



Article

Breeding Strategies to Improve *Miscanthus* as a Sustainable Source of Biomass for Bioenergy and Biorenewable Products

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Abstract: *Miscanthus*, a C₄ perennial grass native to Eastern Asia, is being bred to provide biomass for bioenergy and biorenewable products. Commercial expansion with the clonal hybrid *M. × giganteus* is limited by low multiplication rates, high establishment costs and drought sensitivity. These limitations can be overcome by breeding more resilient *Miscanthus* hybrids propagated by seed. Naturally occurring fast growing indigenous *Miscanthus* species are found in diverse environments across Eastern Asia. The natural diversity provides for plant breeders, the genetic resources to improve yield, quality, and resilience for a wide range of climates and adverse abiotic stresses. The challenge for *Miscanthus* breeding is to harness the diversity through selections of outstanding wild types, parents, and progenies over a short time frame to deploy hybrids that make a significant contribution to a world less dependent on fossil resources. Here are described the strategies taken by the *Miscanthus* breeding programme at Aberystwyth, UK and its partners. The programme built up one of the largest *Miscanthus* germplasm collections outside Asia. We describe the initial strategies to exploit the available genetic diversity to develop varieties. We illustrate the success of combining diverse *Miscanthus* germplasm and the selection criteria applied across different environments to identify promising hybrids and to develop these into commercial varieties. We discuss the potential for molecular selections to streamline the breeding process.

Keywords: *Miscanthus*; Breeding; Perennial Biomass Crop; Wide Hybrids; Bioenergy; Bioeconomy

1. Introduction

Urgent action is needed to reduce levels of carbon dioxide entering the atmosphere and sequester carbon [1]. Climate change active policy makers are looking to the agriculture and forestry sectors to use the large global resource of underutilized marginal lands to massively increase biomass production and utilization and sequester carbon from the atmosphere. Perennial biomass crops (PBCs), including grasses and trees, have desirable sustainability characteristics: high output to low energy input ratios, the ability to be deployed at sufficient scale to impact fossil fuel usage and the ability to be grown economically on lower-grade land not currently needed for food production.

The C₄ perennial grass *Miscanthus*, a close relative of sugarcane (*Saccharum spp*), combines these desirable characteristics and has been grown in trials in a wide range of climates, including temperate and continental climates in Europe and America [2]. The widely grown commercial *M. × giganteus* (*M × g*) is a sterile triploid hybrid collected from sympatric regions in Japan where *M. sinensis* and *M. sacchariflorus* co-exist [3]. Harvestable yields for the standard *M × g* range from 10 to 30 Mg DM ha^{−1} depending on location and inter annual weather variations during the growing season [4]. Early molecular diversity studies in Europe [5] showed there were a handful of different clones of *M × g*, but these represented little phenotypic diversity [6]. While highly productive in some field trials [7,8], a number of experiments showed that the original *M × g* hybrids were particularly susceptible to water deficit [9,10]. With global warming, climatic extremes are likely to become more frequent [11,12] and resilience traits to both too much and too little water need to be incorporated into new hybrids. Breeding programmes need to produce hybrids that achieve high biomass with minimum inputs of water and nutrients and with resilience to both abiotic stresses from extreme weather and biotic stresses from diseases.

Over the past 20 years, *Miscanthus* has received sustained financial support for genetic improvement through breeding [13,14] from a combination of public and private investors. Genetic improvement depends on an understanding of Genotype × Environment (G × E) interactions that determine which combination of traits would potentially result in highest yield. In conventional breeding, breeders use phenotypic selection to drive decisions for cross hybridization. Phenotypic selection, applied to both parents and progeny, focusses on final yield and quality and is normally based on morphological traits, such as shoot height and shoot densities per m².

Underlying these traits are the physiological traits driving growth. Plant growth can be viewed as a biological solar panel: leaves intercept light, capture atmospheric CO₂ and convert the assimilated carbon into structural and non-structural carbohydrates. These physiological processes with complex feedbacks are challenging to use in practical breeding.

This paper describes the practical strategies taken over the past 15 years to move *Miscanthus* breeding, as quickly as possible from wild accessions in Asia into the PBC of choice for large areas of marginal lands.

2. Breeding Strategies

The *Miscanthus* crop is largely undomesticated and thus for any breeding programme there are many potential traits that could be targeted for simultaneous improvement. In establishing the *Miscanthus* breeding programme at Aberystwyth, a long-term goal set in 2006 was to breed resilient *Miscanthus* that could be propagated from true seed [15]. While clonal hybrids have advantages such as fixing traits giving homogenous stands, multiplication rates for clones through rhizome splitting and nodal propagation range from 10–100, while seed multiplication rates are > 2000. It was believed that seeded varieties were the most likely route by which sufficient area of *Miscanthus* plantation could be established to deliver the biomass required to generate a global impact. Resilient crops would allow

Miscanthus to sustain higher levels of yield by matching varieties with many different environments. One major question remained, how in practice could this be done?

The first step taken was to investigate the available trait diversity within *Miscanthus* collections. Diverse *Miscanthus* germplasm was collected from European ‘secondary germplasm sources’ and characterized at a single site near Aberystwyth [16]. The field trial was planted with 240 genotypes and over half of these accessions had known Asian country origins. Using the site of origin data allowed geographical analysis of traits such as flowering time, and improved the understanding of how the spread of *Miscanthus* impacted traits of interest and of where further germplasm collections should be made [17]. Preliminary distribution maps and yield assessments emerged in the literature in 2004 [18]. These early studies highlighted the diversity of traits available within a wide distribution of *Miscanthus* species. In 2006, Aberystwyth began to lead expeditions to collect wild *Miscanthus* germplasm in Asia [19]. Over five years, one of largest *ex-situ* *Miscanthus* germplasm collections in the world was created with germplasm from China, Japan, Taiwan and South Korea. For each collected accession the geographical location was recorded along with local soil conditions, the environmental conditions and altitude corrected “historical” meteorological data [19].

Of the original 240 genotypes studied, two *M. sacchariflorus* accessions towered above all accessions including *M* × *g* displaying exceptional growth vigor and yield potential. Further, *in situ* assessments of yield at collection sites in Asia suggested that some wild accessions might out yield standard *M* × *g* and that these, subject to adequate performance trialing, could be the first new types to be commercialized. This approach was part of the ‘four tracks strategy’ shown in Figure 1.

