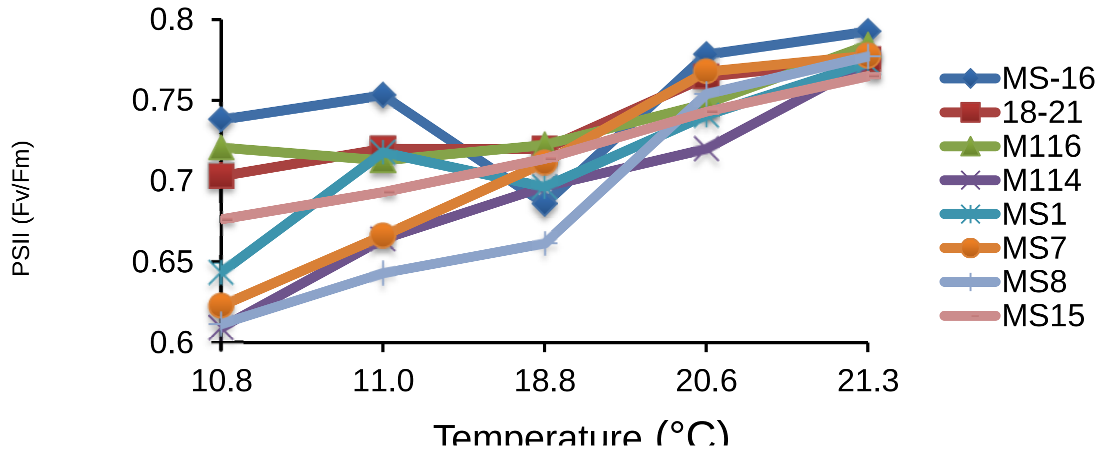


Figure 3.7. Maximum quantum yield of PSII (Fv/Fm) of miscanthus at cold and warm temperatures in the field.

An extensive data collection on leaf photosynthesis of 14 miscanthus genotypes in response to temperature and light was done in climate chambers. There seemed to be no irreversible cold stress induced by growing the genotypes at 14C which for many other C4-crops (e.g. maize) causes damage to the photosystem. Further decrease in growing temperatures (down to 6C) was then conducted with comparison to the C3 festulolium. The data showed a clear advantage of C3 photosynthesis at low temperatures, even though the continued photosynthesis of miscanthus at 6C (at a low level) was a new discovery.

A few other interesting results were that there was a good correlation between the field measured shoot growth rates (done in Sweden, Poland and Denmark) and leaf photosynthesis in climate chamber. And there seems to be a surprising positive correlation between specific leaf area and leaf photosynthesis (Fig. 3.8) which means that the thinner the leaf the higher CO_2-fixation per leaf area. Therefore, these two parameters may be deployed as screening tools for miscanthus productivity in breeding programs.

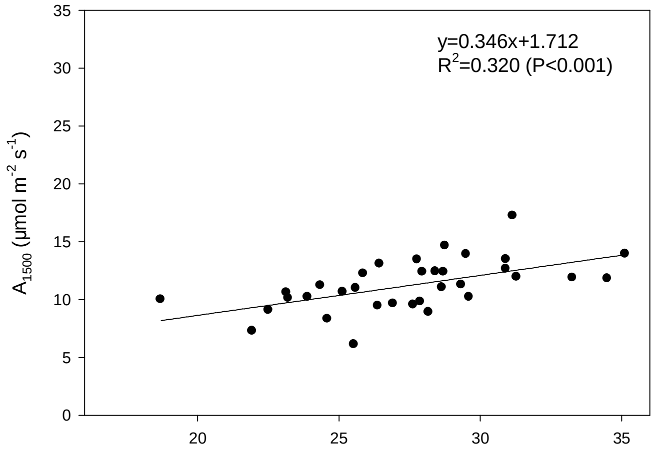


Figure 3.8. Relationship between specific leaf area and photosynthetic rate under cold conditions in miscanthus genotypes.

Leaf samples were taken from the miscanthus genotypes after growing under cold conditions for analysis of cold induced enzymes and carotenoids e.g. PPDK and zeaxanthin, and new immunological methods were developed at the Russian partner. PPDK increase in response to low temperatures is considered to keep the efficiency of C4 photosynthesis at a high level. Fig. 3.9 presents the results of ELISA analysis of miscanthus before and after cold induction.

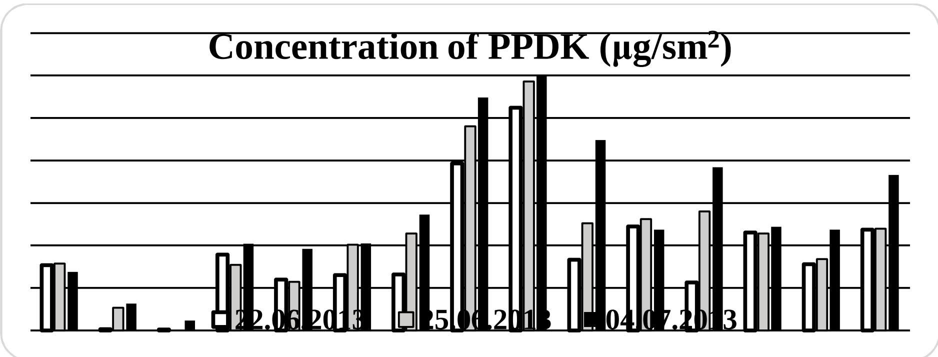


Figure 3.9. PPDK content in Miscanthus at 24C (22.06) and after increasing time at 14C (25.06 and 04.07).

The results show very different responses to temperature. The basic level of PPDK during warm conditions varied significantly, and some genotypes had no significant increase in PPDK concentrations after cold induction. The most pronounced response was observed for EMI 9 (*M. sinensis* hybrid), in which PPDK increase by 240%. For 55 and EMI 3 (*M. sacchariflorus*) the increase in PPDK content was also high (106% and 169%, respectively). Genotypes M114 (*M. sinensis X M. sacchariflorus*), 26S and 39 (*M. sinensis*) had PPDK content increased by about 55%.

Spring frost tolerance

The field collection of miscanthus genotypes in Denmark experienced two very cold nights in May 2012 reaching -3C. Many miscanthus shoots were killed from these conditions but shoots from other genotypes survived the harsh conditions (Fig. 3.10). In *M. giganteus* 20-30% of the shoots survived

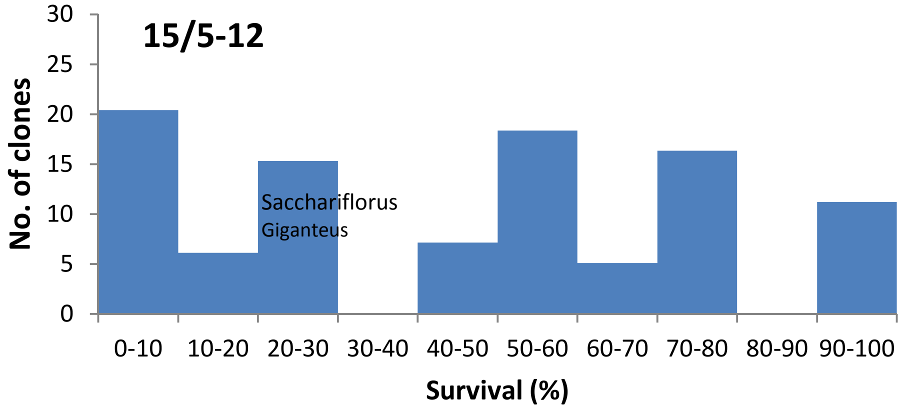


Figure 3.10. Shoot survival after severe frost on May 6-8th.

RNA sequencing experiments

In RNAseq the whole complement of mRNA is transcribed and sequenced. The transcripts of an experimental sample mapped against a reference transcriptome provides information on the level of expression of identified transcripts, derived from read counts, and splicing variant information. The comparative analysis of samples between control and treatments, but also between more susceptible and more resilient accession allows an unbiased view into the response of a plant to abiotic stress, and in whether resilient and susceptible accessions react differently to the same stress.

Reed canary grass samples extracted during the salt stress experiment were converted to cDNA and analyzed in a RNA-seq experiment. A novel reed canary reference transcriptome has been constructed from a cDNA sequenced on the Roche 454 platform. Differentially expressed sequenced were identified in collaboration with IBM research using a novel software, and a first paper on reed canary transcriptomics of salt stress response has been published.

In a greenhouse water stress experiment cocksfoot and reed canary grass were subjected to drought and waterlogging stress. Samples for RNA-seq were taken during the experiment, and in parallel measurements of water content of soil and of various stress-related parameters were taken to assess the extent of stress the plants were experiencing. A similar water stress experiment has been carried out for a range of Miscanthus genotypes, but these samples have not yet been analyzed.

