Optimizing development for improved yield and yield quality in the perennial bioenergy crop *Miscanthus*

A thesis submitted in candidature for the Doctor of Philosophy degree of Aberystwyth University

by

Rebecca Wilson

2019

Institute of Biological, Environmental and Rural Sciences

In collaboration with the BBSRC and Terravesta ltd

2019

DECLARATION

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed…… (Candidate)

Date……..

This thesis is the result of my own investigations, except where otherwise stated. Other sources are acknowledged by giving explicit references. A bibliography is appended.

Signed…… (Candidate)

Date……..

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan and for the title and summary to be made available to outside organisations.

Signed…… (Candidate)

Date……..

Word Count of 67,433

Summary

Miscanthus genotypes exhibit key characteristics that make it an excellent sustainable source of biomass. *Miscanthus* is perennial, requiring few inputs for growth and thus has a highly favourable energy ratio and produces biomass yield for up to 20 years. *Miscanthus* is typically propagated clonally via rhizome which is expensive, and it is difficult to scale up existing and new varieties to plant large areas rapidly. Propagation by glasshouse raised plug plants from seed is a new alternative but requires optimisation from growth in plugs to field establishment and senescence. This study focussed on the optimisation of the first year of a seeded *Miscanthus* stand, from germination and seedling phase to harvest the following spring. Germinating seedlings under mulch film in the glasshouse improved seedling vigour and germination rate, leading to larger plants at field planting, but the larger plants did not reliably yield higher biomass production at harvest, leading to further experimentation for the optimum seedling morphology and growth conditions.

Combinations of plug design and planting date were tested. Establishment success in field was more likely in warmer, wetter conditions earlier in the season, as opposed to dry, summer conditions which increased the risk of plug desiccation, especially under mulch film. Planting environment and post planting care had a significant effect on overall yield, whereas initial plug morphology did not. Increasing glasshouse module sizes from 35cm^3 of soil to 45cm^3 improved plug plant development and planting time flexibility. Experimentation with different seedling morphologies concluded that larger plants with strong root biomass were more likely to survive planting into the field, but correlations with yield were less significant, suggesting that increased establishment percentage should be the first priority when growing seeded *Miscanthus*. The study concluded that individual plant growth over the season is extremely difficult and complex to predict or influence due to a multitude of interacting environmental and physiological factors.

Finally, plant growth regulators were investigated to encourage senescence in a hybrid that had previously failed to successfully overwinter in the UK. Applications of exogenous ethephon at 480g/L stock solution greatly altered leaf colouration in treated plants, suggesting this approach has potential for commercial application; however, the consequences of the induced senescence need further investigation.

1 Table of Contents

Table of Figures

Table of Tables

Acknowledgments

First and foremost, I would like to thank my supervisor Dr Paul Robson for giving me this opportunity in the first place. The time spent developing ideas, going through the execution of experiments and the endless proof reading and helpful chats these last weeks have been invaluable. To my second supervisor Prof John Clifton-Brown, thank you for never being short on ideas, enthusiasm and drive that keep the *Miscanthus* team going. It could not be done without you. Huge thanks also to the BBSRC, Terravesta and Dr Michal Mos for making this project possible. My greatest gratitude to the world class planting team Chris Ashman and Robin Warren. Thank you both for your unrelenting efforts in the field in all weathers, and for putting up with my constant questions and favours. Other special mentions go to Chris Glover, Jason Kam and Danny Awty-Carroll for always being on hand to help when needed, even in one of my last-minute rushes and providing ridiculously clever methods help that I could never have executed (or even thought of). Additional and huge thanks to Laurence Jones for calm and unwavering support to the entire team, for patient instruction and always being a kindly face around the coffee table. I wish him a fantastic retirement, safe in the knowledge that many students including me, are now in a much better position as a result of his help and skills both interpersonally and professionally. Many thanks to Sarah Hawkins for saving me in the lab, particularly at the end, when endophyte infected seedlings just wouldn't co-operate, and thanks to Sue Youell for taking me under her wing at the start. To my office mates Louise Radley, Amanda Holder, Laura Cammarisano and Rosario Iacono I thank you all so much for the help where needed, the chats, laughing and sometimes cursing in the office in-between bouts of hard silent work!