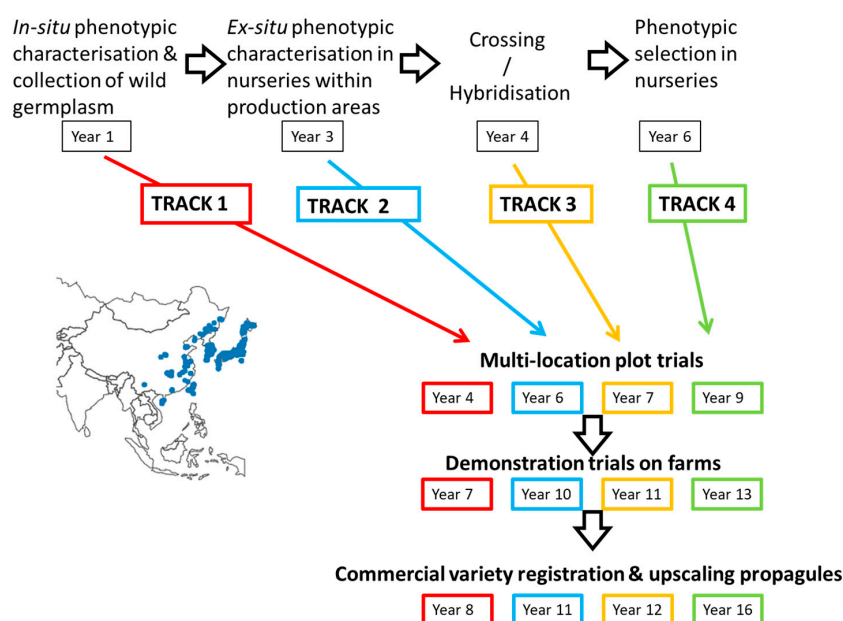


Figure 1. Four interrelated strategic ‘tracks’ for the development of commercial hybrids from wild *Miscanthus* accessions with estimates of minimum cumulative years needed to take wild germplasm through to the commercial hybrids. Inset, a map showing locations of wild germplasm collections.

Track 1. Wild population-based accessions from seed collected in Asia of *M. sacchariflorus*. Accessions were selected *in-situ* with good biomass characteristics and high seed numbers per panicle, which could be efficiently threshed resulting in multiplication rates > 2000 propagules per plant (~ per m²). Track 1 potentially represented the fastest track to expand commercial cropping areas. In spite of this, with three-year yield evaluations at multiplication plot and demonstration scales, Track 1 could only be expected to reach commercial maturity in about eight years. It was also the most risky approach, since G × E adaptations were not tested outside the native environments of the accessions.

Track 2. ‘Outstanding’ wild genotypes of *M. sinensis* and *M. sacchariflorus* were selected *ex-situ* after one year’s growth and successful overwintering. These accessions were upscaled with clonal propagation by *in vitro* tillering and placed into multi-location plot trials for yield and quality evaluation. The Aberystwyth programme recognized the value of outstanding clones because cloning fixes desirable traits, as exemplified by the commercial standard $M \times g$. Track 2 utilized selection of accessions in or near the climatic zones where the biomass was intended to be produced. However, these selections were considered as only ‘intermediate varieties’ because clonal propagation was likely to be too expensive and too slow in producing sufficient clones for thousands of hectares.

Track 3. ‘Outstanding’ genotypes (from Track 1 or 2) were used as parents to produce F1 hybrid progeny. These progeny were assessed directly in multi-location plot trials for yield and quality. Track 3 assumed that outstanding accessions identified from *ex-situ* nursery trials would be good parents.

Track 4. A simple breeding track that used phenotypic evaluation of the progeny of crosses (from within and between species groups) in small scale breeding nurseries trials before further testing in multi-location plot trials. The nursery trials helped inform the choice of parental combinations for upscaling of seed production and were therefore less likely to waste resources in the multi-location plot trialing, but this track was the slowest and required about 16 years.

3. Aberystwyth Experience of Implementing Fast Tracks

Accessions collected from Japan and China in 2007/8 were planted in Track 1 and 2 trials at three locations: UK (Aberystwyth), Germany (Braunschweig) and Italy (Sicily). These locations spanned a wide range of European climates; however, rabbit damage in the UK and irrigation problems in Italy lead to poor stand establishment, and these two sites could not be further used. At Braunschweig, the original track 1 and 2 plots produced high quality yield comparisons between standard clonal $M \times g$ and new germplasm (Figure 2). In the second growth year, three Chinese accessions of *M. sacchariflorus* matched $M \times g$ in producing 10–12 Mg DM ha⁻¹ (data not shown). However, after the second growing season, $M \times g$ produced not only the highest spring harvestable yields (Figure 3) but also the biomass had relatively low moisture content. The Track 1 & 2 trials showed that (i) the very tall (>4 m) Chinese accessions nearly reached their yield potential in two years, (ii) yields from the tetraploid Japanese and diploid Chinese *M. sacchariflorus* were comparable and (iii) $M \times g$ performed better than the new accessions in good growing years in central Germany with plenty of water and sunshine.



Figure 2. A view of a combined Track 1 and 2 field trial in Braunschweig, Germany in late August 2012 (Photo by Kai Schwarz, with breeding technician, Heike Meyer).