A new reed canary transcriptome was constructed from a combination of all reads generated for this experiment, treatments and controls. The new transcriptome comprised three times the number of transcripts of the previous transcriptome (61000 vs 19000 sequences) used for analysis of the salt stress data above, and was applied to analysis of the new water stress data.

In parallel a cocksfoot transcriptome has been prepared as well and used for the analysis of cocksfoot RNA-seq samples. Differentially expressed genes were identified, and a manuscript on the results is in preparation.

WP4: Rapid drying of biomass

The use of hitherto underutilised marginal land for the production of biomass may offer a means of avoiding conflict between the production of food and fuel. Our hypothesis is that biomass yields from marginal lands can be comparable to those from good agricultural land. The objective of this part of our work (Task 4.1) was to quantify yields of biomass yields from marginal land. We also (Task 4.2) examine genetic variation among those species suitable for biomass combustion in order to identify genotypes with good drying characteristics.

Task 4.1: Optimising Biomass Production

Ireland

C3 grasses grown alone as well as in mixtures were grown at a control site (good agricultural land) and three marginal sites in Ireland (dry, flooded and very wet). Miscanthus genotype trials were also established at each of these sites. During the 2013 growing season, the highest grass yields were obtained from the flooding site in spite of the fact that this site had to be re-sown in September 2012 after prolonged flooding during the summer of 2012. Yields from all sites were comparable to, or exceeded, yields from the control site. During the 2014 growing season, the highest grass yields were obtained from the site which suffered from drought stress (dry site) and from the control site (Fig. 4.1). Yields at the flooding site and at the very wet site were lower although Hykor (*Festulolium)* and Bamse (*Phalaris arundinacea*) produced good yields at all sites. In both years, yields from treatments which included a mixture of grass species produced yields which were comparable to or exceeded the yields of single varieties.

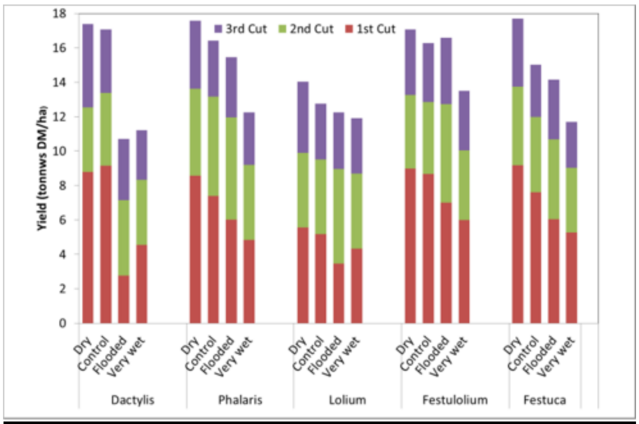


Figure 4.1: Yields of C3 grass species from three grass harvests at each of four sites during the 2014 growing season (selected results).

The Miscanthus genotype trial which was established on the site prone to flooding revealed that Miscanthus has good resistance to flooding. During the 2013 growing season, highest yields were obtained at the very dry site. *Miscanthus sinensis* genotypes performed particularly well on the dry site while the highest yields from the *Miscanthus giganteus* genotypes were obtained from the very wet site. With some exceptions, biomass yields at marginal sites exceeded those at the control site. During the 2014 growing season (Fig 4.2), highest yields were obtained from the very wet site. For many genotypes, yields from this site were at least twice as great as yields from other sites. Highest yields were obtained, in general, from *Miscanthus giganteus* genotypes. *Miscanthus sacchariflorus* genotypes produced their highest yields at the site which was prone to flooding. Apart from these two exceptions, biomass yields at marginal sites exceeded those at the control site.

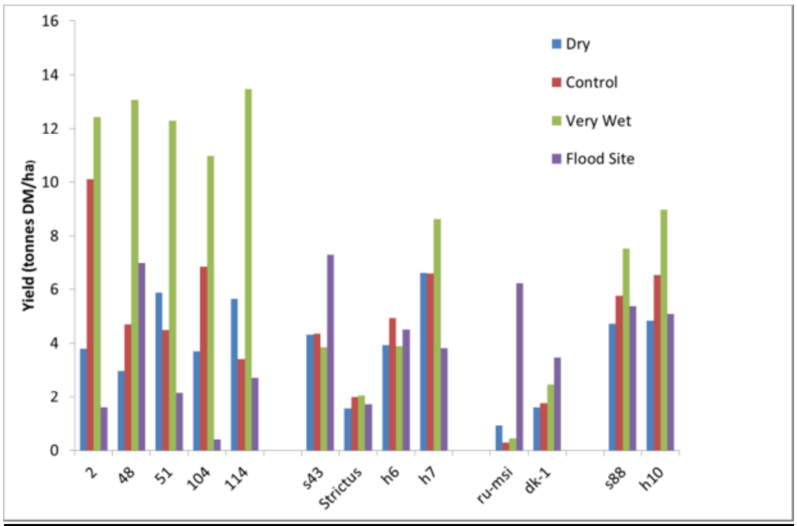


Figure 4.2: Biomass yields of Miscanthus genotypes from four sites harvested in spring following the 2014 growing season (selected results)

From these studies conducted in Ireland, it was evident that yields of C3 grasses from sites on marginal land can equal or exceed those from good agricultural land. *Miscanthus* genotypes survived flooding stress very well and *Miscanthus sacchariflorus* genotypes can be expected to produce the highest biomass yields in sites which are prone to flooding. On very wet sites, *Miscanthus giganteus* genotypes can be expected to produce yields far in excess of those which can be expected from good agricultural soils. We conclude that marginal land can be used to produce biomass for the production of renewable energy, minimising the conflict between the production of food and fuel.

Sweden

The field experiments were situated at two locations in southern Sweden; in Alnarp in the southernmost part of Sweden and in Götala in southwestern Sweden. The aim was to determine how cultivars of grass species grown as sole crops or as intercrops with and without legumes perform regarding biomass yield, as influenced by harvest system and N-fertilization. The crop management strategy was designed according to low-input systems with zero, low or moderate fertilisation levels and a low harvest intensity. This low-input strategy was based on the assumption that soils on marginal land have low production capacity, relatively high yield variability and larger risks for a weak response to fertilisation as compared to fertile soils on agricultural land.In Alnarp, the mean values of yield over three sampling years showed no difference between different levels of diversification (Fig. 4.3). Grass varieties grown as sole crops as well as in mixture, show increased yield as a result of fertilisation while mixture with legumes did not show any significant response to N fertilisation. This confirms that the inclusion of legumes reduces the need for N fertilisation, since they acquire N via symbiotic N_2 fixation. The results were confirmed in the Götala experiment. Biomass yields were higher in the one-harvest system compared to the two-harvest system during two of the three years of the study.

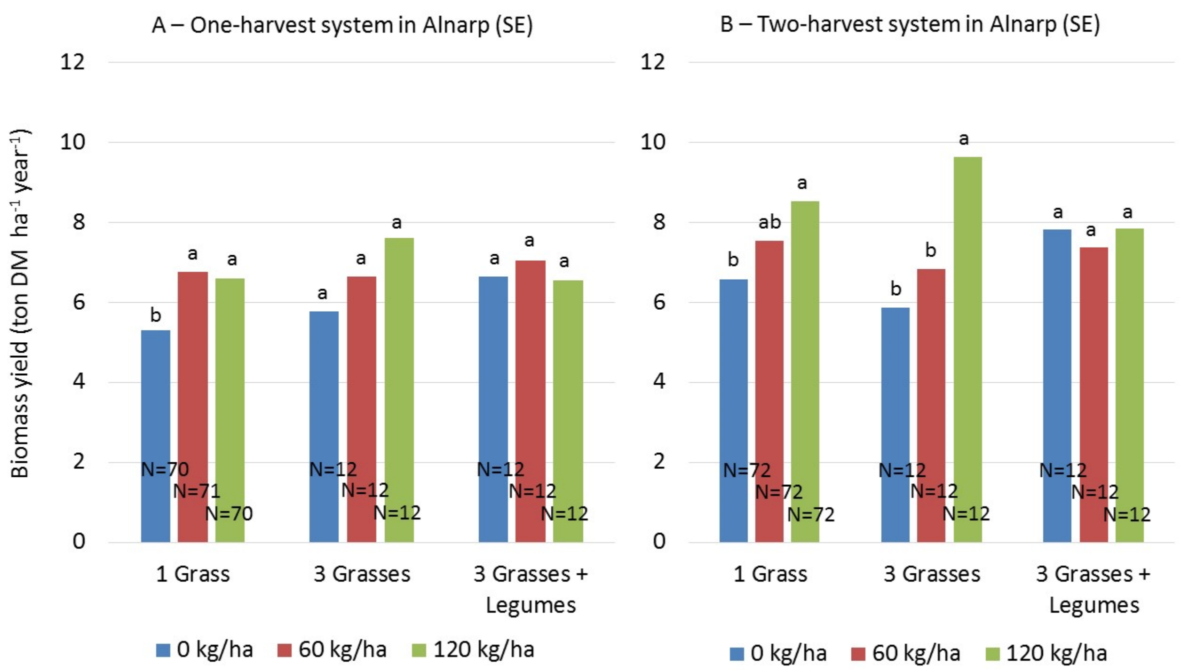


Figure 4.3: Biomass yields from the one-harvest system (A) and the two-harvest system (B) in the field experiment in Alnarp, Sweden.

