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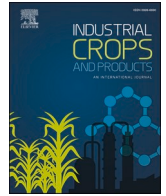**Awty-Carroll, D, Iurato, A, Scordia, D, Schwarz, KU, Scalici, G, Robson, P, Mos, M, Webster, R, Cosentino, S, Clifton-Brown, J and Shafiei, R**

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Achieving hybridisation between Miscanthus species: Commercially-scalable methods to manipulate flowering synchronisation and maximise seed yield

Danny Awty-Carroll [a,b], Antonella Iurato [b,c,d,*], Danilo Scordia [e], Kai-Uwe Schwarz [f], Giovanni Scalici [b,c], Paul Robson [b], Michal Mos [d,g], Richard Webster [a,b], Salvatore Cosentino [c], John Clifton-Brown [b,h], Reza Shafiei [b,i]

[a] *Biological and Environmental Sciences, Liverpool John Moores University, Liverpool L3 3AF, UK*
[b] *Institute of Biological, Environmental and Rural Sciences, Gogerddan, Aberystwyth University, Ceredigion, UK*
[c] *Dipartimento di Agricoltura, Alimentazione e Ambiente – Di3A, University of Catania, Italy*
[d] *Terravesta ltd, Lincoln, UK*
[e] *Dipartimento di Scienze Veterinarie, University of Messina, Polo Universitario dell'Annunziata, Messina 98168, Italy*
[f] *Julius-Kühn-Institut, Braunschweig, Germany*
[g] *Energene Seeds Ltd, Gogerddan, Aberystwyth, Ceredigion SY23 3EE, UK*
[h] *Justus Liebig University Giessen, Germany*
[i] *Division of Plant Sciences, School of Life Sciences, University of Dundee at the James Hutton Institute, Dundee, UK*

ARTICLE INFO

ABSTRACT

Miscanthus is a high-yielding lignocellulosic perennial biomass crop. The low multiplication rate of clonal rhizome propagation is a bottleneck to upscaling plantation areas of feedstock needed to supply and expand the bioeconomy. Novel seeded *Miscanthus* hybrids are currently being developed to overcome this bottleneck by increasing annual multiplication rates from approximately 10 to over 1000 times. We describe a series of field experiments in southern Italy using agronomic methods to optimise multiplication rates through (i) planting configurations and densities, (ii) ratio of seed parents to pollen parents (iii) supplemental pre-dawn misting to increase humidity during pollination. In these trials the seed-bearing *M. sinensis* started flowering 2–3 weeks earlier than the *M. sacchariflorus* pollen parent. Earlier experiments indicated that flowering in *M. sacchariflorus* was mostly determined by photoperiod while in *M. sinensis* it was modulated by endogenous signals. Consequently, a second set of experiments were conducted to delay flowering time in *M. sinensis*: (iv) mid-season stem cut back, (v) oversupply of nitrogen, and (vi) undersupply of water. Across all treatments and years, the multiplication rates varied from 140 to 1300 seeds m^2. Reducing the proportion of the pollen parent plants (*M. sacchariflorus*) from 50 % to 25 % did not reduce seed yield per plant. This therefore increases the seed yield per m^2 and reduces seed production upscaling costs. Flowering time and duration in *M. sinensis* was significantly impacted by mid-season cutting and water stress, but not by nitrogen supply rates. Mid-season shoot cutting reduced number of flowers per plant (77 %), seed quantity (47 %), seed size (46 %), and resulted in seeds with a low germination rate of 39 %. High *M. sinensis* planting densities produced higher seed yields in the first year. However, in subsequent years higher density plots were more susceptible to autumn lodging lowering seed production by loss of panicles. Pre-dawn misting to prolong pollen life and stigma receptivity had no significant effects on seed production. This study demonstrates the importance of flowering time synchronization in the open field for commercial seed production. The limited effect of agronomic efforts to reduce the interspecies flowering time gap emphasises the importance of genetic factors in controlling flowering time. The most impactful intervention to change flowering time and improve parental synchronisation was mid-season cutting, while this method reduced seed production when applied to the seed parent it could be ideal for pollen parents.

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* Corresponding author at: Dipartimento di Agricoltura, Alimentazione e Ambiente – Di3A, University of Catania, Italy.
  E-mail address: antonella.iurato@unict.it (A. Iurato).

1. Introduction

Renewable lignocellulosic feedstocks from biomass crops are key elements in a circular economy and in meeting net-zero greenhouse gas (GHG) emissions targets by 2050 (United Nations Environment Programme, 2015). As biomass crops absorb carbon from the atmosphere during the growing season, they offset the carbon footprint associated with their biomass conversion to heat, electricity, and a variety of bio-products. In this context, *Miscanthus* (*Miscanthus* spp.) offers practical solutions for environmental improvement due to its perenniality, low maintenance, nitrogen fixing capability and crop production longevity of up to 20 years.

Miscanthus grows well on marginal land therefore not competing with conventional arable food crops for land resources (Clifton-Brown et al., 2015; Scordia et al., 2022; Valentine et al., 2012; Winkler et al., 2020; Zhang et al., 2020). However, rhizomatous *Miscanthus* which was generated mainly from a single *Miscanthus* × *giganteus* clone has been the dominant around the world (Clifton-Brown et al., 2017). This could pose several significant challenges for the expansion of *Miscanthus* cultivation. A single cloned genotype carries an increased risk of pathogen or pest outbreak over time especially as the area under production increases annually (Scauflaire et al., 2013). On the other hand, rhizome propagation is labour-intensive and requires considerable resources (Hastings et al., 2017), which is a limiting factor for upscaling *Miscanthus* cultivation. One potential solution would be to propagate diverse *Miscanthus* genotypes from seed rather than relying on rhizomes (Clifton-Brown et al., 2016). Since *Miscanthus* species are typically self-incompatible and exhibit high resistance to inbreeding (Jiang et al., 2017), hybrid plants resulting from seed propagation are genetically diverse. Moreover, an economic assessment indicates that commercial *Miscanthus* cultivation can be scaled up nearly 100 times faster using seeds compared to rhizome propagation (Hastings et al., 2017). Seed-based *Miscanthus* offers a promising option for addressing the challenges associated with the current practices for expanding *Miscanthus* cultivation.

To develop diverse seed-based hybrid varieties for *Miscanthus* cultivation, the *Miscanthus* breeding group at Aberystwyth University collected germplasm from various geographical locations across East and Far East Asia, which was then introduced to the UK. These collections predominantly consisted of species *M. sinensis*, *M. sacchariflorus*, and *M. floridulus* (Clifton-Brown et al., 2019; Huang et al., 2019). These accessions were assessed and selected for breeding seed-based hybrids (Clifton-Brown et al., 2019). Several novel interspecific seed-based hybrids were produced through exploratory hybridizations between the wild accessions. These high-yielding hybrids also expressed interesting features such as drought tolerance (Clifton-Brown et al., 2019), physiological strategies and productive traits (Scordia et al., 2020), and phytoremediation of heavy metal-contaminated lands (Krzyżak et al., 2017).

A major challenge in developing interspecific hybrids is flowering time synchronisation. A gap of just a few days in the flowering time of parental lines can cause a significant damage to both seed yield and quality. This is a significant issue in *Miscanthus* interspecific seed production as the parental lines were collected from diverse geographical regions, often with contrasting climate conditions (Huang et al., 2019). Genetic improvement of parental lines can help to close the flowering time gaps. We previously identified that endogenous signals play a key role in *M. sinensis* flowering time, and the genetic determinants are likely to be governed by aging and gibberellin pathways (Jensen et al., 2021). However, the genetic basis of flowering time remains to be identified for other *Miscanthus* species. A flexible and immediate alternative approach for genetic improvement could involve agronomic methods to manipulate flowering time. For instance, the drought in the pre-flowering stage led to delay the flowering in *Sorghum*, but it could be a risky treatment since if the stress is severe the development of the panicles stops and can lead to lower grain yield (Craufurd et al., 1993). A similar effect from

water stress was observed in *Miscanthus x giganteus* (Cosentino et al., 2007). Cutting back *Miscanthus* was shown to delay flowering, although cutting was performed at early stages of growth (Dong et al., 2021). Oversupply of nitrogen could delay flowering due to increased vegetative growth (Heaton et al., 2009; Maughan et al., 2012). Other agronomic strategies have been employed to optimize flowering in many annual crops through plant density manipulation in grain crops (Duvick, 2005) or the ratio of the seed parents to the pollen parents and drought stress in maize (Wang et al., 2017, 2020), or increase air humidity to improve pollination and seed set in *Sorghum* (Gitz et al., 2015). However, a study addressing these issues in field crossing blocks is lacking for a perennial crop like *Miscanthus* to date.