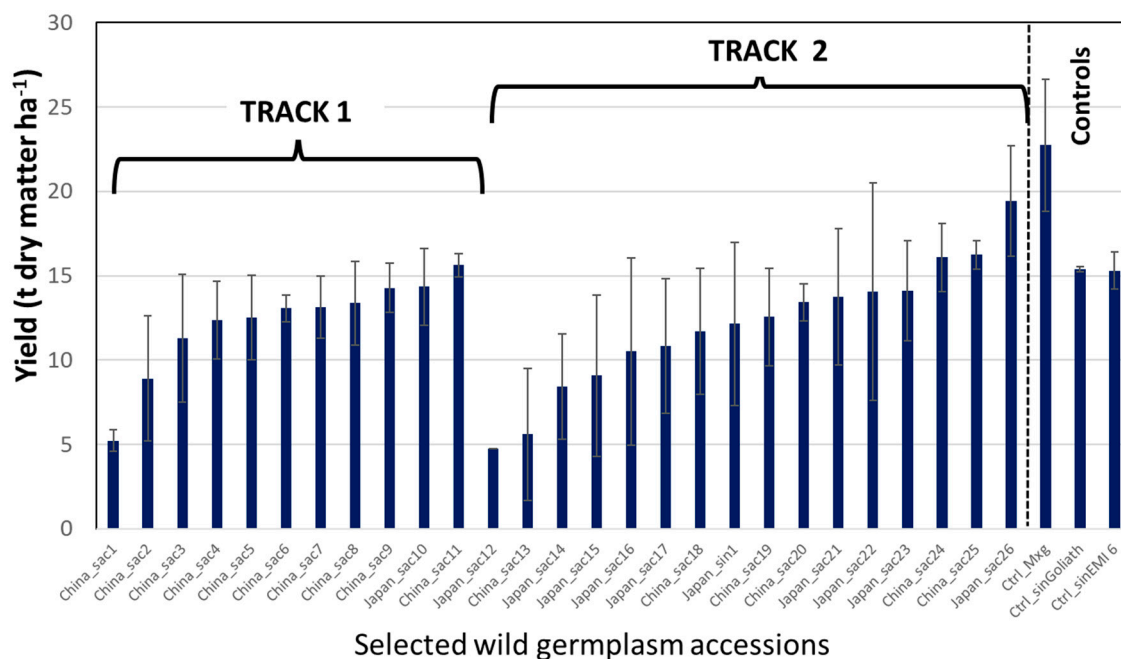


Figure 3. Spring harvestable yield (t dry matter ha⁻¹) for selections from Track 1 (11 *M. sacchariflorus* accessions from seed) and Track 2 (16 selections, cloned by *in vitro* propagation) and three control genotypes (standard *M* × *g*), *M. sinensis* (Goliath and EMI-6, [6]), following the third growing season at the JKI site in Braunschweig (March 2012). Data from three plots of 50 plants with a planting density of 2 plants m⁻², each in a randomised block. The field design is similar to those in [4]. Error bar = +/− 1 SE.

4. Tracks 3 and 4: Improvement through Hybridisation

Twenty-six informative microsatellite DNA simple sequence repeat (SSR) markers developed by CERES Inc. (Thousand Oaks, CA) were used to categorize 240 genotypes in a trial planted in 2005 in Aberystwyth into eight molecular diversity groups. These genotypes showed a wide diversity in flowering times from late June to October, and some genotypes never flowered. To achieve the flowering synchronization needed to test hybridization between diverse genotypes, a range of treatments were used including: (i) delaying spring growth in winter state plants by keeping rhizome at ~4 °C, i.e., lower than the threshold for regrowth; (ii) removing physiologically older stems to stimulate younger ‘shorter’ shoots into maturity; and (iii) using climate controlled environments to precisely control and vary the progression of photoperiod. The latter was the most successful and bagged crosses of several thousand cross combinations were made over a period of five years. These bi-parental crosses were made in every SSR grouping and demonstrated that there was no genetic interspecies barrier (Figure 4).

Figure 4 shows a matrix approach to investigate the crossing compatibility across 240 diverse genotypes. This visualization was automated in our bespoke MySQL breeding database we called “MSCAN”. Each time a cross was attempted and recorded on MSCAN, a red ‘dot’ appeared. When the cross was found to have produced seed, the dot was changed to blue. This dynamic near real-time visualization allowed those making the crosses to see where further crosses should be attempted to explore the available genotypic diversity. In addition to biparental crosses, single panicles were also bagged to test if any of the genotypes were capable of producing ‘self’ pollinated seed. Careful examination of the 1:1 diagonal line in Figure 4 shows that a few genotypes tested appeared to be self-compatible and produced seed that germinated. These tests for selfs, while a useful screen, depend on the quality of pollination control by the materials used to create the crossing bags and were not further investigated using markers, but provide an indication of the potential to identify self-compatible *Miscanthus*.