From the field experiments we can conclude that grass production systems which include legumes can be used on marginal land to produce stable biomass yields with the need for N fertilizer. Such systems have lower requirements of external resources and, thus, low costs and potentially low environmental impact.

Denmark

Field trials of the four perennial grasses and mixtures with legumes were established at two Danish locations (loamy sand at AU Foulum, and coarse sand at AU Jyndevad) in Spring 2012. In 2013, the yield of *Festulolium* was much higher than from the other grasses. Wheat produced 11.5 t/ha dry matter at Foulum when grain and straw yields were pooled although *Festulolium* produced twice as much biomass per area unit compared with wheat. In most cases yields were slightly higher at the better soil at Foulum than at the coarse sand at Jyndevad but not for *Dactylis* and *Miscanthus*. N-fertilised grasses produced higher yields than the mixed grass crops which relied on on biological N-fixation.

Even though the pure grasses were fertilized with high amounts of nitrogen (425 kg N/ha) the crop uptake was even higher in some grasses. In *Festulolium* 562 kg N/ha was taken up and in *Tall fescue* 465 kg N/ha. This leaves very little room for nitrate leaching and denitrification losses which is in line with the very low nitrate levels measured from the grass crops.

Yield in *Miscanthus* increased in 2014 compared with 2013 but was still lower than in the C3 grasses (Fig 4.4). The low *Miscanthus* yield at Jyndevad was likely due to leaching of applied fertiliser in the spring. The whole experiment was irrigated in early May where the C3 grasses were dry on the coarse sandy soil. However, at that time water use had not been initiated in *Miscanthus*, and the irrigation probably leached the nitrate, which became visible as a very pale crop.

The reference grain crops in 2014 were winter barley and triticale with total yields (grain + straw) of 12.4 and 14.8 tonnes DM/ha, respectively. Thus, the grasses did not produce double the yield of grain crops in 2014 but still produced 35-60% higher yields. Grass-clover mixtures without N fertilisation produced yields similar to the grain crops.

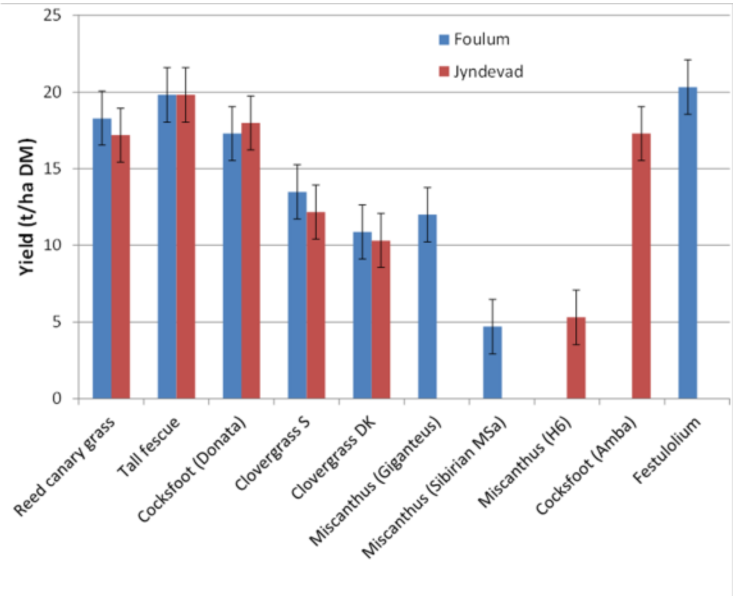


Figure 4.4: Yields (ton/ha DM) in 2014 of grasses and grass-legume mixtures at a coarse sandy soil (Jyndevad) and a loamy sand (Foulum).

From the work carried out in Denmark it is evident that grasses are capable of producing up to double the biomass yield of e.g. barley and wheat. Due to their longer growing season. At the same time nitrate leaching was more than halved in grasses compared with grain crops even though the sole grasses were fertilised intensively. However, the harvested grass removed more than applied while this was not the case in the grain crops. Grass-clover mixtures, that fixed their nitrogen from the atmosphere, produced approximately similar biomass yields as grain crops with almost similarly low nitrate leaching as the sole grasses.

Russia

Work included the search for new potential sources of cellulose that can be efficiently cultivated in various climatic and soil conditions. The search for new potentially renewable cellulose sources was performed using the collection of unique Siberian plants from the Institute of Cytology and Genetics.

Origin of material	Water content, %*	Extractive substances, %	Cellulose content, %	Ash content, %
M. sinensis, leaves	71,2±4,2	12,32±0,95	61,93±1,07	8,70
M. giganteus, leaves	68,7±5,1	13,23±0,64	60,64±0,61	8,98
M. sinensis, stems	75,0±2,7	7,84±1,17	61,35±1,33	6,75
M. giganteus, stems	72,9±1,9	4,80±0,75	62,46±0,49	5,03

The following conclusions can be made:
1) Differences in the studied parameters among species are within statistical error.
2) Miscanthus leaves contain more extractive and ash-forming substances than stems.
3) α-cellulose content is roughly equal in stems and leaves.
3) Plants collected in 2013 contained significantly more α-cellulose than those collected in previous years.

Task 4.2 Rapid Drying of Biomass

Ireland

Grass drying trials were conducted in Ireland in 2013 and 2014 in which differences in varietal drying rates were quantified. In both years, the lowest drying rates were exhibited by the *Lolium* varieties while the highest drying rates were exhibited by the *Dactylis, Festuca and Festulolium* varieties.

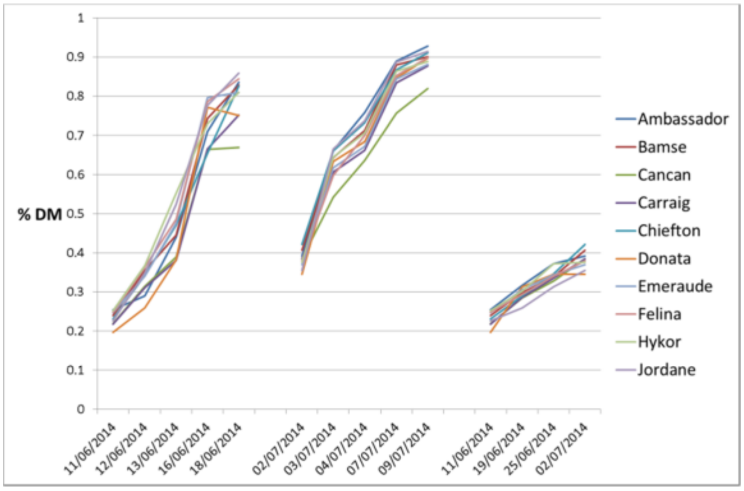


Figure 4.5: Grass drying 2014

Differences in the drying rates of 15 Miscanthus genotypes were quantified during the winters of 2013/2014 and 2014/15. The diameter of aneuploid and diploid genotypes were smaller than those of triploid and quadroploid genotypes. In general, those genotypes with smaller diameters had lower final moisture content. The rate of moisture loss increased with stem diameter (Fig 4.6), genotypes with smaller diameters appeared to have lost much of their moisture before the measurements period commenced. The rate of moisture loss during the period was lowest in diploid genotypes followed by aneuploid genotypes while moisture loss was greatest in triploid and quadroploid genotypes.