Huge special mention must go to the long-suffering Ben Jones for always being there for me no matter how grumpy I got, and endless help with data, harvesting and counting things. I genuinely could not have done it without you. To my mam and my friends for putting up with me these last months and helping with boring grunt work like root washing…I promise I'll have time to do other things with you now!

Finally, a huge thanks to coffee and the Lord of the Rings soundtrack, for both revving me up and calming me down as needed during times of stress.

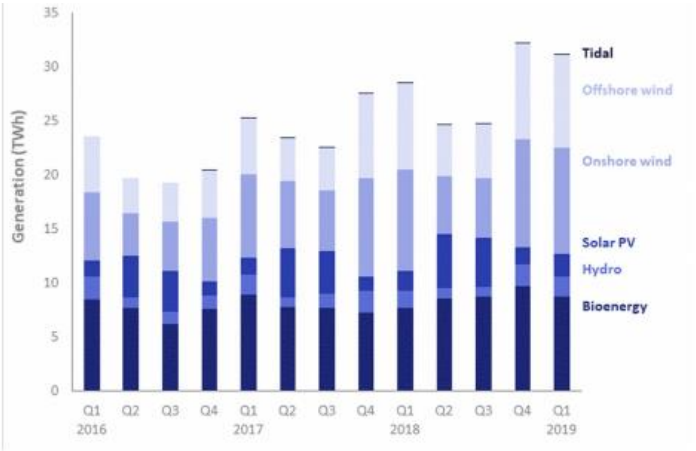
1. Introduction

1.1 Global bioenergy requirements

The alarming rate of climate change and the magnitude of its effects are becoming increasingly apparent and worrying. As stated in a recent publication by Bradshaw and Brook (2014) '*The planet's large, growing and overconsuming human population, especially the increasing affluent component is rapidly eroding many of the Earth's natural ecosystems*'. The current atmospheric concentration of carbon dioxide is expected to increase by almost 50% over the first half of this century (Heaton *et al.*, 2004). In addition to this, the IPCC predicts that cumulative anthropogenic greenhouse gas emissions (GHG) have led to climate warming and extremes of temperature and precipitation levels (IPCC, 2018). It is possible that without additional mitigation methods and intervention, the increase in climate temperature will likely exceed 4 °C by 2100. The resulting climate changes from these extremes will lead to extensive species extinctions, global food insecurity, and constraints on human activities (McCalmont *et al.*, 2015). A major cause of these climate concerns is the continuing combustion of fossil fuels (coal, petroleum and natural gas) which can be attributed to approximately 61.4% of world GHG emissions (Herzog, 2009). Global economic and population growth continue to be the most important drivers of increases in CO_2 emissions from fossil fuel combustion (Pachauri *et al.*, 2014). With the global population expected to reach 10.9 billion by 2100 (Jones and Warner, 2016) we need to provide sufficient food, fuel, shelter and resources to accommodate the increasing lifestyle expectations, whilst simultaneously mitigating climate damage as a result (Valentine *et al.*, 2012). The answer to producing these, with as little cost to the environment as possible, lies partly in the area of sustainable biomass production. The Energy Technologies Institute (Colchin, 2015) suggest that the biomass supplied in combination with carbon capture and storage is the only credible route to achieving negative emissions, in conjunction with other renewable technologies which will be necessary to meet the UKs 2050 GHG emission targets. Biomass production provides a storable and flexible use of fuel which can be readily converted to heat, electricity or liquid transport fuel, and has the potential to remove atmospheric carbon by capture and storage (Colchin, 2015).