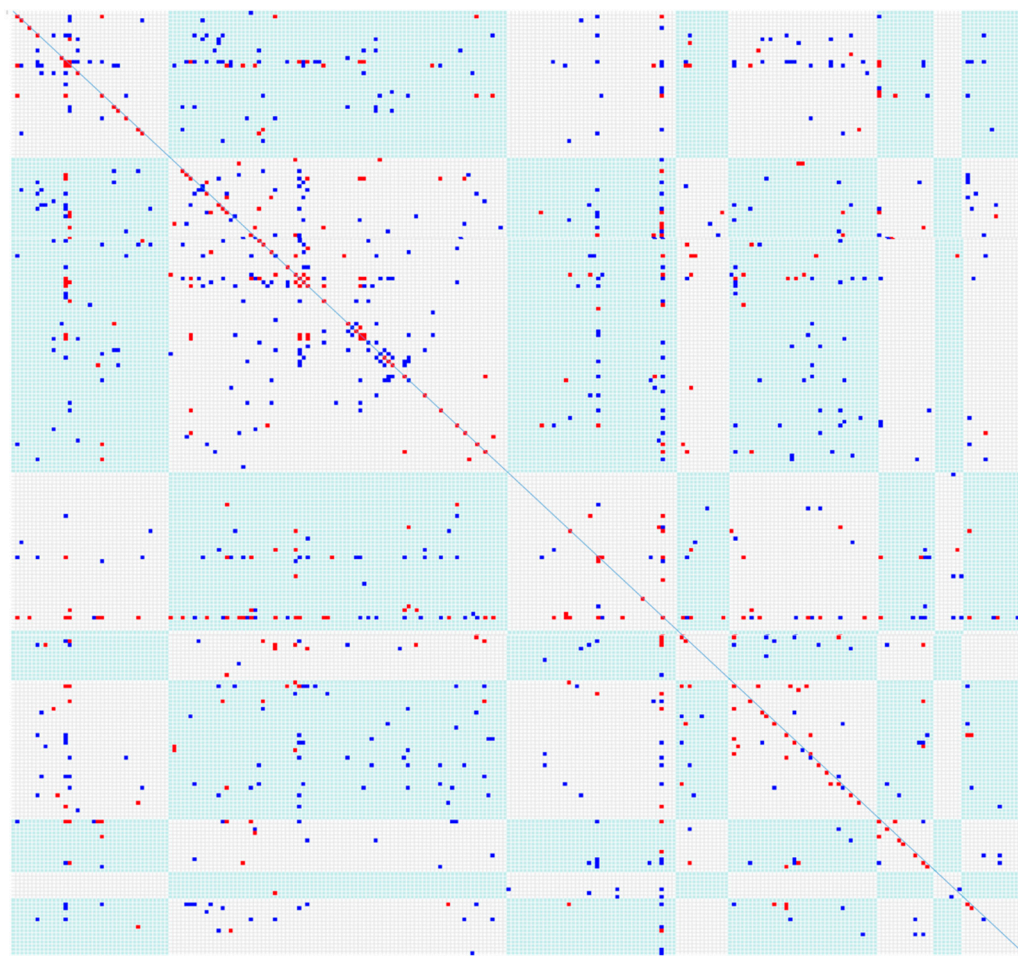


Figure 4. Each row and column represent 240 *Miscanthus* genotypes, sourced from European collections. These were sorted into 8 phylogenetic groups distinguished by microsatellite DNA simple sequence repeats (SSRs) across the x-axis (seed bearing) and y-axis (pollen donor) by the background of alternating light blue and white. Each colored dot represents a cross or a selfing attempt: red for attempted but unsuccessful, blue for successful i.e., the cross produced progeny. Pollination control was achieved by bagging two panicles, one from each parent in pollen proof bags. The diagonal 1:1 line are self-pollinations made by bagging single panicles.

All glasshouse crosses were performed with a mixture of traditional glassine paper and modern non-woven fabric bags. Glassine is light and convenient to use, but is a delicate paper susceptible to physical damage. Crossing bags made from non-woven polyester fibers were more breathable and overall gave a higher seed set than glassine bags [20]; however, polyester bags were heavier and needed to be supported, which greatly added to the time taken to set up crosses. Detailed investigations of the environment effects of different materials used for crossing bags showed very complex interactions: higher light transmission produced high within bag temperatures, especially in glassine paper [21]. Thus, we found the immediate crossing environment, over and above genetic compatibility and flowering synchronization, contributed to the success or failure of the crossing attempt. Therefore the crosses in Figure 4 are indicative, rather than an absolute test of genotypic compatibility. Interestingly, controlling these extra environmental factors clearly showed that crosses could be achieved between all groups in the diversity matrix and in a very small number selfed seed were possible. Overall, we consider the ability to self a negative trait, since the breeding strategies based on F1 hybrids rely on self-incompatibility.

In general, seed from crosses made in autumn were sown in sand and transferred to modular plugs in January/February. Between 2007 and 2012, the plugs were space-planted into nurseries at

Aberystwyth and Braunschweig in May, after the risk of late spring frosts was reduced. Plant losses upon transplanting to the field in Aberystwyth were invariably higher than those in Braunschweig. The combination of low temperatures, marginal land with low soil pH and competition from native grass weeds resulted in high risk, slow establishment with plants typically no taller than 75 cm in autumn. In contrast, warmer summer temperatures and light textured soils in Braunschweig resulted in rapid establishment. Low continental winter temperatures in Braunschweig meant established plants could be screened for cold tolerance of the overwintering rhizomes. The Braunschweig site, in the middle of Europe, turned out to be strategically the right place to make first stage selections for track 4 because the rankings based on phenotypes were consistent with other ‘slower selection’ locations in Europe. Using the Braunschweig site enabled selections of vigorous hybrids to be made after two years that could be promoted to the next phase: multi-location plot trials.

5. Phenotyping in Multi-Location Plot Trials

In 2011, a European project ‘Optimising *Miscanthus*, OPTIMISC’ provided an opportunity to appraise $G \times E$ interactions with both wild and genetically improved *Miscanthus* planted at six locations across a wide climatic range; from cold sites such as Moscow in Russia, warm sites such as Adana in Turkey to mild and high rainfall sites such as Aberystwyth in Wales [22]. Promising clonal wild accessions from Track 2 (nurseries) and some hybrids from Track 4 that looked promising at the end of the first growing season in Braunschweig were selected to be part of the OPTIMISC trial. The capacity to clone plants *in vitro* and raise sufficient plug plants for replicated plot trials at six OPTIMISC locations in 2012, limited plot sizes to 50 plants at 2 plants m^{-2} (25 m^2). These trials showed different morphotypes were largely not specified by environment and that a high performing genotype in one site was mostly high performing across all sites. This may have been because the site of selection in Braunschweig, Germany represented a reasonable amalgam of the extremes of cold and drought experienced across the other diverse Continental European sites. It is difficult to extend this kind of large-scale empirical analysis to more sites, which is where the modelling of yield is important. A growth model was developed and refined that takes meteorological and soil data as input and estimates yield at a spatial resolution of 1 ha. This model was largely developed and parameterised with data from field trials growing $M \times g$. Controlled environments and field experiments have demonstrated that $M \times g$ is sensitive to drought stress in particular, but also chilling stress and that accessions that yield more under abiotic stress have been identified [23,24]. Thus there is identified genetic potential for improvements over the current commercial standard in particular stressed environments. The impacts of abiotic stress such as drought, cold and salinity on the available area of economic cultivation has been modelled [25] and parameters modified to illustrate the potential gains from adding the improved performance of new accessions to $M \times g$ [12]. Important in the political context of gaining support for breeding energy crops spatial models for yield have been extended to identify the particular geographical areas best suited to production at low carbon intensity to produce a more sustainable energy efficient crop ([2], Figure 1).

Five years later, production systems for seeded hybrids had developed at such a pace using developments from the GIANT (Genetic Improvement of *Miscanthus* as a sustainable feedstock for bioenergy in the UK, 2011–2016) and MUST (*Miscanthus* Up-Scaling Technology, 2016–2019) projects, that in 2018 multi-location trials at plot (0.8 ha per site) and field scales (3.6 ha per site) were achievable in the EU project GRACE (Growing advanced industrial crops on marginal lands for biorefineries, 2017–2022).