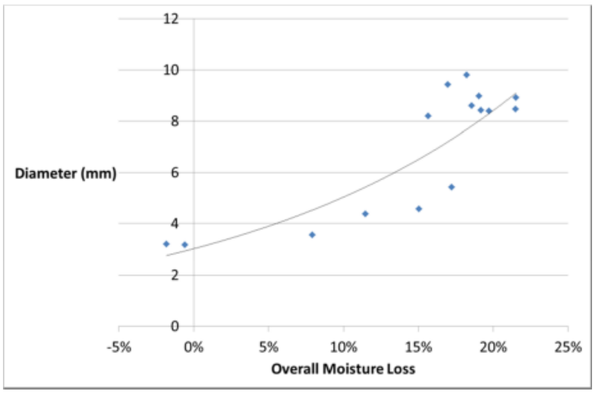


Figure 4.6 Stem diameter vs moisture loss

Partner 9: Polish Academy of Sciences

Six *Miscanthus* genotypes consisted of two *Miscanthus sacchariflorus* (2x and 4x) of Polish origin, two *Miscanthus giganteus* clones (MG-3 and MG-4) of Russian and Danish origin, and two *Miscanthus sinensis* (MS-1 and MS-16) of Polish origin were used in this study which was carried out over the winters (2013/2014 and 2014/2015).

Results 2013/2014

DRY MATTER CONTENT (%)									
GENOTYPE	**17 XI**	**26 XI**	**03 XII**	**09 XII**	**3 XII**	**30 XII**	**13 I**	**01 II**	**II**
MG-3	34.6	42.7	51.6	59.7	50.4	52.6	8.4	65.3	55.4
MG-4	34.8	38.1	55.1	57.6	48.6	55.7	46.5	62.6	56.0
MS-1	37.9	45.7	61.1	63.1	58.2	59.8	62.7	73.1	71.8
MS-16	40.6	47.1	68.2	66.7	58.1	62.3	69.1	74.0	76.3
MSch (2x)	63.0	62.6	75.5	79.7	74.7	78.1	76.9	81.2	83.3
MSch (4x)	35.0	42.8	56.5	57.6	59.0	63.5	56.8	68.0	73.0

In both years, the moisture content in all *Miscanthus* clones exceeded 50% in november, with the exception of *Miscanthus sacchariflorus* 2x clone, which contained significantly more dry matter. In December, January, and February moisture content in all *Miscanthus* clones did not exceed 50%. In February the recorded moisture contents were the lowest in *M. sacchariflorus, M. giganteus,* and *M. sinensis*. The fastest drying rate was exhibited by the diploid *Miscanthus sacchariflorus* in both years.

C3 grass drying trials were conducted in Poland over a three year period. Losses in biomass moisture contents for most cultivars between successive measurements were significant. *Phalaris* cv. Chieftain and Bamse lost moisture at the greatest rate, while the process was slowest in *Festulolium* cultivars.

Moisture loss in Phalaris tended to be both stable and consistent whereas there were considerable fluctuations in moisture content in the cut biomass in the other tested species during the drying process. To conclude, considerable genetic variation between cultivars was observed in terms of the rate of moisture loss in dried biomass.

Years	Time points	Species and variety *Festuca* Emeraude	*Festuca* Jordane	*Dactylis* Ambassadr	*Dactylis* Donata	*Festulolium* Felina	*Festulolium* Hykor	*Phalaris* Chieftain	*Phalaris* Bamse
Y1	T1	31.50f	31.07f	25.32e	26.39e	33.18f	32.41f	22.52d	23.90d
	T2	29.99f	29.81f	24.68e	26.54e	28.77e	29.86e	20.02d	20.97d
	T3	24.54e	24.01e	23.37d	22.76d	27.79e	25.15d	18.00d	16.48c
	T4	19.76d	17.47c	15.05c	16.45c	22.95e	22.53d	14.66c	13.01b
Y2	T1	29.24f	29.41e	24.22e	25.07e	19.61d	21.48	32.66f	31.47f
	T2	26.97e	24.32d	27.28e	21.33d	32.72f	30.33f	22.37e	26.80e
	T3	12.31b	11.81b	12.18b	10.71b	11.72b	11.06b	10.27b	9.87b
	T4	6.37a	3.17a	5.57a	4.28a	4.88a	7.63a	4.87a	2.31a
Y3	T1	25.50d	28.07e	23.07d	29.16f	27.24e	32.53f	33.72f	29.47f
	T2	22.30d	26.12e	23.02d	27.70e	22.62d	25.62e	22.62d	23.64d
	T3	11.54b	13.21c	13.21c	12.37c	12.88b	11.39b	12.73b	11.04b
	T4	12.03b	4.05a	4.05a	5.99a	4.43a	3.99a	7.97b	2.51a

WP5: Dissemination

Website

The website grassmargins.com has been kept up-to-date to communicate the project findings, progress and plans to the general public (i.e. objective 1). This website is maintained with the most up-to-date news and content from the project (http://www.grassmargins.com/), not only in terms of publications but also in terms of educational material.

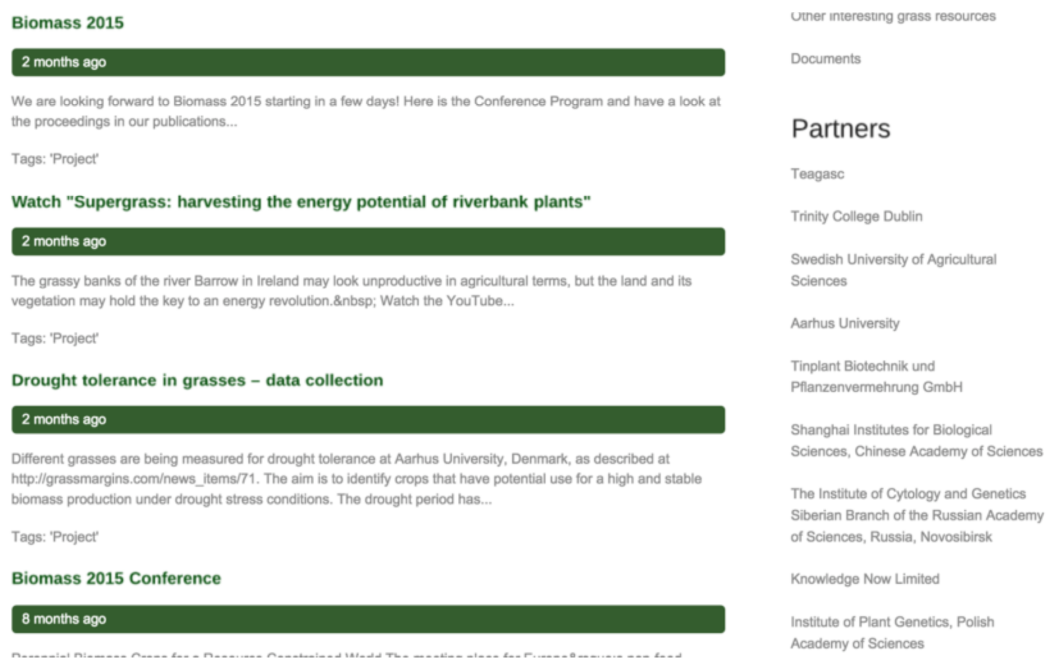


Figure 5.1 - News page

Twitter

Grassmargins has also launched and is maintaining the Twitter (https://twitter.com/grassmargins) account for the project that is being used to disseminate news updates on the project as well as point the public to material that is of general relevance to the aims of the project.

Events

The inter-project meeting and public events held in Dublin in June 2014 were organised by the project management teams of the OPTIMA, OPTIMISC and GrassMargins projects in cooperation with the organizers of FESPB (PLANT BIOLOGY EUROPE FESPB/EPSO 2014 Congress).

A reference to the call for papers can be found at http://t.co/aOoWc20MdG (screenshot below)

The events took place in Dublin Convention Centre and Trinity College in Dublin on June 24-26, 2014 and aimed to provide general public and scientists more information on the state-of-the-art in biomass research and to exchange the knowledge and information between the related EU- and non-EU research projects working with perennial grasses. The event website is: http://europlantbiology.org/ .

In 2015 the project has successfully organised Biomass2015, on 7 – 10 September at the University of Hohenheim in Germany.

The conference was organised together with joint projects OPTIMISC (coordinated by University of Hohenheim/Germany), OPTIMA (coordinated by University of Catania/Italy), WATBIO (coordinated by University of Southampton/UK) in co-operation with the EU project FIBRA, the French BFF (Biomass for the Future) project and the International Miscanthus Society (MEG).

The programme included 6 keynote presentations, 9 themed sessions and side meetings for project consortia.

The abstracts of all presentations was published on the Grassmargins website in DPF format and advertised on Twitter. A book of articles based on the conference presentations and outcomes and a special issue of Global Change Biology – Bioenergy with papers linked to presentations are planned.

More details on program and sessions can be found on the Conference website: www.biomass2015.eu.