Policy makers within the sectors of climate change, are now looking to the agricultural and forestry sectors in order to make best use of underutilized marginal lands on which to grow

biomass and sequester carbon (Clifton-Brown *et al.*, 2019). Renewable energy use in the UK increased by 30% from 2012-2013, supplying 14.9% of UK electricity, of which plant biomass supplied 21.6% (McCalmont *et al.*, 2017). More recent figures suggest bioenergy produced 8.76TWh electricity generation in 2019, a 13.3% increase on the previous year (Figure 1.1). Other forms of renewable energy such as wind, tidal and solar also contribute, but can be less reliable and often require energy storage or back up. Bioenergy has the potential to deliver many forms of energy, leading to more sustainable equivalents of fossil fuels. Energy from biomass growth also has the advantage of having the potential to decarbonize industries which would be difficult to maintain using the other forms of renewable energy, such as transport fuels and manufacturing (Reid *et al.*, 2020). Using *Miscanthus* on lands that are more marginal has the added benefits of potential phyto-remediation in contaminated soils (Krzyżak *et al.*, 2017), soil stabilisation and flood mitigation (Kam *et al.*, 2020), as well as the potential for a new rural business (Clifton-Brown *et al.*, 2019).



	2019 Q1 *TWh*	Percentage change on a year earlier
Renewable electricity generation		
Onshore wind	9.84	+4.8
Offshore wind	8.56	+7.3
Hydro	1.81	+14.6
Solar PV	2.12	+18.7
Bioenergy	8.76	+13.3
All renewables	31.09	+9.2

Figure 1.1 Breakdown of renewable energy generation in the first quarter of 2019. Bioenergy generation has increased by 13.3% from the previous year, largely due to higher uptake from energy plants. Source: Press notice from department of Business, Energy & Industrial Strategy, 2019 (BEIS, 2019)

1.2 The innovation of the biomass fuel crop

Heaton *et al.* (2004) describe the ideal fuel crop as having "*sustained capacity to capture and convert the available solar energy into harvestable biomass with maximal efficiency and with minimal inputs and environmental impacts".* Therefore, it would be vital for crops grown for this purpose to have favourable energy balance; low energy input and high output. They should also have maximum efficiency of light, water and nutrient use, minimal need for cultivation, land use change and pest control, and ideally be a non-invasive species to avoid spread to natural ecosystems nearby (Heaton *et al.*, 2004). There is also a necessity for production of next generation biomass crops to not compromise food security by competing for arable land availability, as well as perform well under a wide range of climatic conditions. One of the largest areas of criticism for the growth of energy crops is their risk of taking over vital arable land and displacing food crops, causing an increased pressure on an already stretched system (Jones *et al.*, 2015).

The genus *Miscanthus* contains species which match the criteria explained above and are valued for their high biomass potential (Clifton-Brown *et al.*, 2008). These perennial crops can grow well with little maintenance on lesser-valued arable land; avoiding competition with food crops, and have high temperature tolerance; growing well in a wide range of climates and soils. It has also been suggested that *Miscanthus* can convert solar energy into biomass energy with up to 30% more efficiency than other crops as a result of its C4 photosynthetic pathway (Zhu *et al.*, 2008). C4 photosynthetic pathway, is more energy efficient than the typically occurring C3 pathway, seen in approximately 85% of known plants, as it produces a higher concentration of carbon, making C4 organisms more adept at managing in low light or low water environments (Campbell *et al.*, 2012). *Miscanthus* plants can remain in the soil producing viable yields each year for up 15 years (Atkinson, 2009). The current leading genotype for use as a biofuel, due to the large yield it can produce is *Miscanthus x giganteus* (*Mxg*). This genotype is a triploid sterile hybrid of *Miscanthus sinensis* and *Miscanthus sacchariflorus.* Trials undertaken using *Mxg* have shown potential for increasing the yield and quality of the species using selection of genotypes. *Miscanthus* breeders at Aberystwyth University are now using this information to guide crosses in a breeding programme to generate high yielding and high quality new genotypes (Clifton-Brown *et al.*, 2008). At present *Miscanthus* is still a relatively new crop in the field of agronomy in comparison to major grain