Standardized phenotyping protocols are needed to compare traits between hybrids within sites, between years, across sites and across different projects. In practice, phenotyping is dependent on the resources, experience and commitment of the teams running the trials. The term schema is used in MSQ and phenotyping schemas were used to standardize protocols. To help those gathering and analyzing the data our system used schema names that were ‘human readable’ and used a ‘CamelText’ format followed by the units of measurement e.g., _cm (centimetres). In Table 1, phenotyping schemas

for a set of ‘growth phenotypes’ have been arranged in chronological order starting from trial planting through to maturity. The appropriate phenotypic measurements adjust slightly as the crop matures. The main reason to collect a phenotype is given in the ‘use’ column. Some phenotypic measurements are made at one or more measurement levels: plant/plot/quadrat. In large field scale trials of several hectares, such as used in the OPTIMISC and GRACE projects, phenotypes used ‘notional’ quadrats (could be patches of rows and columns) positioned appropriately to satisfy random sampling to obtain a representative sample. Protocol details were difficult to communicate across languages and cultures and ‘in field’ training backed up by protocol videos were found to be an effective means to standardize methods. When and what phenotypes were measured depended on the aims of the particular project, the available resources and crop age. Some measurements were considered essential and in tables these are marked ‘mandatory’. These include an assessment of plant losses (gaps) caused mainly by death at transplanting or winter freezing or late frosts after leaf emergence from the overwintering rhizomes in spring. Other traits considered mandatory have a minimum data collection frequency, where more measurements are encouraged. For example, canopy height made at plot level is a simple informative phenotype, but when made regularly it can be used to derive parameterized equations to identify interactions between seasonal growth dynamics and the changing environment [26]. The alignment of these processes with meteorological data underlies the calibration of process descriptions needed to tailor crop models to specific hybrids and thereby improve predictions of crop performance across different countries.

In Europe, biomass harvests are generally performed in late winter/early spring because delaying harvest reduces moisture, nutrient and ash contents. However, there are combinations of hybrid \times environment \times end use applications where earlier harvests are performed before winter senescence [27]. Table 2 shows the schemas for yield determination at harvest. To make harvest assessments small forage harvest machines developed for maize were found to be ideal, but if not available quadrat harvests taken with hedge cutters was a method that was readily standardized across sites. The optimum area for quadrats depended on the plot size and crop morphology. A study in Germany showed yield estimates from quadrats of less than 4 m² could not be reliably scaled to Mg DM ha⁻¹ [28]. Generally, harvest quadrats performed for scientific studies of *Miscanthus* plots were 6–12 m². The quality of the harvest data depended on accurate weighing of the fresh weight of all the biomass in the quadrat (FWQuadrat_kg) and of the subsamples (FWSS and DWSS). Large variations (>10%) in moisture contents within a hybrid, were found to be an indication of low data quality. Particular attention needed to be applied to sources of common errors, mostly due to sub-sample handling between the field and the laboratory where the samples were processed and oven dried. Table 2 provides a list of mandatory and optional harvest-related phenotypes commonly used to assess differences between hybrids and locations. In our experience the splitting of subsamples into leaf and stems, while informative to understand the contribution to high ash contents of the leaf fraction, is often a poor return on investment in early stage trials or young crops. We have concluded that it is better to have a phased approach to the selection process, before leaf stem separations are attempted on a few targeted selections.

Table 1. Phenotyping schema names and protocols developed for *Miscanthus*.

Phenotyping Schema Name	Use	Measurement Level	Protocol	When Performed
SurvivingPlants_pc	To compare establishment and overwintering survival.	plot/quadrat	100 × Surviving plant count divided by planted plants.	July after planting and October (year 1) and following June (year 2)
EHeight_cm	Used to accurately calculate the start of growth.	plant	Measure from first emergence, from soil level to shoot tip/leaf tip.	Weekly March to May (until longest shoot is >40 cm).
FrostDamagePlantParts_0–9	To assess leaf/canopy damage by frosts occurring after spring emergence.	plant	0–9 scale of damage to plant part e.g. leaf where 0 is undamaged.	Weekly March to June.
FrostDamagePlantsinPlot_pc	No. of plants with score of 8, based on above frost score, to assess season start and stop.	plot	Using quadrat of 16 plants with 7 plants affected; $100 \times 7/16 = 44\%$.	Weekly March to June.
CanopyHeight_cm	To compare morphology and create growth curves for models.	plant/plot/quadrat	From ground to where most of the leaves bend to create a canopy.	Monthly, or higher frequency if resources allow.
TotalStems_countperplant	To compare plant morphologies contributing to yield & quality.	plant	Count all stems over 10 cm.	Late autumn (normally October)
BigStems_countperplant	To compare plant morphologies making yield and quality.	plant	Count the number of stems which reach 60% of the CanopyHeight. For spreading plants count within 0.5 m ² (use a frame of 71 × 71 cm).	Late autumn (normally October)
Greenness_0–9	To compare senescence from drought, cold and nutrient deficiency. This helps interpret G × E effects.	plant/plot/quadrat	Visual score where 9 = all leaves dark green, 5 = half of canopy green, 1 = totally brown.	When environmental stress events result in differences. At least in late autumn and again just before spring harvest.
FloweringScore_0–5	This visual score helps identify the window of flowering to understand cross pollination possibilities of different hybrids, resultant seed set, shattering and establishment of volunteer seedlings.	plant/plot/quadrat	Flowering Score (FS) 0-no flowering 1-Flag leaf visible 2-Panicle > 1 cm 3-Start of anthesis (yellow anthers) 4-Main flowering period (flowering intensity >= 50% of ‘big’ stems that contribute to the canopy) 5-Flowering complete (no anthesis on any stems).	Monthly from July and at higher frequencies once flowering has begun.
PlantBasalDiameter_cm	To assess plant maturity and or a measure of potential for invasion by rhizome creep.	plant	The diameter of the ‘tuft’ at plant base.	Late autumn, or just after spring harvest.
PanicleHeight_cm	To understand morphological differences.	plant/plot/quadrat	The height from the ground to the base of the panicle of the tallest flowering stem.	Late autumn when growth has ceased.
LightInterception_pc	Assessing when and to what extend the leaf canopy is intercepting available incident light.	plot/quadrat	The light intercepted by the canopy as measured by a ‘line ceptometer’. 0% no interception. >95% full canopy closure.	March to October.

Units: _pc = percent; _cm = centimeter; _0–9 = visual score.

Table 2. Schemas for parameters used to quantify dry matter yield at harvest from subsampling.