Brochure

A brochure has been produced to illustrate the project and its aims.

Mobile App

An iPhone and Android app have been developed for collecting grass data in the Grassmargins portal, so that it can be kept updated – these will be available shortly on Google Play Store and App Store.

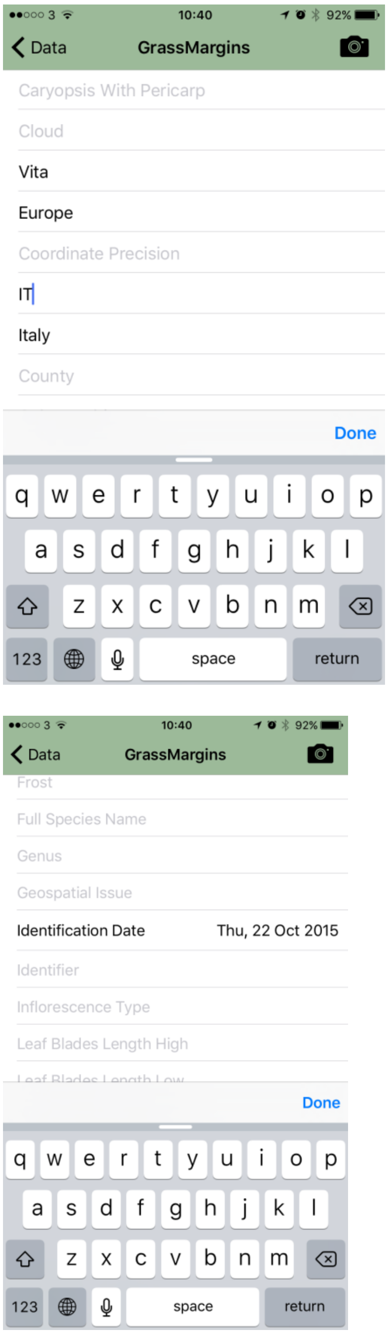


Figure 5.2 - Screenshots of iPhone app to insert new grass instances in the system

Publications

Journals

Haiminen, N., Klaas, M., Zhou, Z., Utro, F., Cormican, P., Didion, T., Sig Jensen, C., Mason, C., Barth, S., Parida, L. 2014. “Comparative Exomics of Phalaris cultivars under salt stress”. BMC Genomics 15(Suppl 6), S18

Hodkinson, T., Klaas, M., Jones, M.B., Prickett, R. and Barth, S. 2014 “Miscanthus: a case study for the utilization of natural genetic variation”. Plant Genetic Resources, available on CJO2014. doi:10.1017/S147926211400094X.

Jones MB, Finnan J, Hodkinson TR (2014) Morphological and physiological traits for higher biomass production in perennial rhizomatous grasses grown on marginal land. GCB Bioenergy.

Song Q, Zhang G, Zhu XG (2013) Optimal crop canopy architecture to maximise canopy photosynthetic CO2 uptake under elevated CO2 – a theoretical study using a mechanistic model of canopy photosynthesis. Functional Plant Biology 40 (2): 108-124

Wang Y, Long SP and Zhu X-G (2014) Elements required for an efficient NADP-ME type C4 photosynthesis. Plant Physiology. 164 (4): 2231-2246.

Book chapters

Barth S, Jones MB, Hodkinson TR, Finnan J, Klaas M, Wang Z-Y (2014) Grasslands for forage and bioenergy use: traits and biotechnological implications. In: Grassland Science in Europe 19 (edited by Hopkins A, Collins RP, Fraser MS, King VR, Lloyd DC, Moorby JM, Robson PRH); Gomer Press Ltd, Wales/UK; p 438-449

Goryachkovskaya T, Slynko N, Golubeva E, Shekhovtsov S, Nechiporenko N, Veprev S, Meshcheryakova I, Starostin K, Burmakina N, Bryanskaya A, Kolchanov N, Shumny V, Peltek S. Perspectives of biotechnological applications of miscanthus in Russia. In: Barth S, Murphy-Bokern D, Kalinina O, Taylor G, Jones MB, (editors): Perennial biomass crops for a resource constrained world, 2015.

Hodkinson TR, Petrunenko E, Klaas M, Münnich C, Barth S, Shekhovtsov SV, Peltek SE. New breeding collections of Miscanthus sinensis, M. sacchariflorus and hybrids from Primorsky Krai, Far Eastern Russia. In: Barth S, Murphy-Bokern D, Kalinina O, Taylor G, Jones MB, (editors): Perennial biomass crops for a resource constrained world, 2015.

Jørgensen U, Lærke PE. Using perennial grasses for sustainable European protein production. In: Barth S, Murphy-Bokern D, Kalinina O, Taylor G, Jones MB, (editors): Perennial biomass crops for a resource constrained world, 2015.

Muennich C, Kørup K, Klaas MJ, Barth S, Bonderup Kjeldsen J, Finnan J, Fonteyne S, Jankowska M, Jørgensen U. Creation of novel cold tolerant Miscanthus hybrids. In: Barth S, Murphy-Bokern D, Kalinina O, Taylor G, Jones MB, (editors): Perennial biomass crops for a resource constrained world, 2015.

Muennich C, Klaas MJ, Barthels V, Gebhardt C. Creation of novel tetraploid Miscanthus sinensis genotypes. In: Barth S, Murphy-Bokern D, Kalinina O, Taylor G, Jones MB, (editors): Perennial biomass crops for a resource constrained world, 2015.

Conference papers (Talks)

Zhu X, Jiao X, Song Q, Jørgensen U. 2015. Modelling crop growth and development for support biomass crop breeding. In: Abstracts from the International conference on "Perennial biomass crops for a resource-constrained world", p. 10.

Jiao X, Sørensen KK, Andersen MN, Lærke PE, Jezowski S, Ornatowski S, Sacks EJ, Jørgensen U. 2015. Photosynthesis in C4 miscanthus and C3 festulolium under cold conditions. In: Abstracts from the International Conference on "Perennial biomass crops for a resource-constrained world", p. 58.

Klaas M, Haiminen N, Cormican P, Grant J, Finnan J, Lærke PE, Jørgensen U, Didion T, Jensen CS, Utro F, Vellani T, Parida L, Barth S. 2015. Responses of perennial bioenergy grasses to abiotic stress. In: Abstracts from the International conference on "Perennial biomass crops for a resource-constrained world" p. 15.

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Klaas, M., Haiminen, N., Utro, F., Vellani, T., Cormican, P., Lærke, P., Jørgensen, U., Parida, L. and Barth, S. 2015 “Uncovering the water stress response of reed canary grass (Phalaris arundinacea) from differential expression of the reference transcriptome” At the Plant and Animal Genome Conference Xiii 10.-14.1.2015 San Diego, California

Haiminen, N., Klaas, M., Zhou, Z., Utro, F., Cormican, P., Didion, T., Sig Jensen, C., Mason, C., Barth, S., Parida, L. 2014. “Comparative Exomics of Phalaris cultivars under salt stress” At the Recomb Comparative Genomics Conference 19.-22.10.2014 New York- Cold Spring Harbour Laboratories

Hodkinson TR, de Cesare M, Prickett R, Jones MB, Barth S (2012) IPSAM, National Botanic Gardens, Dublin, Ireland. Genetic variation in Miscanthus x giganteus (Poaceae) a bioenergy and fibre crop

Hodkinson TR, Prickett R, Jones M, Klaas M, Barth S (2014) IPSAM, University College Cork, April 2014. Utilising natural genetic variation to develop novel biomass grasses for marginal lands

Hodkinson TR, Prickett R, Klaas M, Jones M, Barth S (2014) FESPB/EPSO Plant Biology Europe, Conference, Dublin, Ireland. Keynote talk (KN030) Utilising Natural Genetic Variation to Develop Novel Biomass Grasses for Marginal Lands

Klaas M, Cormican P, Michel T, Velmurugan J & Barth S (2014) Genotyping of a collection of Miscanthus spp. accessions. Presented at Plant and Animal Genome XXII, San Diego, 10th-15th January, 2014

Manfred Klaas1, Niina Haiminen2, Filippo Utro2, Tia Vellani1, Cora Muennich3, Thomas Didion4, Christian Sig Jensen4, Laxmi Parida2, Susanne Barth1 (2014) Exploring differentially expressed genes and pathways under drought, flooding and salinity conditions in reed canary grass (Phalaris arundinacea), an autopolyploid C3 forage and bioenergy grass species

Posters

Klaas, M., Cormican, P., Michel, T. and Barth, S. 2014 “Genotyping by sequencing of a collection of Miscanthus spp. Accessions” At the Recomb Comparative Genomics Conference 19.-22.10.2014 New York- Cold Spring Harbour Laboratories

Kirsten Kørup, Xiurong Jiao, Helle Baadsgaard, Mathias N. Andersen, Poul Erik Lærke, Uffe Jørgensen, Thomas Prade, Stanisław Jeżowski, Szymon Ornatowski, Robert Borek, (August 2013), Leaf photosynthesis and cold tolerance in Miscanthus genotypes. Poster session presented at the 16th International Congress on Photosynthesis Research, St. Louis, MO, USA.