Another important objective in the design of a crossing block is to enhance seed yield and improve the economic viability of hybrid seed production. This can be achieved through optimization of the seed-to-pollen parent ratio. A balanced 50:50 ratio is often suboptimal in open-pollinated grasses. Increasing the proportion of the seed parent beyond 50 % can significantly enhance seed production per unit of land. For instance, a study conducted in maize identified a 9:1 seed-to-pollen parent ratio as optimal (Wang et al., 2020). Similarly, in *Sorghum*, the optimal combination was suggested to be 7:1, as a seed-to-pollen parent ratio of 8:1 resulted in minor reduction in seed yield (Cisneros-López et al., 2017). However, it should be noted that the spacing between parental lines also plays a crucial role. In *Sorghum*, for example, a notable decline in seed yield per panicle was observed when the distance between parental lines reached 4.5 m (Cisneros-López et al., 2017). These observations highlight the critical importance of both the seed-to-pollen parent ratio and the distances between parental lines in optimizing hybrid seed production. It is essential to determine these factors for each species and new hybrid variety.

This study is the first exploration of *Miscanthus* field seed production in Europe for two key species specifically developed for the European climate to produce interspecific hybrids. Thus, we examined the effects of several agronomic practices aimed at manipulating the flowering time of the parental lines. Our goals were to enhance the synchronization of flowering time between the two parents and investigate the subsequent impacts on seed yield and quality.

2. Materials and methods

2.1. Phenotyping

Flowering development stages were closely monitored in order to assess the synchronisation between the parental lines and determine if seed set was improved due to agronomic treatments. First year phenological flowering assessments were carried out as previously described (Jensen et al., 2011) and according to the Biologische Bundesantalt, Bundessortenamt and CHemische Industrie (BBCH) scale of *Miscanthus* × *giganteus* (*M×g*) morphological development stages (Tejera and Heaton, 2017). For the subsequent years, a custom protocol, an extension to the BBCH scale was developed where twelve stages were defined to characterise flowering time in more detail (Table 1).

Flowering stages were recorded weekly using the flowering scale in Table 1 indicating progression from flag leaf to seed set as shown in Fig. 1. Sample quadrats were collected from the field, size and level of treatment isolation depended on the trial and year. When the seed was ripe the total number of panicles in each quadrat were counted, and the seed collected. In all cases, this data was used to calculate panicles and seed m^{-2} for each experimental plot as an average of the amount of seed from the *M. sinensis* (seed) parent only.

2.2. Experimental setup

Trials were conducted on the experimental farm of the University of Catania in Sicily, Italy (37°24'32.436"N, 15°3'36.72"E, 10 m AMSL) with a vertic xerochrepts soil type and a clay texture (Soil taxonomy,

Table 1

A table of flowering scores used in 2017 & 2018. The subset of scores used in 2016 are highlighted in grey. With a comparison to the BBCH scale M×g development stages (Tejera and Heaton, 2017).

Description	Flowering score	BBCH
Any flag leaf emergence	1	40
10 % of plants/tillers have flag leaves	*1a*	*41+*
> 50 % of plants/tillers have flag leaves	1b	41+
Some plants in a plot have panicle > 1 cm	*2*	*51*
> 50 % of plants in a plot have panicle > 1 cm	2a	51+
First visible anthers on panicles	*3*	*60*
Stigma visible	3a	61–69+
Anther visible	3b	61–69+
Swollen or colour change	3c	61–69+
50 % of stems reached anthesis	4	69+
> 75 % of stems reached anthesis	4a	69+
Flowering complete	5	70

USDA) (Cosentino et al., 2007, 2014). Throughout the trials, all the meteorological conditions were recorded by a weather station connected to a GP1 Data Logger (Delta T devices, Cambridge, United Kingdom). Heat accumulation expressed in degree days (°Cd) was calculated using the McVicker equation (McVicker, 1946), with a default base threshold temperature of 10°C which is standard for C_4 such as *Miscanthus* (Clifton-Brown et al., 2011; Jensen et al., 2013; Stewart et al., 1998).

Crossing blocks (as described below) were established in spring, in soil that was ploughed in autumn at 40 cm depth and disk harrowed in spring at 20 cm depth before transplanting occurred. Clonal rhizomes generated from parental lines [seed parent (*M. sinensis*) and pollen parent (*M. sacchariflorus*)] were planted employing appropriate experimental designs. Fertilizers were neither applied at transplant nor during crop growth unless detailed in an experiment below. Weeds were mechanically controlled, when necessary, throughout the growing seasons. After rhizome establishment, plantlets were kept well-watered through a drip irrigation system, by restoring 100 % of maximum
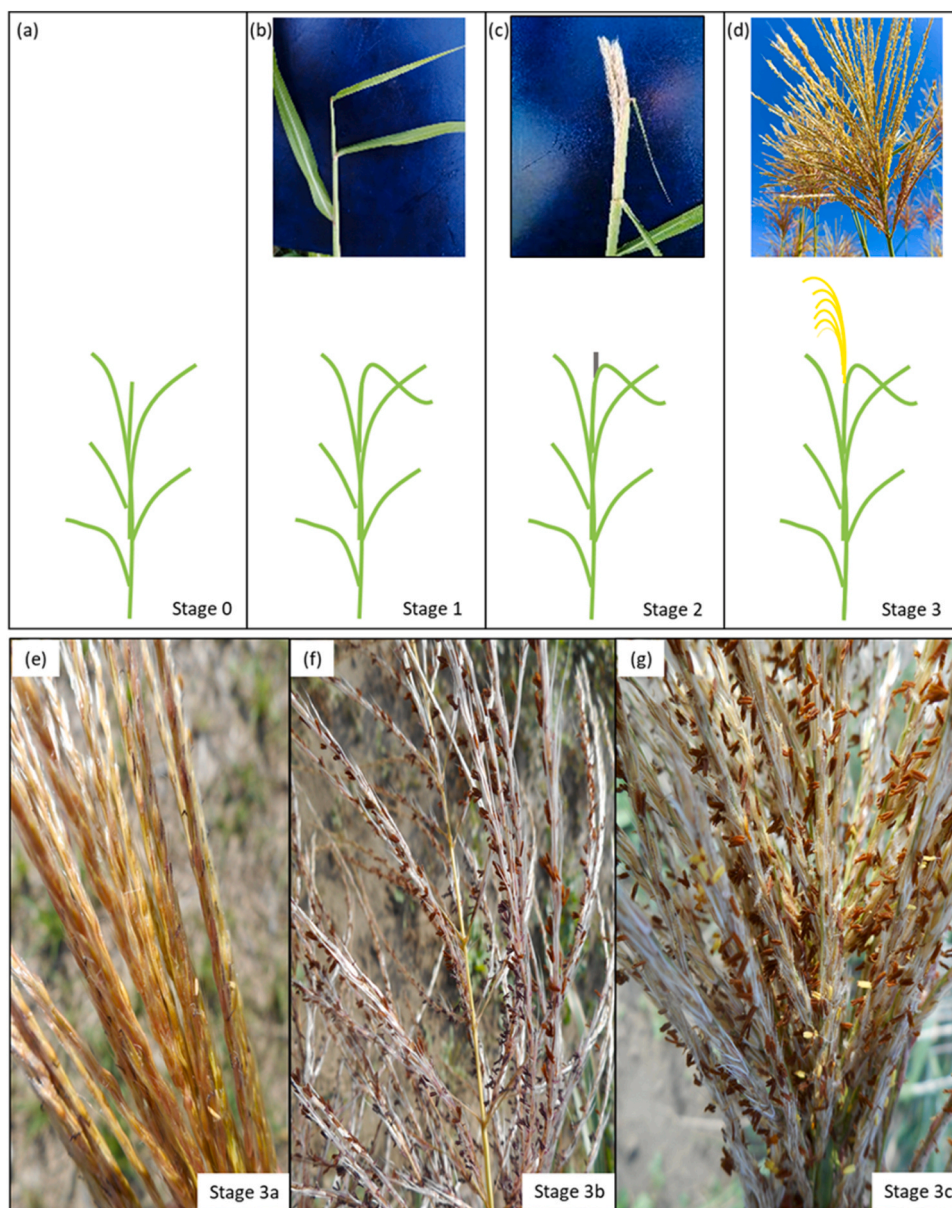

Fig. 1. Miscanthus flowering stages recorded in the field. a) Stage 0: Vegetative stage. b) Stage 1: Flag leaf emergence. c) Stage 2: Panicle emergence stage. d) Stage 3: Full panicle emergence. e) Stage 3a: Stigma visible. f) Stage 3b: Anthers visible. g) Stage 3c: Anthers visible.

evapotranspiration (ETm) according to Cosentino et al. (2007).