Phenotyping Schema Name	Use	Protocol
QuadratArea_m2	Yield assessments are based on sampling area - here called a 'QuadratArea'.	Minimum sample area for <i>Miscanthus</i> is 6 m ² . In practice quadrats may not be used and the harvested area is calculated from the number of rows and columns of plants harvested in a plot and the plant density.
FWQuadrat_kg	Bulk 'fresh weight' from the 'QuadratArea_m2' harvested.	The fresh weight in kg of the stems cut within the QuadratArea_m2 at a cutting height of 5 cm above ground level. Often measured immediately 'on field' using a tripod and hanging balance method.
FWSS_g	Fresh Weight of a subsample to calculate the moisture content and dry matter yield; samples may be used for compositional analysis.	A typical subsample is ~200 g fresh weight of randomly selected whole stems, or from well mixed chip. FWSS_g should be determined as quickly as possible after the FWQuadrat_kg to avoid moisture losses.
DWSS_g	Needed to calculate the moisture content and dry matter yield.	Oven dry subsample to constant weight, at 105 °C is most reliable, but lower temperatures are acceptable, as long as drying is complete, for certain compositional analysis.
MoistureContent_pc	MoistureContent_pc is a key biomass quality characteristic.	$= 100 \times (\text{FWSS}_g - \text{DWSS}_g) / \text{FWSS}_g$.
QuadratDW_kg	QuadratDW_kg is an intermediate in upscaling measurement of yield.	$= \text{FWQuadrat}_kg - \text{FWQuadrat}_kg \times (\text{MoistureContent}_pc / 100)$
Yield_tDWperha	Expressed as tonnes dry matter per ha, or in SI units 'Mg ha ⁻¹ '.	$= (10,000 / \text{QuadratArea}_m2 \times \text{QuadratDW}_kg) / 1000$
FWLeafSS_g	Improves yield assessment by calculating 'leaf share'.	Quantify leaf lamina, not leaf sheath which is difficult to separate from the stems. Should be determined as quickly as possible after the FWQuadrat_kg.
DWLeafSS_g	Improves yield assessment by calculating 'leaf share'.	FWLeafSS is dried to constant weight at 105 °C. 40–60 °C may be used if carbohydrate analysis is required.
MoistureContentLeaf_pc	A key biomass quality characteristic.	$= 100 \times (\text{FWLeafSS}_g - \text{DWLeafSS}_g) / \text{FWLeafSS}_g$.
FWStemSS_g	To assess proportion of yield due to stem.	Includes stems (culm) and leaf sheath; measured as soon as possible after the FWQuadrat_kg to increase accuracy of moisture content data.
DWStemSS_g	To assess proportion of yield due to stem.	The FWStemSS is dried to constant weight at temperatures 105 °C. 40–60 °C is recommended for analysis of cell wall carbohydrates (lignin and cellulose).
MoistureContentStem_pc	To assess proportion of yield due to stem.	$= 100 \times (\text{FWStemSS}_g - \text{DWStemSS}_g) / \text{FWStemSS}_g$
LeafBiomassProportion_pc	To assess proportion of yield due to leaf.	$= 100 \times (\text{DWLeafSS}_g / (\text{DWLeafSS}_g + \text{DWStemSS}_g))$
StemBiomassProportion_pc	To assess proportion of yield due to stem.	$= 100 \times (\text{DWStemSS}_g / (\text{DWLeafSS}_g + \text{DWStemSS}_g))$
StemBasalDiameter_mm	Impacts senescence rate and moisture content, harvesting and baling efficiency.	Measure the basal diameter of ~five randomly selected stems.
StemWallAtBase_mm	Impacts senescence rate and moisture content, harvesting and baling efficiency.	Measure at base of ~five randomly selected stems.

Units: _pc = percent; _mm=millimeter; _cm = centimeter; _g = grams; _tDWperha = Mg dry weight per hectare.

6. Selection Criteria and Methodology

The phenotyping described above was used to quantify performance attributes of progeny from crosses along a phased screening chain illustrated in Figure 5. These are the hurdles that progeny must clear in a long race to become a winning hybrid and commercial variety. The first phase is early plant development and survival. *Miscanthus* has small seed, high germination temperatures and slow initial rates of growth following germination [29]. Therefore, before transfer to the field environment, growth in small compost plugs is the current standard method used to establish *Miscanthus* seedlings.