Sørensen KK, Lærke PE, Sørensen HB, Andersen MN, Jørgensen U. 2015. Drought tolerance in perennial grasses. In: Abstracts from the International Conference on "Perennial biomass crops for a resource-constrained world", p. 48.

Jiao X, Sørensen KK, Prade T, Jezowski S, Ornatowski S, Andersen MN, Lærke PE, Jørgensen U. 2014. Differences in leaf photosynthesis, specific leaf area and cold tolerance for 14 Miscanthus genotypes. Abstract Book of Poster Presentations P. 377. Plant Biology Europe FESPB/EPSO Congress, Dublin, Ireland. 22-26 June 2014. http://europlantbiology.org/wp-content/uploads/2014/06/Posters-Final-18.6.2014.pdf

Lærke PE, Karki YK, Sørensen HB, Sørensen KK, Jørgensen U. 2014. Screening genotypes of candidate energy grasses for flooding tolerance. Abstract Book of Poster Presentations P. 234. Plant Biology Europe FESPB/EPSO Congress, Dublin, Ireland. 22-26 June 2014. http://europlantbiology.org/wp-content/uploads/2014/06/Posters-Final-18.6.2014.pdf

Sørensen KK, Jiao X, Baadsgaard H, Prade T, Jezowski S, Ornatowski S, Borek R, Andersen MN, Lærke PE, Jørgensen U. 2013. Leaf photosynthesis and cold tolerance in Miscanthus genotypes. Poster session presented at The 16th International Congress on Photosynthesis, St. Louis, USA.

Thesis

MSc Thesis TCD 2012

Phylogenetics of the grass genus Phalaris and population genetics of P. arundinacea L. a potential bioenergy crop

Amanda grace Philpot

Supervisor T.R. Hodkinson

Msc Thesis TCD 2013

Natalia de la Torre Rodriguez

Genetic resources of Phalaris

Supervisor T.R. Hodkinson

B.A. (Mod)Plant Sciences Thesis 2015

An investigation into the phylogenetics of Phalaris through molecular analysis of the nITS gene region.

Tegan Parkes

Supervisor T.R. Hodkinson

B.A. (Mod)Plant Sciences Thesis 2015

An investigation into the phylogenetics of Festuca grass species

Jane Goldrick

Supervisor T.R. Hodkinson

Other

Dr. Olena Kalinina: “Protocol of the workshops and Perennial Grasses project meetings in Dublin, Ireland, June 24-26, 2014”, 2014.

Termansen M, Gylling M, Jørgensen U, Hermansen JE, Hansen LB, Knudsen MT, Adamsen APS, Ambye-Jensen M, Jensen MV, Jensen SK, Andersen HE, Gyldenkærne S 2015. GRØN BIOMASSE. DCA - Nationalt center for fødevarer og jordbrug. DCA Rapport Nr. 068, 38p.

Barth S, Klaas M, Burke B, Doyle D & Finnan J (2013) Perennial grasses for marginal soils. TResearch 8(3), p 42-43

Papers submitted

Can miscanthus C4 photosynthesis compete with festulolium C3 photosynthesis in a temperate climate? Jiao, Xiurong; Kørup, Kirsten; Andersen, Mathias; Sacks, Erik; Zhu, Xinguang; Lærke, Poul; Jørgensen, Uffe. Submitted to Global Change Biology Bioenergy.

Creation of novel cold tolerant Miscanthus hybrids. Cora Muennich, Kirsten Kørup, Manfred Klaas, Susanne Barth, Jens Bonderup Kjeldsen, John Finnan, Simon Fonteyne, Marta Jankowska, Uffe Jørgensen. Submitted to “Perennial biomass crops for a resource-constrained world” Springer book.

Using perennial grasses for sustainable European protein production. Jørgensen, U & Lærke, PE. Submitted to “Perennial biomass crops for a resource-constrained world” Springer book.

Papers in preparation

Manevski, K., Jørgensen, U. & Lærke P.E. 2015. Biomass productivity of innovative cropping systems for biorefinery over three seasons at two locations in Denmark (in prep. – probably for Industrial Crops and Products)

Low temperature leaf photosynthesis of a Miscanthus germplasm collection correlates positively to shoot growth rate and specific leaf area. Xiurong Jiao, Kirsten Kørup, Mathias Neumann Andersen, Karen Koefoed Petersen, Thomas Prade, Stanisław Jeżowski, Szymon Ornatowski, Barbara Górynowicz, Idan Spitz, Poul Erik Lærke and Uffe Jørgensen. To be submitted to Annals of Botany.

Drought tolerance in eight perennial grasses. K. Kørup, P.E. Lærke, M.N. Andersen, H. Baadsgaard, K. Kristensen, C. Muennich, E.S. Jensen, L.-M. Mårtensson, U. Jørgensen. To be submitted to Journal of Experimental Botany.

Discrimination of 13C and water-use efficiency in different varieties of the C3 grasses Dactylis glomerata, Festuca arundinacea and Phalaris arundinacea. Mårtensson, L-M, Carlsson, G, Körup Sörensen, K., Jörgensen, U., Prade, T, and Jensen, E.S. To be submitted to Annals of Botany.

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Modelling the productivity in the perennial grass festulolium in northern Europe. Xiurong Jiao, Qingfeng Song, Xinguang Zhu and Uffe Jørgensen. To be submitted for European Journal of Agronomy.

Abstract

“Crop modeling for agriculture and food security under global change” MACSUR symposium in Berlin, March 2016.

Guests and media

“Supergrass: harvesting the energy potential of riverbanks plants” – YouTube Video - https://youtu.be/uC8tz-bkA6Q (SCREENSHOT)

“Elephant Grass” – Youtube video - http://www.youtube.com/watch?v=z0IdAd9L9sw&feature=youtu.be (SCREENSHOT)

A King: “Grass as the new biofuel” – article about Grassmargins. http://www.youris.com/Mobility/Biofuels/Grass_As_The_New_Biofuel.kl (PDF)

S. Barth. Grassmargins as a EU Success Story. http://ec.europa.eu/research/infocentre/article_en.cfm?id=/research/star/index_en.cfm?p=ss-grassmargins&calledby=infocentre&item=Countries&artid=31579&caller=SuccessStories (PDF)

Hodkinson (2013) Seminar given to the School of Biology and Environmental Science, University College Dublin, Ireland. Miscanthus systematics and evolution

Master students excursion to field experiment from the Aarhus University master course “Energy crop production” September 2015.

Visit to Aarhus University by PhD student Simon Fonteyne, ILVO, Belgium on May 4. 2015. Interaction on chlorophyll fluorescence measurements.

Ph.D.-students from the international Ph.d. course ”Modelling climate effects on crops and cropping systems” visiting field experiments at Aarhus University on 28. August 2015.

Interview of Uffe Jørgensen on national radio on the potential production and use of perennial grasses for protein and bioenergy supply, DR1 Orientering, 22 SEP. 2015, http://www.dr.dk/radio/ondemand/p1/orientering-2015-09-22/#!/ (PDF)

Interview of Uffe Jørgensen on regional radio on the potential production and use of perennial grasses for protein and bioenergy supply, DR MV May 2015.

Field visit at Aarhus University by 10 journalists from agriculture and business media causing 3-4 stories in various media, April 2015.

Potential Impact, including the socio-economic impact and the wider societal implications of the project so far

The definition of marginal land is often discussed and may differ in different settings and with time. Most often it is understood as land of low productivity for the traditional agricultural crops or land that is difficult to access in an efficient way. However, the stricter environmental regulation of EU agriculture and the higher concern towards sustainability, nature preservation etc. also means that some current agricultural areas might be marginalized because they cannot be sustainably managed with current agricultural cropping systems.

To minimise competition between the production of food and fuel, the Grassmargins project sought to identify novel varieties of perennial grasses which show high and stable yields when grown on marginal land. Marginal land was defined as land of poor quality which yields poor returns for the farmer. Hitherto, the potential of marginal land for the production of biomass feedstock had not been quantified and remains largely unutilised or underutilised for this purpose.