The three crossing blocks planted (Trials a, b & c) have numbers that follow the name to describe the experimental year where needed:

2.3) Increasing seed per area of crossing block: **2.3.1)** Increasing the ratio of seed parents to pollen parents (Trial a1 & a2); **2.3.2)** Manipulating seed parent planting densities (Trial b1, b2 & b3).

2.4) Application of agronomic practices (Trial c): **2.4.1)** Increasing relative humidity for pollination and seed set (Trail c2); **2.4.2)** Cutting back seed parent (Trail b3); **2.4.3)** Nitrogen application (Trial c3 & c4; **2.4.4)** Irrigation reduction to induce drought stress (Trial c4). With 2.4.2 – 2.4.4 using a method of flowering delay.

For the purpose of this study, parental lines of a commercially promising *Miscanthus* hybrid, PCH-14, were investigated. All described crossing blocks are crossing a *M. sacchariflorus* genotype (pollen parent) with a *M. sinensis* seed parent to make a commercially relevant hybrid *Miscanthus* names PCH-14. For the first time in *Miscanthus* seed production, we aimed to optimise seed production at field scale. Given the complexity of the experimental designs and number of treatments, we concentrated resources on trials producing this hybrid for four years. There are several promising hybrids of *M. sinensis* x *M. sacchariflorus* (Awty-Carroll et al., 2023). However, parental lines of PCH-14 expressed the greatest flowering time gap compared to the other lines, thus the information learned from PCH-14 could potentially be applicable to the other crosses including those from other breeding programmes globally.

2.3. Increasing seed per area of crossing block

The hypothesis was that higher number of seed plants per area of the crossing block could lead to more seed production from *Miscanthus* hybrids. In this context, two field trials were set up in 2016 with the aim of increasing the number of seed parents.

2.3.1. Increasing ratio of seed parents to pollen parents (Trial a)

Compared four proportional levels of the seed parent (*M. sinensis*) to the pollen parent (*M. sacchariflorus*), at 1.5:1, 2:1, 2.5:1 and 3:1 in two replications. Blocks of pollen parent consisted of two rows across the length of trial plot and the rows of seed parents increased proportionally (Fig. 2). The seed pollen was planted at 1.5 plants m^{-2} while the seed parent was at 4 plants m^{-2}. This trial was conducted for two years (2016–2017). In the second-year parts of the pollen parent (*M. sacchariflorus*) were cut back to equalise height and stimulate tillering.

In the first year, 10 plants were selected at random excluding the edge row and phenotyped to assess the number of panicles and seed traits. In the second year the plot was less traversable, and the first linear meter of the rows was collected as a subsample to allow a calculation of the amount of seed per m^2. This change in methodology was due to the density, particularly as the planting density had resulted in lodging of the *M. sinensis* during the second year, reducing the ease of access to the plot.

2.3.2. Manipulating seed parent planting densities (Trial b)

Here we tested the hypothesis that by growing plants closer together more seed can be produced per m^2 of crossing block. The trial consisted of three densities of the seed parent (*M. sinensis*) planted at 1, 2, and 4 plants m^{-2}, and pollen parent (*M. sacchariflorus*) at 1.5 plants m^{-2}. Each block was 25 m^2 with three randomised replications (Fig. 3). The trial was conducted for three years (2016–2018). During this period, panicles were collected to calculate the panicle numbers, seed characteristics and seed yield m^{-2}. In the third year due to lodging the number of tall plants per plot was reduced. This was done through the testing of a cutting treatment to change the flowering time; this was randomly assigned to half of each block (Fig. 3).

2.4. Application of agronomic practices (Trial c)

A multi-factorial trial was designed to assess the impact of environmental conditions on flowering time of the parental lines, seed yield and quality (Fig. 4). The design of the experiment consisted of up to two treatments per year in three randomised replications. Clonal rhizomes generated from parental lines were planted in rows at a 1:1 ratio and the planting distances were the same for both parental lines at 0.66 plants m^{-2}. The trial ran from 2015 to 2018 with experiments conducted in the second, third, and fourth years. Plant heights were not measured in the fourth year due to the density the crop had achieved. The treatments are detailed below.

2.4.1. Increasing relative humidity for pollination and seed set (Trial c2)

The hypothesis was that a dawn misting regime would lead to improved pollination and thus better seed quantity and quality. In the second and third years (2016 & 2017) the three cross sections of Trial c2 received three misting treatments at 6–7 am; a) Ground level, b) Canopy level (3 m), and c) Control with no misting. Relative humidity data were recorded using TinyTags (Gemini Data Loggers Ltd, Chichester, UK), which subsequently were averaged for each hour of the day from October 17th to November 7th and used for analysis.
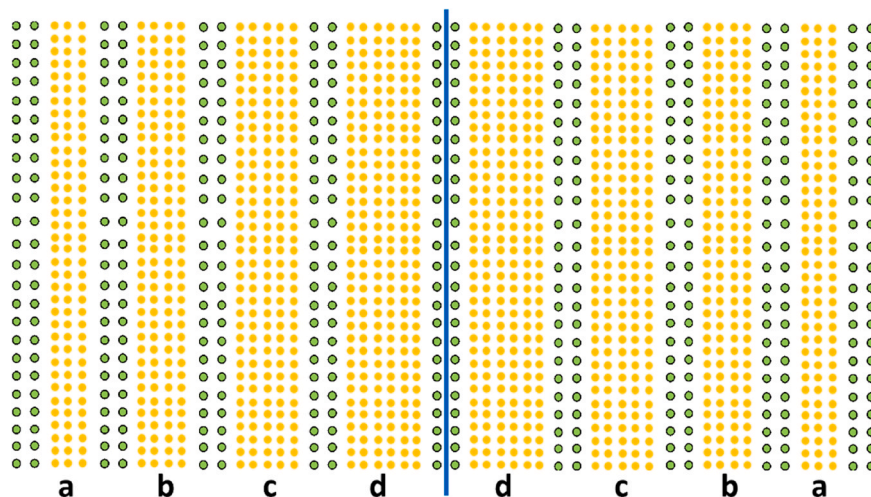
Fig. 2. Layout of Trial a, Green circles represent the pollen parent (M. sacchariflorus) and orange circles the seed parent (M. sinensis) in two-replications with parents in proportional ratios of a) 1.5:1, b) 2:1, c) 2.5:1 and d) 3:1Vertical blue line splits the two-replication.
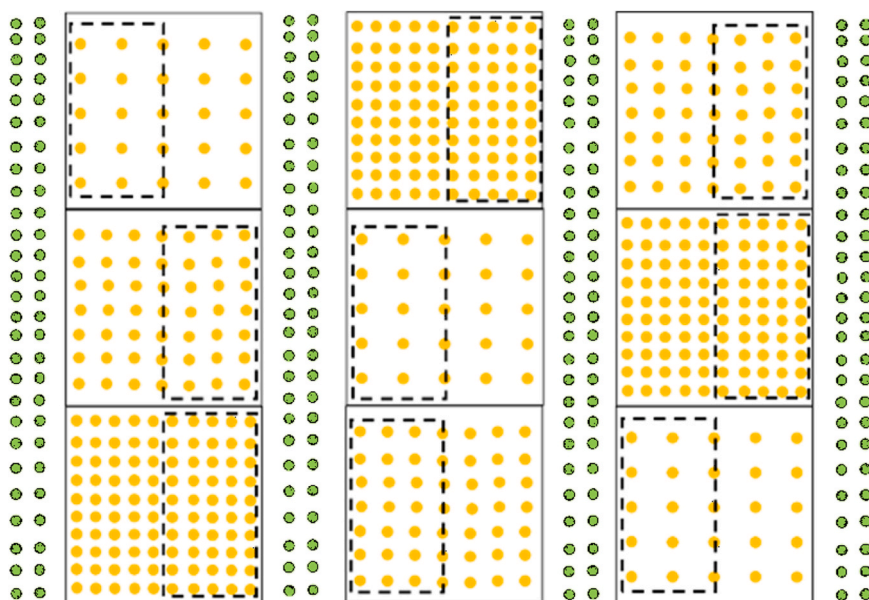
Fig. 3. Layout of Trial b. Pollen parent (M. sacchariflorus) is represented by green circles, and seed parent (M. sinensis) by orange circles. The densities used were 1, 2, and 4 seed parents per m^2, replicated three times into three 25 m^2 blocks. In year 3 there was a seed parent cutting treatment shown by black dashed lines over a random half of each plot within the crossing block.
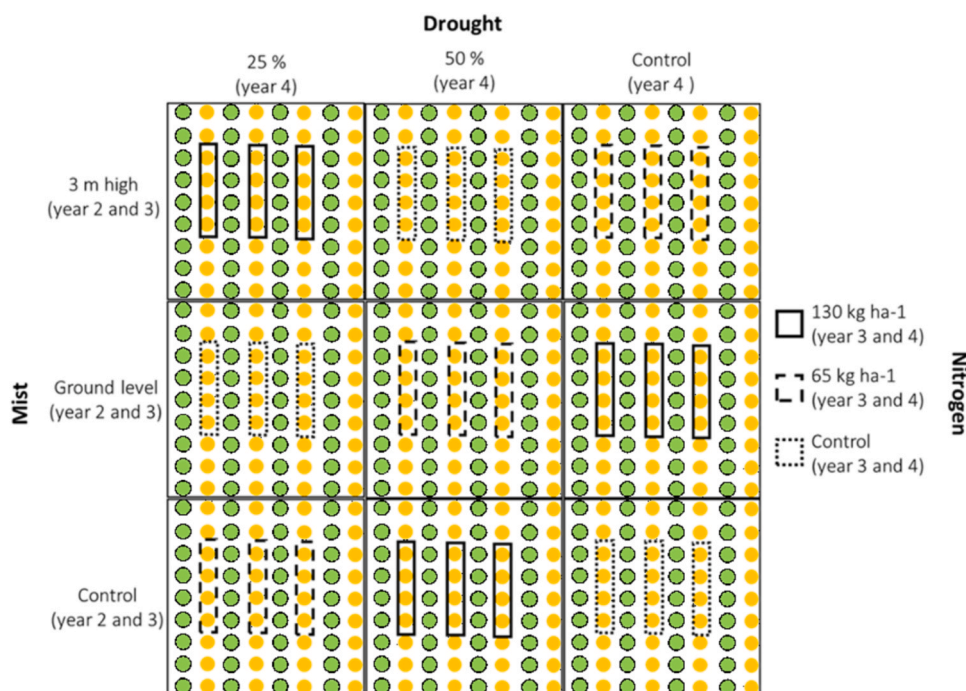