species, and as such still has a large scope available for domestication and improvement. Propagation costs and patchy establishment combine to make *Miscanthus* an expensive choice for some farmers (Xue *et al.*, 2015). An economic review conducted in 2016 surrounding the area of *Miscanthus* production, highlighted the major barrier to *Miscanthus* cultivation as being the uncertainty that farmers face when considering any profitability involved with investing in this new crop (Witzel and Finger, 2016). Additional issues include the large variability in yield seen across *Miscanthus* plantations. This unpredictable discrepancy between the expected or potential yield and the actual annual yield, which can vary as a result of weather events, plant survival, and plant growth, further decreases the likelihood of uptake of *Miscanthus* within the farming community. Remedying this requires high density planting using homogenous, uniform crops of the best type of germplasm available (Atkinson, 2009). It is also vitally important that farmers can access support throughout the process from deciding if *Miscanthus* could be a suitable choice for their land, to the logistics of planting and growing, and through to the infrastructure required to sell the final product. Companies such as Terravesta are invaluable for this area, as their goal is to get farmers on board with the crop, and to provide knowledgeable on farm support, machinery and skills, before ultimately purchasing the harvested product, posing as a mediator between farmer and end user. Power stations such as Brigg renewable energy plant in Lincolnshire are committed to the cause, and it is hoped more green energy plants will be coming on board in the near future.

Of course, increases in the demand for the product requires increasing land being turned over to *Miscanthus*. Establishment of such scale will require the technology and germplasm to mass-produce large numbers of the plants. Therefore a key area for improvement is minimization of costs of the initial plant material, whilst ensuring a potential to establish a high number of plants at the optimum time (Atkinson, 2009). In order for *Miscanthus* to be considered a viable business option for farmers and growers, it is necessary to come up with more innovations to improve vital agronomic traits such as establishment time and costs. Being able to produce a wider genetic base of varieties which can withstand the varying abiotic and biotic stresses which plants may encounter in the field would also be beneficial. Due to the longevity of the life of the crop stand (up to 15 years), the land must be committed long term, and unlike annual species, a farmer cannot maximize farm profits by changing

species regularly, to follow market prices (Hastings *et al.*, 2017a). The ultimate goal for *Miscanthus* researchers therefore is the development of high yielding genotypes with great resilience that are also both economically viable both temporally and monetarily; supporting the transition from a relatively unknown crop into a practical and large-scale business and environment opportunity.

1.3 *Miscanthus* propagation and the seeds of change

Over the past 25 years, field trials over Europe have shown that hybrid genotypes such as *Mxg* exhibit the combination of both high yield potential and minimal inputs in a wide range of soils and climates(Clifton-Brown *et al.*, 2017). To establish a biomass supply and planting density large enough to come close to meeting the government's target for biomass action plan, there is a great need to develop an intensive and high throughput plant production line. A number of different propagation methods are available for *Miscanthus* (see Table 1.1), but any attempts to optimise the system will only be of use if the new system outperforms the tried and tested (Boersma, 2013). Most widely used and reliable at the present time for growth of new *Miscanthus* plants is the rhizome production system. The popular hybrid *Mxg* is a sterile clone and as such the only propagation option is vegetative and clonal, leading to high establishment costs and lower multiplication rates than would be ideal(Clifton-Brown *et al.*, 2017). Clonal varieties have long been the staple system of *Miscanthus* production due to a number of factors, including; the formation of suitably uniform and homogenous plantations, good establishment in the field due to the nutrients available in the rhizome section for the developing plantlet. Additionally, this method affords better control to the farmer, who can also use their own farm equipment. However, in order to upscale production to the levels necessary for use as a staple biofuel, the clonal system of reproduction is no longer viable with higher than ideal costs, low multiplication rates and intensive labour requirements (see Table 1.1). As such, steps have been taken over the past ten years in Aberystwyth University, with the help of other leading institutions in the field, to attempt to develop a more rapidly multiplied system with lower production costs, using hybrids capable of seeded reproduction. The crucial advances that have been made and methodology behind them can be found in a review, the primary author of which being prominent *Miscanthus* researcher Professor John Clifton-Brown (Clifton-Brown *et al.*, 2017). The review outlines the vital advantages that could be attained by using seeded cultivars of *Miscanthus*. These include

the aforementioned high multiplication factors in comparison to current methods and the simpler logistics of transportation and storage. Additionally, this method could lead to the faster introduction of newer cultivars exhibiting a higher level of resilience to abiotic stresses and improved biomass traits, as breeding plays a key role in development of new and enhanced cultivars. New hybrid seed production significantly reduces establishment cost to below £900 a hectare for direct sowing innovations (Hastings *et al.*, 2017a).