At the start of the project, our assumption was that yields from marginal land would be somewhat lower than yields from good agricultural land. However, the results of the project have shown that grass yields obtained from marginal lands are not always lower than yields from good agricultural land. Moreover, in certain circumstances, yields from marginal land can exceed those from good agricultural land. This was particularly the case for Miscanthus grown on wet land. Additionally, grass yields on marginal land can be stimulated by the addition of high levels of nitrogen fertilizer while minimising nitrate leaching compared to annual crops but nitrogen levels can be reduced by the addition of legumes to grass mixtures. This result is also very significant as restrictions on the use of high nitrogen levels on annual crops due to failure to meet nitrate directive limits could turn land which was previously profitable for crop production into land which is marginal for crop production. However, our results have shown such lands can continue to be productive with low levels of nitrate losses if the land use is changed from crop production to the production of perennial grasses.

Our identification of the potential of marginal land for bioenergy production has significant implications for the economy and society within the European Union. The fact that additional land resources can be used to produce good yields of biomass feedstock means that EU policies on renewable energy, land use and the European bioeconomy can be pursued without restrictions on the availability of land or because of conflicts between the production of food and fuel. The availability of, previously unused or underutilised, marginal land for the production of biomass can benefit the European economy and, more specifically, the European bioeconomy with concomitant societal benefits. At regional and locals levels, the additional potential of land previously classified as marginal can be expected to bring economic benefit to parts of the European Union where wealth from land use was limited. This, in turn, can be expected to bring social benefits to these areas resulting from increased employment and local opportunity.

One such example is the Danish fulfillment of the Water Framework Directive, which is very difficult to obtain in certain areas of the country. Here nitrate leaching is high, and soils are drained so that the leached nitrogen reaches nitrate sensitive fiords and belts directly when lost from the field. These areas can be very productive and of high agricultural value. However, if not cropping systems are optimized to obtain a very significant reduction in nitrate leaching, these areas may have to be taken out of production or may have to be turned into extensive grazing areas of low economic value. We have therefore also investigated the production of grasses for energy or biorefinery utilization on good agricultural land at high-input conditions with the aim of investigating if more sustainable systems could be developed that can keep current agricultural business on land that is in risk of becoming marginalized. Our results have shown that on sandy soils in these regions nitrate leaching will be reduced by approx. 50 kg N/ha when annual crops are substituted by perennials even though these are still fertilized.

Thus the Grassmargin field experiments have partly investigated low input system with a major focus on delivery of increased biodiversity as well as low environmental impact. Partly we have investigated high-input grass production with a major focus on sustainable intensification, i.e. high yields and low environmental impact. The high-input systems have been compared with current high-input grain and seed production systems.

On the high-input grass production we conclude, that these systems seem to be able to increase agricultural productivity in non-water-limited areas significantly compared with existing systems (Jørgensen & Lærke, 2015). And that the grass may be utilized in green biorefineries for production of protein feed for monogastric animals, grass fibre feed for cattle and energy production from surplus fibre and waste water following protein extraction. Especially biogas, ethanol and HTL oil (hydrothermal liquefaction) are bioenergy technologies well suited to convert moist fractions from green biorefining of grasses. And the production of liquid or gaseous biofuels that can be used in the transport sector is more valuable for the future energy system than the production of heat and power, which can be produced in a number of other ways from e.g. solar and wind.

The growing global resource demands call for sustainable intensification of agriculture in order to produce more with less environmental impact. Easier said than done this will require an integration of societal, agronomic and environmental perspectives (Struik and Kuyper, 2014). Kuyper and Struik (2014) concluded that “sustainable agriculture is a contested concept and significant sustainable intensification requires a radical rethinking of agricultural production”. It is often questioned if it is realistic to simultaneously close yield and resource efficiency gaps in agricultural production (van Noordwijk and Brussaard, 2014), and it is stated that in most European agriculture only limited yield improvement is possible, while on the other hand further sustainability improvements are required to meet our environmental goals (van Grinsven et al., 2015;van Ittersum, 2015). However, whilst this is largely true of many existing agricultural chains that are already highly optimized, the potential for a change into more resource efficient cropping systems and for rethinking their utilization in view of the emerging biorefinery developments and bioeconomic thinking is largely unexplored.

Grassmargin results confirm earlier findings that in humid temperate regions grasses and legumes can capture solar radiation more efficiently than annual grain and seed crops, in which a considerable part of the growing season is used for crop ripening, harvest, soil tillage and sowing (Cadoux et al., 2014; Dohleman and Long, 2009; Pugesgaard et al., 2015). Potentially, biomass yields can be doubled and the potential is expected to increase with climate change (Jørgensen et al., 2012). Most promising are grasses with C3 photosynthesis in northern (Manevski et al., 2015) and C4 grasses in southern regions (Cadoux et al., 2014).

The EU is heavily dependent on protein feed imports – with over 30 Mton of soybean products imported annually (de Visser, 2013; Parajuli et al., 2015). If protein from locally produced grass crops can be efficiently and economically extracted and formulated into feed (or food), a significant share of the soy could be substituted (Jørgensen & Lærke, 2015). This is especially interesting for sectors wishing GMO-free feed products or products with a better environmental profile than soy. Especially in organic agriculture further growth of the sector will be facilitated if locally grown green protein can be supplied. This is also because green biorefineries can utilize and valorize grass clover fields that are the "engine" of organic agriculture, and at arable organic farms they are today often only used as green manure.

The large feed protein deficit in EU has caused the EU Parliament motion (Häusling, 2011) that calls on the Commission to support research into breeding and supply of protein crop seeds in the EU to promote on-farm feed production. The European Innovation Partnership on 'Agricultural Productivity and Sustainability' has reviewed the possible protein crops for the whole of EU with a major focus on grain legumes (EIP-AGRI, 2014). The leaf protein potential is briefly mentioned and the high water content of the raw biomass is mentioned as a major obstacle that calls for local use, but there is no real analysis of the possible developments. The EIP-AGRI report also lists a number of EU-projects on protein crops which are almost exclusively on grain legumes and not on green biorefinery. In the light of the major win-win perspectives of changing from annual into perennial cropping systems and of utilizing surplus grasslands for production of high-value protein there is an obvious need for renewed focus on prospects of this whole chain – especially under humid northwestern EU conditions. Grain legumes may still be more warranting under drier and warmer conditions.

The research on green biorefining in Europe has been scattered so far, has not covered the whole chain of optimization and has not included the major environmental benefits from conversion into more sustainable cropping systems in the analysis (McEniry & O´Kiely, 2014). However, the huge potential benefits and markets to address with this possible new agro-industrial development calls for joining experiences and setting up joint scenarios and analyses. This should form the basis for subsequent demonstration and proof of concepts.

In order to fulfill the demands of European policies and directives e.g. the Water Framework Directive, the EU Climate Policy, the Soil Quality Directive and the greening of the CAP, there is a large pressure on agriculture to reduce its losses and emissions to the environment. At the same time the rising global population and increased wealth, the Renewable Energy Directive and the need for more job creation in rural areas demands for increased production of food, bioenergy and high-value products. Our results indicate that non-water limited parts of Europe will be able to double crop production per unit of land and halve the emission of environmentally negative substances, with smaller but still significant improvements achievable in other regions. Furthermore, the establishment of green biorefineries in rural areas will create jobs, both directly and via the supply chains stimulated.

Agricultural GHG emissions need to be reduced from both non-CO2 emissions and from land use change (LULUCF). According to the latest EC paper on the future policy framework for climate change to 2030, the reduced emission from agricultural LULUCF will be achieved by production of more perennial energy crops and grassland (European Commission, 2014). Development of green biorefineries to increase the value from these crops is consistent with this ambition. And extraction of proteins or other food components before the remainder is used for energy avoids the negative influence of iLUC on the perceived GHG benefit from biomass crops.

Even though water quality will be improved by cropping systems with increased growing season, water quantity (surplus for ground water and river discharge) may be reduced due to higher annual evapotranspiration. However, new research shows that not all perennial crops increase water use (Ferchaud et al., 2015). In addition there is an increased water infiltration capacity in pastures compared with annual crops (Franzluebbers et al., 2014), which can reduce the loss by water run-off. Still, the potentials for securing a water supply for a long growing period are highest in humid northwestern Europe, even though optimizations may also be feasible in water-limited areas such as the use of winter-grown green biomass (Tsiplakou et al., 2014).

Permanent soil cover contributes to creating a more resilient cropping system that will reduce soil erosion and facilitate soil water infiltration from increasingly heavy rainfall incidents with climate change. Grasses will stop growing during drought but will speedily recover when rain falls (drought escape strategy), and our results have shown marked genotypic differences in both drought and flooding tolerance (Klaas et al., 2015; Lærke et al., 2014).