Fig. 4. Layout of Trial c. The misting treatments (Trial c2) were applied in the three horizontal sections in the second and third year: first section no mist (control), second section ground level mist, third section received 3 m high mist. The drought treatment (Trial c3 & c4) was applied in the three vertical sections in the 4th year: left 25 %, middle 50 % and right 100 % (control) of evapotranspiration. The internal boxes show the nitrogen treatments applied in year 3 and 4: solid line for 130 kg ha^{-1}, dashed for 65 kg ha^{-1} and dotted for Control (no nitrogen). This shows the nine sections the trial was divided into and the sub-sampling zones (boxes of 4 plants). Pollen parent (M. sacchariflorus) is represented by green circles, and seed parent (M. sinensis) by orange circles. The plan alternates rows of M. sacchariflorus and M. sinensis at 1.5 m spacing. (n = 3).

2.4.2. Cutting back seed parent

Trial b3 (Fig. 3) was used again for a 3rd year (2018), this time to test cutting to delay flowering the *M. sinensis* seed parent was cut back as a method of delaying flowering to synchronise the parents. The cutting treatment was expected to delay flowering, cutting was much later (mid-July) than the cutting of the *M. sacchariflorus* which can be done to decrease height and increase panicle numbers. This cutting was carried out on one half of each plot 18 days prior to the estimated start of flowering. This time-frame was chosen to pre-empt flowering induction based on Jensen et al. (2013). To give the best chance of success cutting was done more severely than in *M. sacchariflorus* (to a height of 40–50 cm). This was only tested in one final year of the trial due to the long-term effects of vegetative cutting on *Miscanthus* (Kiesel and Lewandowski, 2017).

2.4.3. Nitrogen application

The hypothesis was that nitrogen application has been observed delaying flowering in *Miscanthus* × *giganteus* (Heaton et al., 2009), this would probably be due to the ease of more vegetative growth which is stopped after flowering. The effect of nitrogen on flowering has not been studied in *M. sinensis* or *M. sacchariflorus* individually.

In the third and fourth years (2017 & 2018 of Trial c3 & c4, Fig. 4), nitrogen fertiliser factor was added to delay flowering, in combination with two other factors. In the third year in combination with a misting treatment and in the fourth year in combination with a drought treatment. Three levels of nitrogen were used (0, 65, 130 kg N ha^{-1}) by arranging a nine block Latin square design with the misting and drought treatment (year three and four, respectively). Four subplot sections per block (highlighted in Fig. 4) were used to test the nitrogen treatment. The amount of seed per m^2 was calculated in the fourth year from an 80 panicle sub-sample.

2.4.4. Irrigation reduction to induce drought stress

The response to drought has been extensively studied in maize, and it has been seen that water deficit can delay growth stages affecting both flowering time and seed set characteristics (Wang et al., 2017). In the fourth year (2018) the misting was removed from Trial c (Fig. 4) and the three blocks had a drought treatment applied to delay flowering. This treatment was only applied for a single and final year due to the potential for compound effects of drought treatments on perennial crops. In Trial c4 the drought treatments used were 100 % of evapotranspiration (control), 50 % of evapotranspiration and 25 % of evapotranspiration, replaced through a per plant watering system using drippers with a flow rate of 8 l h^{-1}, 4 l h^{-1}, and 2 l h^{-1}, respectively. The 100 % water requirement was calculated using a Penman-Monteith calculation (Monteith, 1965). A drought was also applied to the neighbouring *M. sacchariflorus* side of crossing block. This was due to the trial design (with alternating rows), the aim was not to delay flowering in *M. sacchariflorus*, but the flowering of *M. sacchariflorus* was monitored.

2.5. Seed characterization

All seed measurements were produced using the MARVIN system (GTA Sensorik GmbH, Neubrandenburg, Germany), which takes a sub-sample of up to 500 seeds to weigh and photograph in a high contrast environment. This was used to determine the number of seeds, average seed length and width and the Thousand Seed Weight (TSW). Germination rate was determined following incubation at 25°C on tissue using the method described in Awty-Carroll et al. (2020) but using lower irradiance constant light (~15–30 μmol m^{-2} s^{-1}). The total germination percentage was estimated at seven days.

2.6. Statistical analysis

Data was analysed using R Statistical Software application (R Core Team, 2022). If data followed a Gaussian distribution the experiment was analysed using an ANOVA. If the data did not follow a Gaussian distribution simple transformations were tested and are stated in the results where used. If it was difficult to transform data to a Gaussian distribution a Kruskal-Wallis test was used and this is stated. Where trials b, and c used Latin-square based designs they were analysed with row and column as factors. Where an ANOVA was used and identified a significant factor a *post hoc* Tukey's HSD was run using the 'agricolae' package (de Mendiburu, 2021). Seed metrics were all tested together with a MANOVA and if significant were broken down into individual tests.

3. Results

3.1. Meteorological conditions

The thermal time and temperature profile was similar across the four years of field trials, with a slight drop in temperature and thermal time in 2017 (Fig. 5). There was high rainfall during flowering (August-October) in 2015 and 2018. Heavy rainfall can affect pollination and thus seed set, as well as disrupting the effectiveness of low irrigation drought treatments.

3.2. Increasing seed per area of crossing block

3.2.1. Increasing ratio of seed parents to pollen parents (Trial a)

In Trial a1 (the first year) 40 plants were monitored for heights and the number of panicles. In this year, there was a statistically significant decrease ($P < 0.01$) in the number of panicles per m^2 with the increasing ratio of *M. sinensis* to *M. sacchariflorus*, but this did not affect the quantity of seed per plant ($P = 0.67$). As the ratio of *M. sinensis* seed parents increased from 1.5:1–3:1, the seed per m^2 increased by 41 % Table 2).

In the second year the quantity of PCH-14 seed produced did not significantly vary between the different ratios and the number of panicles per m^2 was not significantly affected when tested with a Kruskal-Wallis rank sum ($P = 0.32$). The ratio modifications did not affect flowering time or plant height and made no significant difference to the germination or seed size (Table S1).

3.2.2. Manipulating seed parent planting densities (Trial b)

In Trial b densities of 1, 2, and 4 seed parents per m^2, within 25 m^2 blocks were tested. In the first year the amount of seed per m^2 was significantly affected by the density ($P < 0.05$), this increased approximately doubling as the number of plants doubled, showing a near 1:1 increase in seed with planting ratio which would allow for more seed production in the same area. Interestingly, this was not the case for the panicle count which was lower in the first year and was unaffected by the number of plants per m^2 ($P = 0.57$). This provides a benefit to pollination as the panicles are in a dense zone next to the pollen parents. The *M. sinensis* plants were not significantly taller in the first year at the higher densities (Table 3).

In the second year the amount of PCH-14 seed produced and the number of panicles per m^2 were significantly different (both $P < 0.05$). The seed were slightly but significantly less ($P < 0.01$) at higher densities (Table 3).