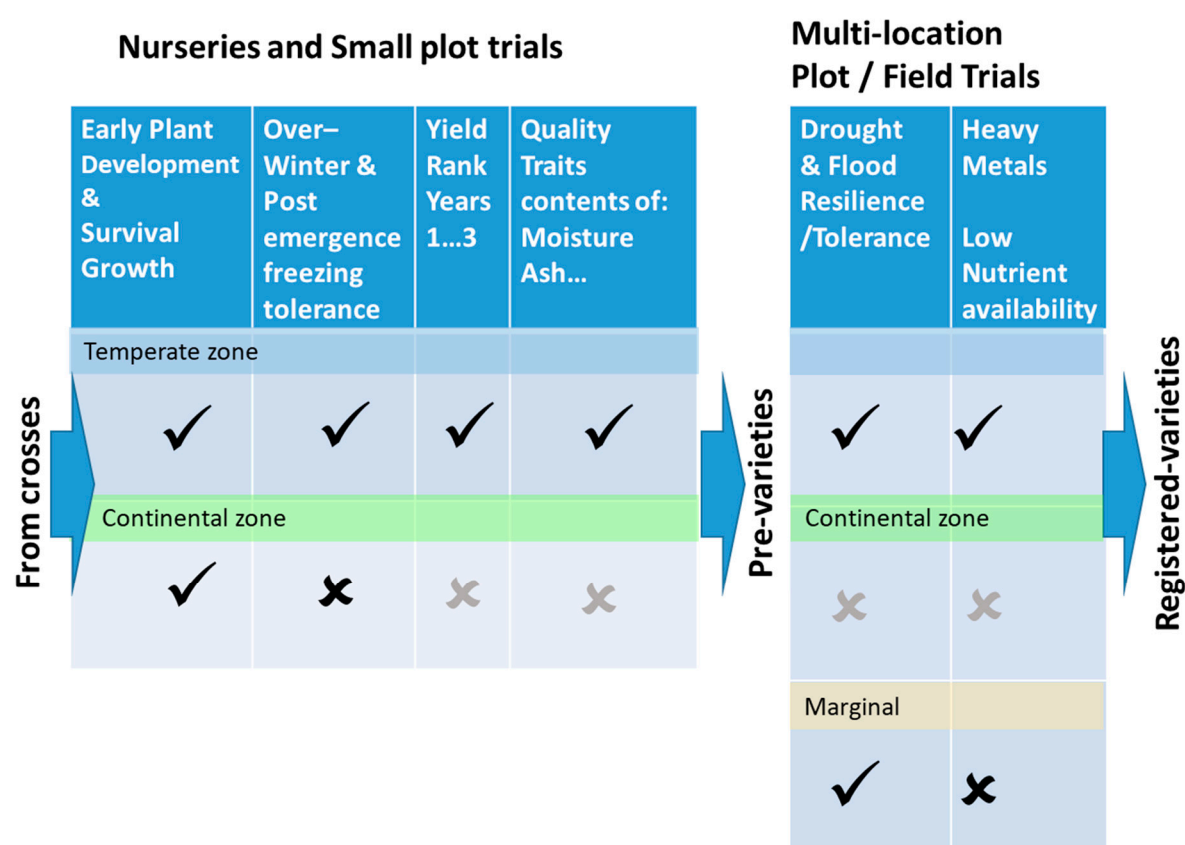


Figure 5. A schematic showing the different phases of selection applied to the progeny of crosses from successful transplantation, sufficient first season growth to overwinter through to the production of biomass yields above 10 t by the end of the second year with low ash and moisture content. Once a fail (an 'X') occurs in the selection sequence, the hybrid is eliminated. The extra steps indicated for pre-varieties illustrates further resilience testing related to likely stresses encountered on marginal lands. The resilience tests include tolerance to flooding, drought, low nutrients and heavy metal contaminants in the soil and apply to both potential parents and progeny.

The counts of surviving plants to derive 'SurvivingPlants_pc' (Table 1), were performed at about six weeks after planting, and again at the end of the growing season. There are many reasons why plug plants may fail to establish, including immature or unhardened seedlings, poor plug to soil contact and prolonged drought, grazing and weed competition. We have found that most of these establishment risks can be managed by covering the plants immediately after transplanting with a thin mulch film which degrades after 6–8 weeks. These mulch films prevent moisture loss and increase the growth rates by raising the soil temperatures about 4 °C [29]. Film coverings also protect the crop from grazing hares/rabbits, bird damage and late frosts but may stimulate weed growth and there are concerns over microplastics entering the soil. Solutions to both these problems are being actively researched in the current GRACE project by using bio-herbicides and biomass-derived biodegradable films. In our

experience, the risk reduction and acceleration in establishment from the use of film outweighs its economic cost. Any establishment problems are compounded in later years.

An adequate first year growth rate is needed to reach a level of physiological maturity to produce sufficient rhizome growth to maximise the likelihood that the plant will survive over winter. Young plants have small amounts of immature and shallow rhizome making them more susceptible to sub-zero soil temperatures than older plants. By phenotyping in October, including a further plant count, plant losses within the growth season and during winter can be distinguished. Rapid above ground measurements of canopy height (CanopyHeight_cm) and stem counts per plant (BigStems_countperplant) were normally used to assess early plant development and maturity before winter, but occasionally onerous estimates of the below ground biomass and freezing tests have been made in *Miscanthus* [30]. Sometimes fast growing hybrids died during the first winter in Braunschweig, and it was noted that this often coincided with a lack of autumn senescence. Successful overwintering is crucial, but a standardized field treatment, to test overwintering, is practically unattainable because the depth and timing of subzero temperatures and soil moisture varies from year to year. Braunschweig's continental climate was reasonably consistent and trials there were successful in identifying resilient germplasm in 2010/11 for the OPTIMISC multi-location trials which included cold winter sites in Ukraine and Moscow. However, plants making it through to the second year in Braunschweig were not guaranteed cold tolerant for locations and years with exceptionally severe winters.

Once plants survived the first winter, the rate of plant development depended on growing conditions and interactions between hybrid, temperature, water availability and soil texture. On light sandy soils, in warm locations mature maximal yields were reached by the end of the second year. However, on heavy clay soils and cool climates, *Miscanthus* may require up to 5 years to reach mature yields. Pragmatically we have used an assessment of yield after two years in nurseries before promotion to replicated multi-location plot trials. There are so many longer term factors that could impact yield but are difficult to include in a breeding programme, such as the effects of initial planting densities on long term yields. Long-term trials are essential for environmental and economic analyses, and these at present rely on a few long term yield series from $M \times g$. However, longer term trials with more diverse high yielding germplasm will be needed to mitigate against potential problems not encountered within short term screening programmes.

The screening of novel germplasm and different breeding tracks (Figure 1), the extensive hybrid crossing programme (Figure 4) and phenotyping across sequential selection criteria (Tables 1 and 2 and Figure 5) used in the breeding programme led from Aberystwyth has identified new competitive *Miscanthus* hybrids. Figure 6 shows a side by side comparison of one such new clonal hybrid, GNT-10 and the commercial standard $M \times g$, planted in replicated plot trials in Lincoln (UK) and in Poznan (Poland) in 2014. In Lincoln, during the second and third growing seasons rainfall patterns ensured growth without water deficit and $M \times g$ performed as expected reaching ~ 10 Mg DM ha⁻¹, while GNT-10 out-yielded $M \times g$ by almost one third. Meanwhile at a site near Poznan on a light sandy soil long drought periods occurred during mid-growing season in both the second and third years. The calculated soil moisture deficits in Poznan reached < 200 mm for 6–8 weeks, way below estimated plant available water capacity of 70 mm in the soil profile exerting a strong drought. Under the drought prone conditions at the site in Poznan growth of $M \times g$ was severely restricted producing only ~ 2 Mg DM ha⁻¹, five times less than GNT-10 which produced ~ 11 Mg DM ha⁻¹.