Table 1.1. Summary of various *Miscanthus* propagation methods and their relative merits and drawbacks

Propagation method	Description	Advantages	Disadvantages	Multiplication rate	Average cost (€/ha)	Reference
Rhizome planting	Manual splitting and dividing of harvested Miscanthus rhizome nodes to establish multiple new clones derived from one parent.	• Widely available and commercially mature method • Good establishment and winter success (>90%) • Farmer can use own propagated material for future establishment	• Clonal – low genetic variability • Labour intensive • Rhizome easily spoiled during harvest and storage • Risk of disease spread from infected mother plant • Low multiplication rate compared to demand	Low - 1:10 parental plant division	Medium – 1,500-3,375 €/ha	Xue et al., (2015) Atkinson (2009) Clifton-Brown et al., (2016)
Rhizome propagation in plugs	Using rhizome nodal sections to produce plantlets in trays	• Effective at producing uniform plants • More easily managed due to clonal development • Better establishment than direct field planting	• Clonal material more vulnerable to pathogens • Monoculture – lacks genetic diversity as with rhizome • More labour and materials intensive	Low – 1:30	High - 4300 €/ha	Atkinson (2009) Clifton-Brown et al., (2016)
Micropropagation	In vitro propagation using small quantities of plant material in controlled conditions to produce new clonal plantlets using callus culture	• Disease free plant production • Germplasm conservation • Fast production of high number of clones	• Most expensive method • Clonal material as above • Requirement of good care for acceptable field establishment	Very high - 1:960	High - 6320 €/ha	Xue et al.,(2015)
Direct seed sowing	Applying seed directly to field via drilling or basic sowing	• Very high multiplication • Cost effective • Newer varieties can be bred • Current equipment can be used effectively • Lower risk of invasion if planting non-seeded hybrids	• Need great care in the field before and after sowing for good establishment • Seed production restricted to high temperature regions • Very low field germination (<10%) • High risk of weed competition	Very high – 1:1172	Lowest – 1,508 €/ha	Xue et al., (2015) Clifton-Brown et al., (2016)
Seed derived plugs	Growing seed in modular plug trays in a glasshouse environment until plantlets are large enough to withstand field planting	• Allows all the advantages of direct seed sowing • Increased germination • Better establishment • Less plant loss in the field	• More labour and cost intensive • Requires a large scale glasshouse phase • Glasshouse energy inputs detract from net energy gain from yield • Often 20-40% of plugs without seedlings unless good attention to detail	Very high – 1:1172	Medium - 2100 €/ha	Clifton-Brown et al., (2016)

Seeded reproduction is well established in many crop varieties and the method of direct sowing is inexpensive and widely used across other agricultural grasses and cereals (Clifton-Brown *et al.*, 2011). Attempts at seeded *Miscanthus* propagation can be seen in an experiment by Christian *et al.* (2005b) using the fertile genotype *Miscanthus sinensis*. The varying methods included pelleted and unpelleted seed for seed treatment, and drilling or broadcasting as the two different ways of sowing. Results from this study indicate that drilling and unpelleted natural seed was significantly more successful than the coated seeds sown by broadcasting on the soil surface. Overall establishment was reported as low however, and it was concluded that this could not be a viable alternative to clonal propagation until further agricultural improvements had been established.