In the second and third year, there was a significant ($P < 0.01$ & $P < 0.05$ respectively) decrease in the average seed overall size at higher planting densities. However, individual seed metrics (weight, length, width, area) were only significantly smaller at higher densities in the third year (Table 4).

Also, in the third year due to the lodging, the number of tall plants per plot needed to be reduced. This was done using a cutting treatment designed to change the flowering. When the treatments were analysed using two-way ANOVAs the number of panicles per m^2 was significantly higher at higher planting densities (log transformed, $P < 0.05$). Though panicles per plant are the opposite with fewer per plant at high densities (Table 3). The amount of seed produced per m^2 was significantly ($P < 0.01$) increased under increasing density when tested by two-way ANOVA. When the seed per panicle was analysed, there was significantly ($P < 0.01$) more seed in panicles at four plants per m^2 even when accounting for the cutting treatment done in the same year on half the plot (Fig. 3) which prevented the lodging and may have boosted seed numbers. Planting density did not affect flowering time in all three years, in the first two years the day of anthesis was the same (Table 3). Density did not affect seed quality as there was no significant effect on germination in either the second or third years.
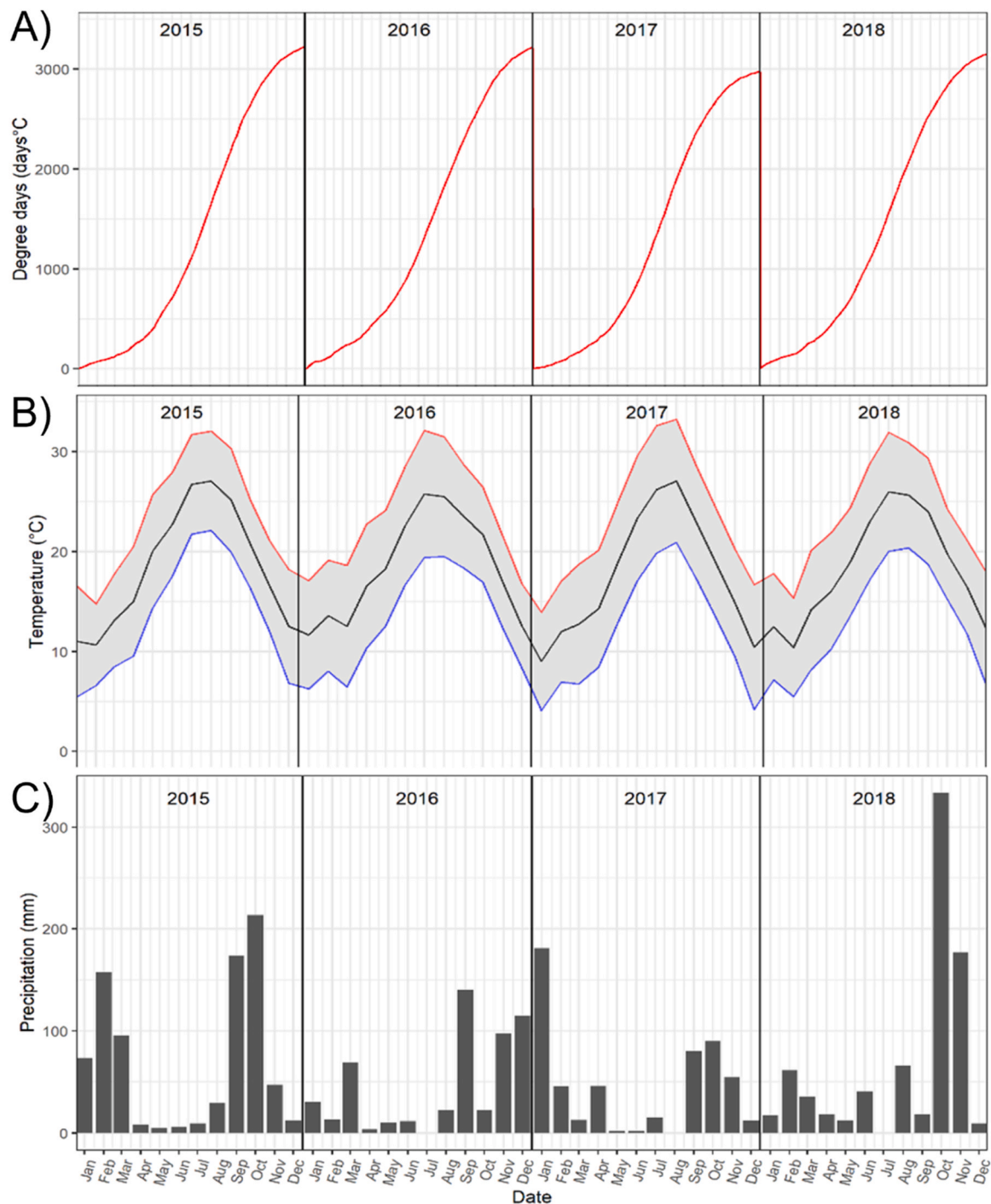
Fig. 5. Summary meteorological data: A) Degree days accumulation from a base temperature of 10°C. B) Daily mean, min and max temperature. C) Monthly precipitation over 4 years.

3.3. Application of agronomic practices (Trial c)

3.3.1. Increasing relative humidity for pollination and seed set

In the second year (with misting) the mist applied increased seed per m^2: Ground mist by 11 % compared to the control and 3 m mist by 18 % ($P < 0.05$) (Table 5). However, in the third year of Trial c, misting did not significantly affect the seed per m^2 ($P = 0.45$) when tested with a two-way ANOVA. The misting did have a significant positive effect on the seed size (Table S2) in the 2nd year tested with a MANOVA of seed size, weight, length, width and area ($P < 0.001$). There was no positive effect from the misting on the germination rate when tested under laboratory conditions.

Flowering time as measured by the first day of anthesis (Table 5) was not significantly affected by the misting ($P = 1.0$ & $P = 0.84$ in the 2nd or 3rd years, respectively). The climate data from the loggers did not show a noticeable effect from the morning misting (Fig. 6), this cast doubt on the effectiveness of misting in field conditions.

3.3.2. Cutting back seed parent

Cutting the *M. sinensis* (seed parent) significantly ($P < 0.001$) delayed anthesis (Fig. 7); however, the cutting did result in a significant (log transformed data, $P < 0.001$) reduction in panicle numbers, dropping

Table 2

Average panicle and seed produced per m^2 within Trial a varying the proportions of seed parent and pollen donor plants as ratios 1.5:1, 2:1, 2.5:1 and 3:1.

Year	Panicles (per m^2)			Seed (g m^{-2})		
& Ratio	Mean	±	SE	Mean	±	SE
1st year						
1.5:1	8	±	0.2*a*	0.44	±	0.12
2:1	6	±	0.6*ab*	0.63	±	0.2
2.5:1	4	±	0.1*b*	0.45	±	0.12
3:1	5	±	0*b*	0.62	±	0.12
2nd year						
1.5:1	40	±	6.8	0.57	±	0.18
2:1	26	±	7.8	0.53	±	0.17
2.5:1	19	±	4.8	0.31	±	0.08
3:1	30	±	14.5	0.63	±	0.17

† Results from 2 years (2016 & 2017). HSD shown when significant ($n = 2$).

from an average of 60–10 panicles m^{-2} (Table 3). This drop in panicle numbers in the treatment blocks also explains a drop in seed m^{-2}, which was significantly affected when tested with a two-way ANOVA for density and cutting treatment ($P < 0.01$ & $P < 0.05$). Seed per panicle was significantly ($P < 0.001$) higher in cut plants, approximately triple; this was particularly prominent in the highest density plants which had lots more seed per panicle when cut. There was also a significant interaction between cutting and density ($P < 0.001$).

There was a significant drop in germination ($P < 0.001$) (57–35 %) in the third year on the parts of Trial b with cutting. Other seed metrics showed significant effects on the seed of the cutting treatment (Table 4). The seed from cut plants were smaller ($P < 0.01$). When individual metrics were tested seed were shorter ($P < 0.01$) and thinner ($P < 0.001$)

with a smaller area ($P < 0.001$) and weighed less ($P < 0.01$).

3.3.3. Nitrogen application

In the third and fourth years in Trial c there was not a significant ($P = 0.54$) effect of nitrogen on the flowering time as measured by the first day of anthesis (Table 5, Fig. 8).

Nitrogen had a significant ($P < 0.05$) reduction on the height of the plants in the third year, from 2.23 ± 0.9 m to 2.12 ± 1.5 m. The height was only reduced at the highest nitrogen level, equivalent to 130 kg ha^{-1}. In the fourth year the plant heights were not recorded due to the impenetrability of the plot after 4 years of growth.