Following extraction of the easily soluble protein from grass biomass, the fiber fraction is a valuable cattle feed with a high digestibility (some addition of molasses may be necessary (Klop et al., 2015)), containing protected proteins that will pass the rumen un-degraded. Thus, simple decentralized separation in high-value feed fractions for both mono- and polygastric animals can create value from locally grown biomass and reduce the need for import (Seppala et al., 2014). For fish production large amounts of soy, wheat and sunflower are used today, and this sector may be boosted with delivery of a more sustainable feed.

The fibre fraction may also be converted into chemicals or energy. One interesting product will be xylitol which is a pentose sugar alcohol that exhibits anticariogenicity (the inhibition of bacteria that cause tooth decay) and also has potential as a low calorie sweetener suitable for diabetics.

Quality criteria for crops for green biorefinery may be quite different from those of crops for direct feed use. The grass species *Festuca* and *Festulolium* are more stress tolerant and can be more productive than the currently preferred *Lolium* (Humphreys et al., 2013) which has a superior direct feeding value. However, this may change after physical treatment and separation in a biorefinery. Such knowledge may impact the grassland species composition towards a much more resilient and productive grass production in Europe (Humphreys et al., 2013). Furthermore, the content of high value components of the biomass, which are so far not included in breeding strategies, will have to be investigated as a basis for new breeding programs targeted to the bioeconomy.

Grassland in the EU-28 in 2010 represented more than 40% of the utilized agricultural area (Eurostat 2015) but its utilization and productivity varies dramatically, and data are often limited (McEniry & O'Kiely 2014). We expect that 2-3 ton/ha dry mass of protein rich feed can be extracted from high-productive grass and/or legumes (Termansen et al., 2015). This means that the 71 million ha grassland of the Europe-28 provides a potential resource for production of 140-210 Mton protein feed if productivity was optimized, and that the current EU import of soy products may be substituted by grass and legume based protein feed produced from only 15-20% of the current EU grassland area.

In some intensive agricultural regions of Europe a change from current annual crop rotation into high-productive grassland will benefit both environment and total productivity. The fiber fraction, after extraction of the easily extractable soluble protein for local use in pig, poultry or fish production, can possibly be used as cattle feed (Klop et al., 2015). But the fiber fraction may also be utilized for material or energy purposes (e.g. lactic acid for the bioplastic industry, xylitol as a diabetic sugar replacement for the food industry or biogas for the energy sector). Sugar alcohols such as pinitol can be used in existing nutraceutical industries. Because total productivity per ha will be increased and part of the harvest is used for feed (or may be directly for food) we expect no indirect Land Use Change (iLUC) from the change but an increased volume of biomass to use for the increasing demands for food, materials and energy. This is a more sustainable option than the current production of rape seed biodiesel and dedicated energy crops, which incur a significant iLUC effect (Tonini and Astrup, 2012).

Also, existing under-utilized grasslands may be exploited via green biorefineries and deliver products of higher value than is possible with traditional grassland management (McEniry & O'Kiely 2014). This can be very important for developing new rural businesses in grassland-rich areas where livestock production is decreasing, and the conversion of permanent grassland into crop rotation is not feasible due to pedoclimatic conditions (too cold or too wet) or because it would induce significant loss of soil carbon. For Ireland alone it is estimated, that an annual resource of 1.7 million t DM of unutilized grass is available, and that with improved management this could be increased to 12.2 million t DM annually (McEniry & O'Kiely, 2014). Invasive species and trees may be regulated by regular mowing of the semi-natural grasslands (Nielsen et al., 2014). Grasslands of high nature value that are threatened by eutrophication can be nutrient depleted by harvesting and utilizing the nutrient rich grass, which will subsequently increase floral diversity (Hald & Nielsen, 2010). The surplus nutrients can, after biorefining into e.g. feed or biogas, be recycled to regular farmland and abate the application of mineral fertilizer or improve the possibility to expand organic farming.

The development of Green Biorefinery technologies, increased total agricultural productivity and the development of high-value products will create jobs and development in rural areas. A Danish case study calculated a creation of 12-26,000 new permanent jobs in agriculture, logistics and biorefinery industries from an increased utilization of 10 Mton of biomass (Gylling et al., 2013).

When environmental challenges (e.g. implementation of the Water Framework Directive, agricultural GHG mitigation (the EU climate policy), and reduced pesticide use) can be solved by choosing grass production systems with increased productivity instead of, as most often proposed, by reduced intensity of production, this will ensure continued rural business opportunities on farms and other business' and security for having a crop production to recycle current amounts of animal manure – especially in nitrate sensitive areas.

Costs incurred by bio-refining will be offset by increased biomass production, product flexibility, new product streams and reduced environmental impact. However, implementing whole new production chains is difficult and often not cost-competitive until innovated over a longer period. Additionally, there is a significant policy element as the environmental benefits will not deliver a direct economic return. Internalizing environmental and socioeconomic benefits of the establishment of green biorefinery based on grass biomass will be pivotal to point out the best region, scale and product focus for initiating subsequent full-scale proof of concept, and to initiate a discussion on the necessary policies to support the development.

Exploitation of results

Our results showed significant species and interspecies variation in abiotic stress tolerance. In example tall fescue was the most tolerant and reed canary grass the least tolerant species to salt stress during growth of established grasses. Cocksfoot appeared from drought experiments under controlled conditions to be the most tolerant species – especially the variety Sevenup. However, when grasses were sown in a drought and salt affected field trial tall at the company DLF, fescue and festulolium varieties performed the best. The drought and salt tolerance during germination thus needs special attention in further work in order to fully exploit the results in practice.

Miscanthus and reed canary grass were the most flooding tolerant grasses while cocksfoot was very sensitive to flooding. It was surprising that miscanthus was so flooding tolerant and this warrants use of the species at flooding prone sites or for safe application of wastewater.

Cold and frost tolerance was only tested within miscanthus and showed a promising variation to include in breeding for better performance in cool climates. The heredity of cold tolerance was investigated on 881 offspring from 13 different crossings performed by the company Tinplant between *M. sinensis* and *M. sacchariflorus*.

Winter survival was registered on offspring from crosses between *M. sinensis* x *M. sinensis* and *M. sinensis* x *M. sacchariflorus*. The winter survival was 79 and 91% in Ireland and Denmark, respectively. The offspring were planted according to the origin of parents. The survival rate within each group varied from 33 to 100%. For seven crossings all offspring survived the winter in both Ireland and Denmark. In August 2015 the shoot length of the offspring varied between 28 and 203 cm. An offspring from crossing of *M. sinensis* S 94 with *M. sinensis* D-60 had the longest shoot. The average shoot length of offspring from this crossing was the highest of all crossings with 167 cm. As all offspring from this crossing also survived the winter in both countries they are interesting for future investigation and cultivation, and we will follow its performance (as well as its siblings) closely over the coming years with a possible commercialization in view. If this crossing provides offspring with good long-term performance and productivity it opens the possibility of preparing seed lines for propagation in order to overcome the costly, and difficult establishment of miscanthus by plantlets or rhizomes.

Another new finding in the project may be exploited as a screening tool for miscanthus productivity in breeding programs. We found a good correlation between the field measured shoot growth rates (done in Sweden, Poland and Denmark) and leaf photosynthesis in climate chamber. And we found a surprising positive correlation between specific leaf area and leaf photosynthesis, which means that the thinner the leaf the higher CO2-fixation per leaf area. These phenotypic characters are easy and low-cost to measure on large breeding populations.

The perspectives of utilizing grasses for green biorefining for protein feed etc. are further investigated at Aarhus University (http://dca.au.dk/en/research/biobase/). Apart from extending the studies initiated in Grassmargins there is now constructed pilot plants for protein extraction and for HTL-oil production, and grass protein and fibre are tested as animal feed for monogastric and polygastric animals, respectively. In a broader context the concept is also included in a large research and innovation project (www.biovalue.dk) that includes a number of large, medium sized and small enterprises (e.g. Hamlet Protein) that looks into the commercial aspects of using grasses for sustainable intensification and new product developments.

Another commercial activity is on utilizing miscanthus for thatching to supplement the use of common reed. This use is slowly increasing in Denmark (www.miscanthus.dk, www.straatagetskontor.dk) but the high costs of establishment from *M. sinensis* plantlets is a serious bottleneck to the development. The chairman of the Thatchers Society, Søren Vodder together with Lars Sommer, Vitroform (provides miscanthus plantlets for the thatching market) and representatives from Aarhus University visited Tinplant during the project period in order to discuss optimizations and cost reductions of the propagation methods.

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