A vital factor associated with ability of *Miscanthus* to set productive or viable seed and germinate is the climate under which the plant is grown. *Miscanthus* originated in the tropical and subtropical climates of East Asia with a wide climatic range, allowing it to be suitable for establishment further afield in Europe and North America (Lewandowski *et al.*, 2000). Varying *Miscanthus* genotypes perform better under differing environmental conditions and as such work has been undertaken to develop new genotypes suitable for varying uses and environments (Clifton-Brown *et al.*, 2017). In light of the poor results seen in previous sowing experiments, a study was undertaken on the thermal requirements necessary for good germination over a wide range of *Miscanthus* accessions, in a comparative study with other directly seeded crops including switchgrass (*Panicum virgatum*), reed canary grass (*Phalaris arundinacea*), maize (*Zea mays*) and perennial ryegrass (*Lolium perenne*) (Clifton-Brown *et al.*, 2011). Results of these trials showed that in comparison to the other species, the number of seeds germinating at the lower temperatures of 5-10 °C was greatly reduced in *Miscanthus*. Lag time to 50% germination increased in *Miscanthus* as the temperature decreased, indicating a great sensitivity to lower temperature. In a study in China it was discovered that seeds of *Miscanthus* genotype *Miscanthus lutarioriparius* germinated best at temperatures between 30 – 35 °C (Xiang *et al.*, 2018). This leads overwhelmingly to the conclusion that seeded *Miscanthus* germination in the field would likely only be successful in more Southern latitudes. Low temperatures seen in the field during spring times may be a highly limiting factor using current genotypes in Northern Europe. This is not the only limiting factor; as issues such as soil moisture content and seed to soil contact will also play a role, it is one

barrier that requires urgent attention. Clifton-Brown *et al.* (2011) stated that if accessions could be found with lower temperature requirements, then exploitation of this trait via breeding would be an important long-term objective. In the shorter term they state that changes to crop agronomy and practice could be used to great effect, using materials such as fleeces or films laid on top of the soil after sowing.

Work on both the short and long term objectives has progressed rapidly since 2011 due largely to the collaborative project 'GIANT LINK', undertaken by partners in the UK, US, and Europe (Clifton-Brown *et al.*, 2015). The project ended in early 2016, culminating in the achievement of many of its goals including the exploitation of the genetic diversity of *Miscanthus* by way of thousands of paired crosses in order to produce more temperate-hardy genotypes. Other achievements include the establishment of large scale seed production methods, the ability to produce large stands of *Miscanthus* by way of seeded plug plant propagation, and the overcoming of many other technical barriers that made *Miscanthus* an unpopular choice for many growers (Clifton-Brown *et al.*, 2017). Out of the many accessions produced during the project, three of the best performing types are currently undergoing field trials across Europe. These three seed-based genotypes are chosen as a result of growth performance and yield in relation to the widely used clonal *Mxg* as a benchmark for good performance. All recent studies related to seeded varieties conclude that there is still a considerable amount of improvement needed for successful field growth. Of particular interest is the establishment and germination rates in fields in the more temperate climates, as the cheaper option of direct seeding is still considered impossible for much of Northern Europe (Anderson *et al.*, 2015, Ashman *et al.*, 2018). For the seeded varieties to meet the criteria of being the cheaper option for propagation, it is essential that this financial saving gained is not then negated by the logistics necessary to encourage good establishment e.g. irrigation and/or glasshouse costs

1.4 Acceleration of establishment to revenue

As mentioned previously, *Miscanthus* plants are able to remain in the ground for many years, annually producing yields. While this promise of annual, profitable yield would sound ideal to a potential grower, a review by Witzel and Finger (2016) highlights the drawbacks of what they call the 'establishment to revenue period'. This can be a period of up to four years, during which time yield will be highly limited as the plantation grows into full yield capacity, at which

point it will reach a regular level of high productivity (Sherrington *et al.*, 2008). For this period the costs of establishment; which include initial planting costs, maintenance of crops, and costs of initial harvests, will not be mitigated by the revenue generated by the yields and the farmers' income will suffer. *Miscanthus* is a highly front loaded crop with the majority of costs occurring in the establishment year (Hastings *et al.*, 2017a). The aims of this project will include the manipulation of establishment time for young *Miscanthus* plants. This will include working alongside the Aberystwyth University *Miscanthus* breeding team to attempt to extend the time the plants spend growing by way of earlier establishment in glasshouses. There are currently unanswered questions related to the extended growth potential of *Miscanthus* seedlings which could hold the key to reducing the amount of time until a first harvestable yield by as much as a year. A primary aim of the *Miscanthus* breeding efforts is to achieve economical yield by the end of the second growth season, as opposed to the third or fourth, allowing a faster return on investment for growers. In order to facilitate this change from the standard establishment time frame of April/May and alter it to an earlier January or February start, one part of this project will analyse the extent to which the plants growth is determined by the environment.