There was no significant effect of nitrogen on the germination of seed produced in the 3rd or 4th year ($P = 0.09$, $P = 0.22$); however, there was a notable increase (16 %) in the chance of un-germinated seeds going mouldy with higher nitrogen.

In the third and fourth year of Trial c there was no significant effect of nitrogen on the number of panicles or seed produced per m^2 (Table 5). Also, in the third-year nitrogen did not significantly ($P = 0.09$) affect the weight, width, length or area of the seeds (Table S2). The only significant effect on the seed from nitrogen in the third year was an interaction with the misting treatment on the seed length, size, and weight (all $P < 0.01$). This appears to show that plants with high nitrogen and misting produced the bigger seeds, while significant this effect was small (Table S2).

3.3.4. Irrigation reduction to induce drought stress

In the fourth year of Trial c plants subject to higher drought intensities showed signs of yellowing (Fig. 9). Drought did produce a significant ($P < 0.05$) flowering delay of 3 days in the 50 % and 25 %

Table 3

The impacts of pollen recipient (M. sinensis) density and cutting on growth, flowering, and seed production traits.

	Anthesis (DoY)				Plant height (m)			Panicles (Per m^2)				Seed (g m^{-2})			
Plants m^{-2}	Mean	±	SE		Mean	±	SE	Mean	±	SE		Mean	±	SE	
1st year															
1	273	±	0		1.88	±	0.04	5	±	0.8		0.28	±	0.05	*b*
2	273	±	0		1.93	±	0.03	4	±	0.3		0.44	±	0.06	*ab*
4	273	±	0		1.96	±	0.01	4	±	0.3		0.91	±	0.02	*a*
2nd year															
1	283	±	0					61	±	5.5	*b*	0.46	±	0.07	*a*
2	283	±	0					83	±	11	*ab*	0.33	±	0.07	*ab*
4	283	±	0					90	±	12.2	*a*	0.30	±	0.08	*b*
3rd year															
1	263	±	0.7	*-b*				41	±	3.2	*a-a*	0.42	±	0.08	*b-a*
1 (cut)	273	±	2.3	*-a*				9	±	4.4	*a-b*	0.22	±	0.12	*b-b*
2	261	±	0	*-b*				63	±	6.2	*ab-a*	0.59	±	0.09	*b-a*
2 (cut)	275	±	0	*-a*				6	±	1.5	*ab-b*	0.14	±	0.03	*b-b*
4	266	±	3.3	*-b*				75	±	15.5	*b-a*	0.85	±	0.34	*a-a*
4 (cut)	273	±	2.3	*-a*				13	±	1.5	*b-b*	0.63	±	0.04	*a-b*

†Trail b years 2016–2018. Plant heights where only taken in the first year due to lodging. HSD shown when significant, first and second factor separated with a dash. (n = 3)

Table 4

Seed analysis of changing pollen recipient (M. sinensis) density and cutting (to delay flowering).

	Weight (mg)				Length (mm)				Width (mm)				Area (mm^2)			
Plants m^{-2}	Mean	±	SE		Mean	±	SE		Mean	±	SE		Mean	±	SE	
2nd year																
1	1.07	±	0.07		2.31	±	0.07		0.96	±	0.02		1.90	±	0.08	
2	0.97	±	0.02		2.26	±	0.03		0.92	±	0.01		1.79	±	0.04	
4	1.03	±	0.01		2.34	±	0.01		0.92	±	0.01		1.87	±	0.03	
3rd year																
1	0.95	±	0.09	*a-a*	2.17	±	0.03	*a-a*	0.87	±	0.03	*-a*	1.63	±	0.07	*a-a*
1 (cut)	0.87	±	0.13	*a-b*	2.13	±	0.07	*a-b*	0.77	±	0.03	*-b*	1.50	±	0.10	*a-b*
2	0.98	±	0.03	*ab-a*	2.20	±	0.00	*ab-a*	0.90	±	0.00	*-a*	1.63	±	0.03	*ab-a*
2 (cut)	0.72	±	0.06	*ab-b*	2.00	±	0.06	*ab-b*	0.77	±	0.03	*-b*	1.40	±	0.06	*ab-b*
4	0.86	±	0.09	*b-a*	2.10	±	0.00	*b-a*	0.85	±	0.05	*-a*	1.50	±	0.10	*b-a*
4 (cut)	0.72	±	0.02	*b-b*	2.03	±	0.03	*b-b*	0.77	±	0.03	*-b*	1.33	±	0.03	*b-b*

†Trail b years 2017 and 2018. LSD shown when significant, first and second factor separated with a dash. (n = 3)

Table 5

The impacts of morning misting for pollination, water reduction to induce drought and nitrogen application, flowering, and seed production traits of M. sinensis.

Year		Anthesis (DoY)				Panicles (Per m²)			Seed (g m⁻²)		
Main treatment **2ⁿᵈ year**	**Sub treatment**	**Mean**	**±**	**SE**		**Mean**	**±**	**SE**	**Mean**	**±**	**SE**
Misting											
Control		273	±	0		26	±	2.5	1.08	±	0.12
Ground mist		273	±	0		27	±	2.9	1.20	±	0.2
3 m mist		273	±	0		29	±	2.6	1.32	±	0.08
3ʳᵈ year											
Misting	**Nitrogen**										
Control	Control	283	±	0.4		44	±	5.5	0.69	±	0.2
Control	Low	283	±	0		25	±	2.4	0.66	±	0.07
Control	High	283	±	0.4		32	±	3	0.45	±	0.03
Ground mist	Control	283	±	0.4		27	±	2.1	0.47	±	0.04
Ground mist	Low	282	±	0.9		29	±	3.4	0.37	±	0.02
Ground mist	High	286	±	0.9		39	±	6.5	0.75	±	0.26
3 m mist	Control	283	±	0		26	±	0.4	0.51	±	0.06
3 m mist	Low	284	±	0.6		40	±	1.9	0.63	±	0.15
3 m mist	High	283	±	0		22	±	3.3	0.50	±	0.05
4ᵗʰ year											
Drought	**Nitrogen**										
Control	Control	273	±	1.1	b-	46	±	5.3	0.72	±	0.25
Control	Low	274	±	1	b-	22	±	5.1	0.33	±	0.16
Control	High	271	±	1.7	b-	41	±	16.7	0.79	±	0.42
50 %	Control	275	±	0.6	a-	33	±	4.4	0.41	±	0.09
50 %	Low	275	±	0	a-	24	±	4.5	0.31	±	0.05
50 %	High	275	±	0	a-	32	±	2.7	0.61	±	0.04
25 %	Control	275	±	0	a-	21	±	1.6	0.27	±	0.04
25 %	Low	274	±	0.5	a-	25	±	1.5	0.35	±	0.05
25 %	High	275	±	0.8	a-	28	±	6.7	0.47	±	0.16

†Results from Trial c for years 2016, 2017, & 2018. LSD shown when significant, first and second factor separated with a dash. (n = 3)
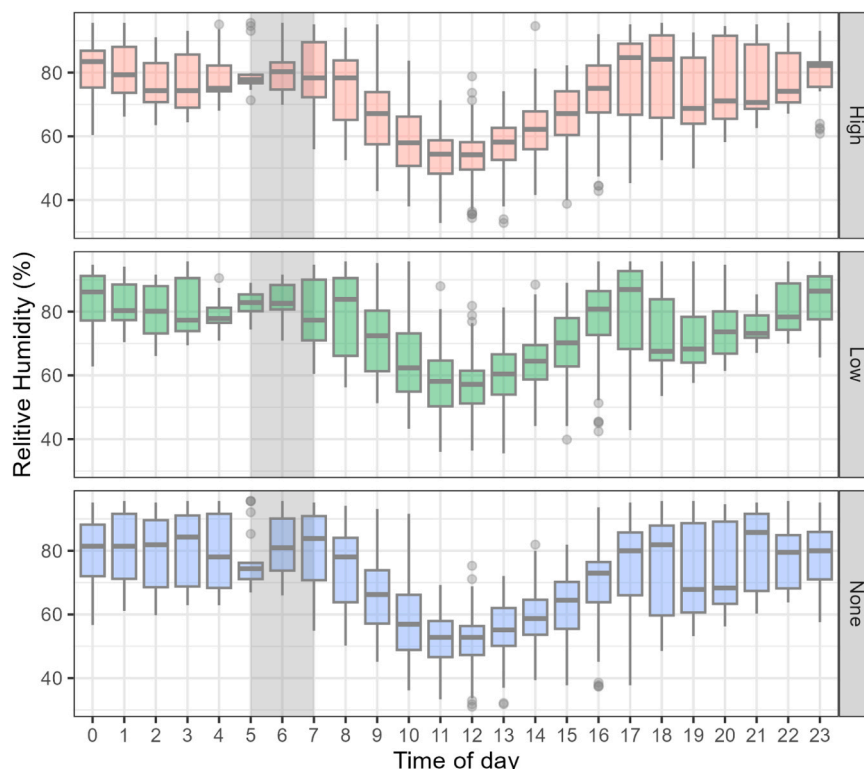

Fig. 6. The relative humidity throughout the day at canopy height in central areas of three misting treatments during the 21 days preceding panicle harvest in Trial c (2017). Treatments high (3 m) in red, low (ground level) in green, and no misting in blue. A grey band has been added to show the misting time.

watering treatments. There was no significant effect on germination of either *M. sinensis* or *M. sacchariflorus* seed which remained around 41 % when tested in seven-day germination tests. There was also no significant effect on seed size or weight from drought alone (Table 6). There was an interaction with nitrogen that suggested the plants may produce

bigger seed in difficult conditions.