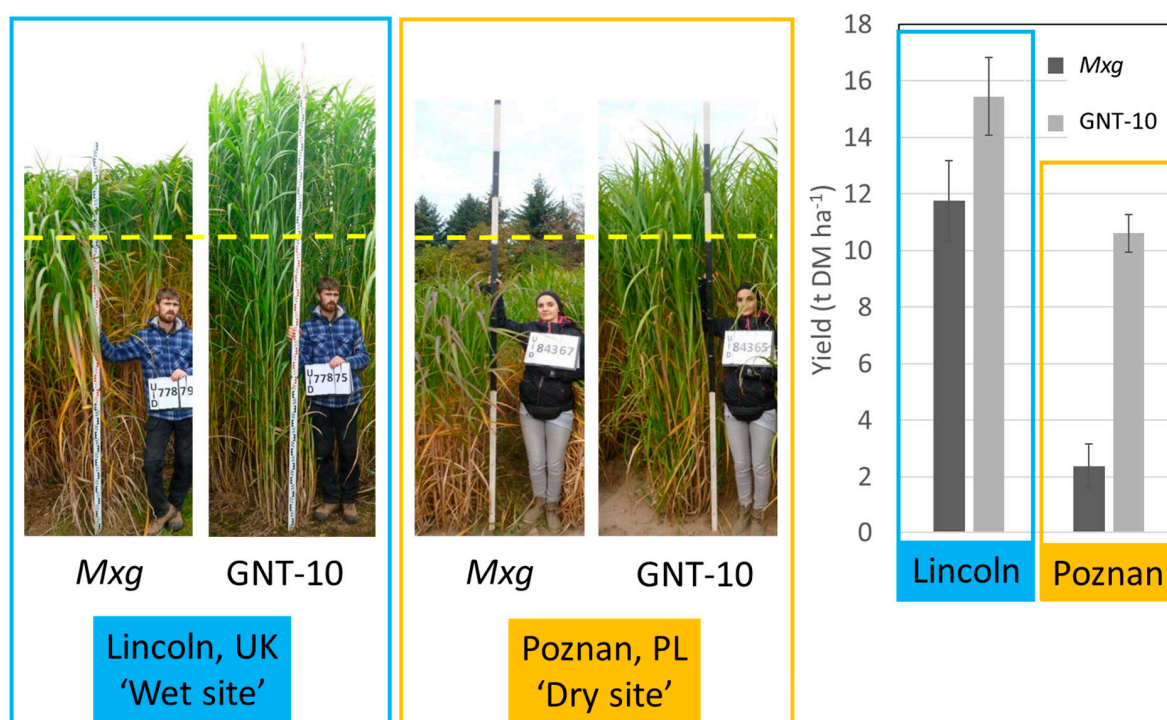


Figure 6. A side by side comparison of average yield in years two and three combined for a novel clonal hybrid 'GNT-10', from the breeding programme at Aberystwyth, and the commercial standard $M \times g$. Plants were grown in replicated plot trials in Poznan (Poland) and in Lincoln (UK), photos are of the second (PL) and third (UK) years following planting respectively. In Poznan, there were long drought periods with soil moisture deficits below 200mm for several weeks in the growing seasons, the resulting growth in $M \times g$ was severely restricted. In Lincoln, there was adequate water supply and $M \times g$ performed as expected but was still out-yielded by GNT-10. A yellow dotted line shows 2m above ground level. Error bar = ± 1 SE.

Such large differences in productivity, particularly when water is limited, are game changers, especially when considering cultivating biomass crops on marginal land to achieve carbon sequestration, phyto-remediation, soil stabilization, and providing new business opportunities for rural economies. A recent EU project entitled 'Miscomar' (*Miscanthus* for contaminated and marginal land) showed that novel *Miscanthus* hybrids, with similar water conservation traits to GNT-10, when grown on metal contaminated land excluded heavy metals over a period of three years [31] suggesting *Miscanthus* could be used to bring contaminated land back in to useful production. The approach taken thus far in domesticating *Miscanthus* reflects the early days of domesticating a crop that had attracted relatively little research funding. The perennial nature and complex genome are challenges for phenotyping and genetic analysis. The performance of a natural wide hybrid suggested potential gains were possible from wild accessions but this was not realised from the collections made. To make progress pragmatic decisions were made including to "cross the best with the best". Markers and conventional cyclical species improvement programmes are being developed but from such an initial paucity of information the generation of multiple interspecific hybridisations generated both potential product and information about which species might be most successful parents.

7. Conclusions and Future Improvements in *Miscanthus* Breeding

Biomass yield has been a particular focus for improvement; however, *Miscanthus* is a relatively undomesticated crop and there are potentially many traits that could be optimised [16]. Many trials have shown *Miscanthus* is well adapted to regions where it is non-indigenous. There are concerns that fertile *Miscanthus* hybrids could become an invasive weed in lower latitude warmer climates

that stimulate flowering where the risk of seed shatter a volunteer establishment is higher. Although field trials performed to date indicate the risk is quite low, breeding sterile triploid hybrids would virtually eliminate invasive risk by seed, and could help speed up the adoption of *Miscanthus* by policy makers and growers. Manipulating ploidy or improvements to triploid germplasm using transgenic manipulation also represent possible routes to develop future improvements. *Miscanthus* has proven comparatively recalcitrant to tissue culture in our experiments at Aberystwyth; however, others have reported success at generating synthetic polyploids [32] and in generating stable transformants [33].

The speed of breeding programmes obviously affects economic cost but, imperative to biomass crops in particular, speed also affects the potential global impacts that such crops can make in the current climate emergency. We attempted to increase the speed of selection using the Track 1 and Track 2 approaches described above but found that competitive germplasm was generated by using the more conventional but slower breeding schemes. Markers and genomic selection may be used to reduce the length of breeding cycles. A particular challenge to the application of molecular selection is that *Miscanthus* has a large complex genome including a recent duplication [34] and few available genomic resources. Resources may be adapted from closely related species such as Sorghum [35,36] but the *Miscanthus* breeding programme at Aberystwyth has developed and tested the potential for *de novo* molecular resources to impact breeding. A high resolution genetic map including all 19 linkage groups was produced [37] and genome wide association studies highlighted the potential of genomic selection by focusing on 138 genotypes of *M. sinensis* species [38,39]. Clark et al. [40] have further demonstrated the potential of this approach using populations phenotyped at different sites. We have recently demonstrated the potential of genomic prediction and index selection to optimise selection across multiple target traits in *Miscanthus* [41]. The power of predictive models may be improved when more individuals are included and our early studies are being extended across larger populations in excess of 800 genotypes from the collections at Aberystwyth.

In conclusion, the breeding foundations have been laid well for *Miscanthus* over the long time periods necessary with the combination of public and private funding. The upscaling of improved seed-based hybrids is ongoing, and technical solutions are being found for every known barrier. These hybrids can be commercially scaled up over a few years if real, stable market opportunities emerge in response to sustained favourable policies. The contributions of growing and using *Miscanthus* for socioeconomic and environmental benefits are clear on paper, but in practice these are still being worked out with regionally diverse actors along the biomass value chains. While innovations in the GRACE project focus on Europe, the opportunity for biomass value chains span large areas globally, including countries in Sub-Saharan Africa, China and the Americas. The large-scale deployment of *Miscanthus* in all these areas needs developments outside the breeding arenas, but we feel confident that our experience and the resources developed over the past 15 years of working with *Miscanthus* will allow us to develop a globally impactful biomass crop.

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