Drought treatments in the 4th year of Trial c did not significantly affect panicles or seed amounts produced by *M. sinensis* plants (Table 5). There was no significant effect of drought on panicle production in *M. sacchariflorus* plants which produced 4±1.5, 5±0.8, 3±0.7 panicles
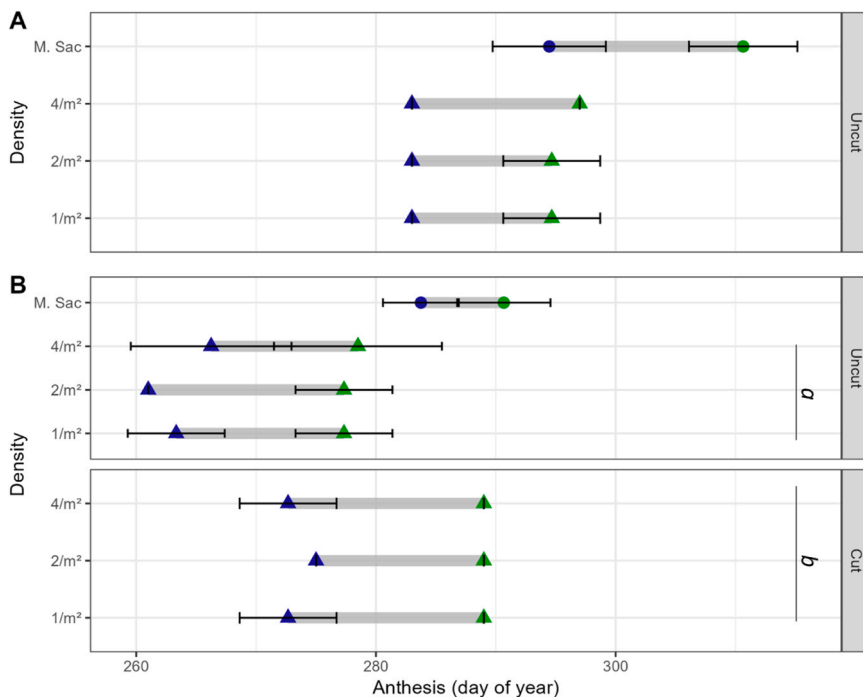
Fig. 7. Mean start day (blue) and end day (green) for anthesis with standard deviation, in Trial b. All densities represent the *M. sinensis* seed parent with the *M. sacchariflorus* pollen parent added above for reference A) is the second year with all plants uncut and some lodging, and B) is the third year with cutting added to delay flowering and reduce lodging. Significant differences in the seed parents flowering time are shown with Tukey's HSD letters.
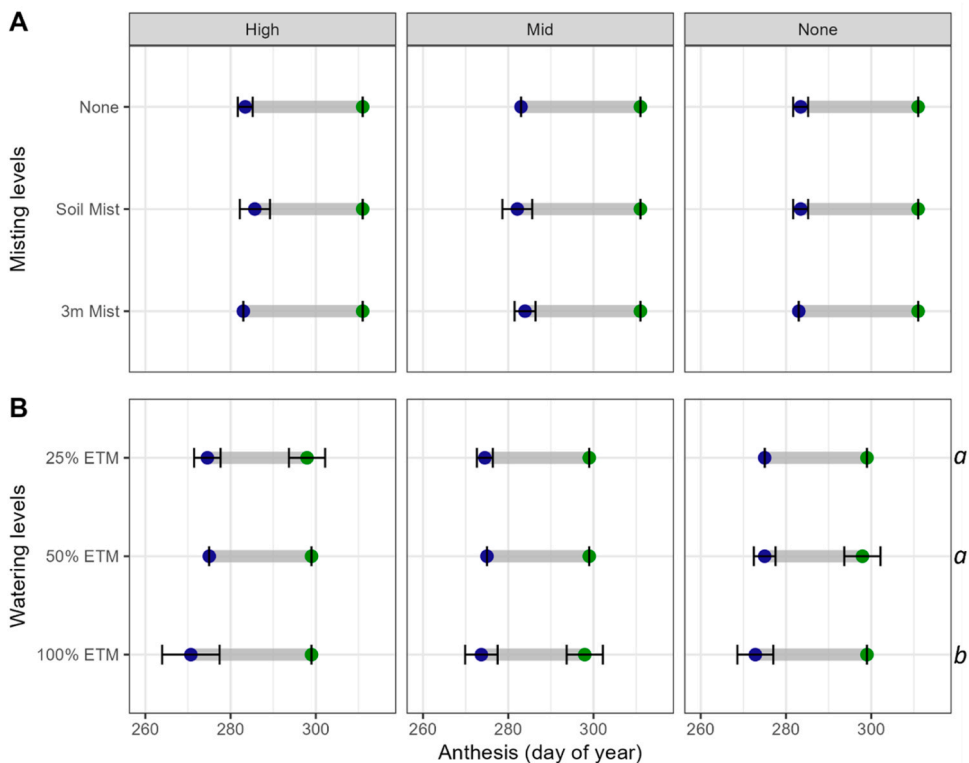

Fig. 8. Mean start day (blue) and end day (green) for anthesis with standard deviation, in Trial c. All data represents the *M. sinensis* seed parent A) is the third year with nitrogen (high, mid and none) and misting, and B) is the fourth year with nitrogen (high, mid and none) and watering reduction. Significant differences in the seed parents flowering time are shown with LSD letters.

m^{-2} at 25 %, 50 % and 100 % evapotranspiration restoration, respectively (Table 6). While the seed and panicles m^{-2} were broadly the same, there was a notable decrease in the amount of PCH-14 seed per panicle at 25 % evapotranspiration replacement, however, this effect was not significant (P = 0.07).

Fig. 9. Plants of the drought treatment in the pre-flowering stage. Treatments are applied from left to right in the following order: 25 %, 50 % and 100 % of evapotranspiration restoration.

Table 6
The effect on the pollen parent (M. sacchariflorus) of reduced watering to induce drought as a percentage of evapotranspiration restoration, on panicle numbers, seed quantity, and seed size.

	Panicles (per m^2)			Seed (mg m^{-2})			Seed (mg panicle^{-1})		
Control	3	±	0.7	43	±	11	13	±	1.53
50 % water	5	±	0.8	58	±	12	13	±	2.87
25 % water	4	±	1.5	28	±	21	5	±	2.19
	Seed weight (mg)			Seed length (mm)			Seed Area (mm^2)		
Control	0.33	±	0.01	2.12	±	0.03	1.32	±	0.03
50 % water	0.33	±	0.01	2.10	±	0.00	1.35	±	0.03
25 % water	0.34	±	0.02	2.17	±	0.03	1.40	±	0.00

†Results from Trial c4 (2018). Means given ± SE with $n = 4$.

4. Discussion

4.1. Improving seed yield

Miscanthus originates from Asia in climates with higher humidity than found in Sicily. China, Taiwan and Japan all have relative humidity values approximating 80 % for the month of July, whereas Catania in Sicily has an average July relative humidity of 49 %. Pollen production, viability, stigma receptivity and pollen germination have all been shown to be negatively affected by low levels of relative humidity (Pacini and Dolferus, 2019). To increase relative humidity at the site of pollen production and to attempt to increase pollen transfer and germination, a dawn misting regime was incorporated to improve pollen transmission between plants. High humidity has been shown to improve pollination and seed set in *Sorghum* (Gitz et al., 2015) and therefore has been speculated to similarly increase seed set in *Miscanthus* (Clifton-Brown et al., 2018). However, in Trial c2 it did not increase the amount of seed produced. Misting it did improve the size possibly due to faster pollination though this did not follow through to germination. This may have been due to the lack of a large increase in humidity (there was little to no effect measured by humidity sensors) or the droplets from the misting being too large to simulate a real morning mist.

Planting the plants closer together (Trial b) which has been used to increase grain yields (Duvick, 2005) or increasing the ratio of the seed parents to the pollen parents to allow a higher percentage of the space for seed production (Wang et al., 2020). Trial a increased seed production per m^2 at least for the first year. This may not be a long-term method for increasing the amount of seed because the *M. sinensis* genotype easily lodged after the first year at higher planting densities. This may increase stress and resource competition among the plants, but it was hypothesised that the increased density would overcome this and produce a net increase in panicle numbers. There was a less than 1:1 increase in the amount of seed per m^2 in trials a and b with the extra seed parents per m^2. High densities could be used with caution, to increase seed yields per ha, the *M. sinensis* is not ideally suitable for this as its less woody stems are prone to lodging. Lodging likely reduced the seed increase at higher densities, and when lodged the panicles can be under the foliage preventing pollination and lowering seed quantities. Commercially lodged plants would also be difficult to harvest without cutting the plants. Densities would need to consider the harvest machinery requirements, as harvesting without cutting the crop is best for sustainable yields due to green cutting sensitivity (Kiesel and Lewandowski, 2017). In addition to this *Miscanthus* seed is harvested in

autumn before senescence is completed, so the nutrient mobilization could be interrupted.

The parental ratio (Trial a) experiment produced approximately the same or more seed when increasing the number of *M. sinensis* rows per pair of *M. sacchariflorus* rows. This trial establishes the ability to be able to put multiple rows of seed parents between rows of pollen parents, without a drop in pollination due to the distance and barrier of the other plants. There was some issue with lodging being worse with more rows of *M. sinensis* seed parent, which makes this trial design not practical for commercial upscaling because of the interior of the plantation being largely inaccessible due to the high planting density. Nonetheless, with changes to plant spacing and paths it is able to provide a method for maximising seed production over land area. This ratio design was only tested on *M. sinensis* so the taller *M. sacchariflorus* may be able to have fewer rows between pollen parents as it would act as more of a barrier.

On average in Trial c PCH-14 produced 0.37 g m^{-2} of seed in 2017, but as an early leading *Miscanthus* seed-based hybrid with poor flowering synchronisation, PCH-14 was used to test seed set and flowering synchronisation improvements that could be used when upscaling *Miscanthus* seed production. Ideally, the amount of seed produced should be more than 1 g m^{-2} of seed in order to provide enough seed for plug plants (Ashman et al., 2023) for the upscaling rates that make seed useful (Hastings et al., 2017). In 2018 PCH-14 control plants produced 0.26 g m^{-2} of seed, a large improvement on some of the 2018 experimental data [from Trial c] but still lower than the 2017 average and 4× less than the target 1 g m^{-2} of seed (Table S2).

4.2. Improving flowering synchronisation

Cutting *Miscanthus* has been shown to delay flowering by Dong et al. (2021). In Trial b cutting was timed to maximise the delay in flowering and cutting the *M. sinensis* gave a 10-day delay in all flowering stages. This was the most successful method of flowering synchronisation improvement. Cutting *M. sinensis* in any commercial use-case would need to be improved to mitigate the negative effects on the number of panicles and seed produced. For crosses, unlike this one, where the seed of interest is harvested from the *M. sacchariflorus* side, delaying the *M. sinensis* by cutting may be an excellent option. As on the pollen parent side even if cutting caused a plunge in the number of panicles should still leave sufficient pollen produced to fertilise many seed. Cutting should work less effectively on *M. sacchariflorus* as there are a more limited number of stems per plant and far fewer small shoots around the base of the plant that grow after cutting. Applications of this method should also

be designed cognisant of the known weakening of *Miscanthus* when repeatedly green-cut in summer (Kiesel and Lewandowski, 2017), the number of panicles may continue to fall year on year as the plants are cut. The cutting was only monitored for one year which is a limitation, however due to the compound effects of cutting over time it would be difficult to test without a custom trial design.

The addition of nitrogen fertilizer had no detectable effect on the flowering time of the plants. While the effect of nitrogen on flowering was noted by Heaton et al. (2009) and Kim, D-S (pers. comm.), this was only in *Miscanthus × giganteus*, while *M. sinensis* and *M. sacchariflorus* have not previously been studied. While the plants with high nitrogen were a bit shorter suggesting the plant may have stopped growing sooner, maybe ready for flowering, this difference was small. Nitrogen may have had an inadequate spatial application accuracy, as the per-plant application, when watered will mobilise the nitrogen spreading it to more of a field application.

Cosentino et al. (2007) reported a delay in flowering from drought in *Miscanthus × giganteus*, which is the opposite of many annuals that initiate emergency flowering during the onset of severe drought as a survival strategy. Drought delayed flowering in *Miscanthus* was also confirmed in the field in Braunschweig, Germany (Kai Schwarz, pers. comm) and in pot trials in Aberystwyth, UK (Louise Radley pers. comm.). The start of flowering to the onset of pollination was often shorter, while drought resulted in a net delay in most genotypes studied. Similar delays have also been observed in *Sorghum*, where the drought in the pre-flowering stage delayed flowering initiation (Craufurd et al., 1993); which is not that surprising since *Miscanthus* is closely related as a chromosome doubled *Sorghum* (Mitros et al., 2020). The small, but significant effect from the irrigation treatment in *M. sinensis* was not seen in *M. sacchariflorus*. This strongly rhizomatous (and deeper rooting) type may have required a more intense drought to produce the desired change in flowering time and synchronisation. Drought intensity was particularly difficult to control due to above average autumn rainfall in 2018 in Catania. Further, if drought stress was used to improve flowering synchronisation it would need to be used on the pollen parent to prevent a reduction in panicle number and seed set per m^{-2}. Pollen does not lose its viability with drought as limiting water did not significantly affect seed amounts produced by *M. sinensis* while not delaying flowering. In practice, water supply treatments are difficult to apply in field conditions where both parents are in close proximity and the rainfall patterns are unpredictable.

4.3. Conclusions

This is the first study attempting to optimise *Miscanthus* seed production in crossing blocks not single plants. Gains in seed production can be through the use of parental ratios and densities particularly in the first year but lodging as a setback must be considered. Therefore, parental ratios are the easiest thing to change while leaving the plants with room and harvest paths. Improvement of flowering synchronisation will also greatly improve seed-set and while more difficult to manipulate than expected cutting would be worth further investigating to optimise the cutting time and severity. Treatments such as misting may be better left to solve by choosing the crossing location with a natural environment that produces a high seed set.

The difficulty in improving flowering synchronisation also shows that if a location has consistent synchronised flowering, small changes to watering, fertiliser, or stresses are unlikely to desynchronise the flowering. Which gives commercial crossing blocks a lot of potential for long-term seed production. As well there are strategies like partial cutting of rows (i.e., in a crenelated manner) that could reduce lodging or provide cutting without depletion of nutrients. Cutting a few pollen parents at different times could hedge against flowering desynchronisation, with unusual weather.

4.4. Recommendations

- Plant multiple rows of seed bearing parents per row of pollen parent, up to 6 until further study of pollen distance.
- Not to plant *M. sinensis* for seed production at more than 1 plant per m^2 to save lodging, and consequent loss of the flowering shoots and airborne pollen exchange.
- To use a harsh cut back (30–50 cm) around 20 days prior to flowering (flag leaf) to make small < 2-week adjustments in flowering time of the pollen parent.
- Cutting could be done in a crenelated manner and alternated each year to avoid the nutrient and carbohydrate cycling problems associated with green cutting Miscanthus.
- To further optimise irrigation and nitrogen applications to improve seed production amounts and quality.

CRediT authorship contribution statement

John Clifton-Brown: Writing – review & editing, Conceptualization, Funding acquisition, Project administration. **Salvatore Cosentino:** Resources. **Danny Awty-Carroll:** Writing – original draft, Supervision, Investigation, Formal analysis, Data curation. **Reza Shafiei:** Supervision, Project administration, Methodology, Conceptualization, Writing – review & editing. **Danilo Scordia:** Writing – review & editing, Supervision. **Antonella Iurato:** Writing – review & editing, Investigation, Data curation. **Giovanni Scalici:** Investigation. **kai-uwe Schwarz:** Conceptualization. **Michal Mos:** Methodology, Conceptualization. **Paul Robson:** Supervision. **Richard Webster:** Writing – review & editing, Investigation, Methodology.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: John Clifton-Brown reports financial support was provided by UK Research and Innovation. Michal Mos reports a relationship with Terravesta that includes: consulting or advisory. A co-author (Michal Mos) now runs a Miscanthus seed company and was working for Terravesta during the experiment. The corresponding author (Antonella Iurato) worked for Terravesta for a short time after the experimentation in 2019–2020 The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2024.119116.

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