Yield in *Miscanthus* is a very complicated trait, but one which has potential to be improved by knowledge of the best agronomy methods and genotype breeding. The amount of time spent growing and the longevity of canopy duration can be a major determinant of yield; as the amount of light captured increases, so do subsequent rates of photosynthesis. Increasing yield is of great importance for the success of this crop, as it has the potential for greater profitability and higher rates of carbon mitigation (Robson *et al.*, 2013b). There are currently large gaps in the knowledge base for creating a protocol of best practice to produce an early crop. Research is now being done at Aberystwyth University testing the performance of plug plant seeded varieties when sown and planted at different times early in the year. The aim is to conclude whether planting earlier will allow for an economical yield from harvest by the end of the second year, or if the earlier planting has a negative effect on plant performance. The hypothesis being that extending the time in field will increase the physical space, nutrients and degree days available for each plant, allowing maximal growth efficiency from an earlier time point, and therefore resulting in enhanced relative growth rate, and plants reaching their maximum potential by the end of the season.

Much work on *Miscanthus* to date has involved the use of vegetative propagation and the genotypes that reproduce this way. Due to the recent innovations seen at IBERs, the move to a seeded plug plant-based propagation method is yet still in its infancy and there is therefore little literature based on the subject. Much of the agricultural knowledge required to create these protocols will be taken from other varying forms of *Miscanthus* growing, and methods which have been shown to be effective on species similar to *Miscanthus*. There is currently much interest in the use of mulch films when young plants are planted into the field (Ashman *et al.*, 2018). A recent paper from Ireland also suggests that the current timespan of three years plus for the crop to reach full potential is an economic barrier to overcome (O'Loughlin *et al.*, 2017). O'Loughlin *et al.* (2017) based their research on the planting of rhizomes as opposed to seeded plugs, but the aim was still the same; to accelerate growth of first year accessions. The use of mulch film increased the establishment rates, average heights, stem numbers and biomass yield in the first growth season, with residual effects carrying on into the second. The film works by increasing the surface temperature of the soil beneath it, creating an improved climate for the plants to thrive in and help protect from spring frosts. Strategies to speed up maturation and development in crop plants have been well developed in many other species using similar techniques to those currently being trialled in *Miscanthus* plug plants. Easson and Fearnehough (2000) released a paper discussing the effects of plastic mulch films (Samco, 2014) and the effects of sowing dates and cultivar on the yields and maturity in maize in Northern Ireland. Amongst many crop species, there is a goal to grow efficiently on not just the favourable land but also the marginal land, and the development of earlier maturing cultivars within maize plants has allowed growing area in Ireland to expand significantly (Easson and Fearnehough, 2000). A vital factor in determining the potential of an area of land is the amount of Ontario heat units (OU) the land receives over a year. Higher yields can be achieved under more favourable weather conditions for the majority of species; however, there will often be seasons under which the cooler temperatures can have a negative impact on the subsequent growth and yield of a specific crop. Despite this, there is still a requirement for good economical yield in a timely manner for these crops, and as such gaining an understanding of measures which could be put in place to mitigate for climatic fluctuations is of utmost importance. Low spring temperatures can have a highly negative effect on the survival and growth of young plants in particular. When attempting to extend the growing season to allow for greater or earlier yields, the strategy of earlier planting can

be extremely effective. In doing so, however, there is a greater risk of exposing immature plants to colder climatic conditions (Easson and Fearnehough, 2000) to which they may be less capable of surviving. Therefore, strategies such as the use of mulch films are very popular throughout agriculture for helping maintain warmer temperatures beneath the film. In order to achieve the best results, work is required to devise best practice protocols regarding optimum times of planting, film laying, type of films, and crucially the timing of film removal. It is the development of this agronomy and knowhow which will help pave the way for the next steps in *Miscanthus* plug trials (Clifton-Brown *et al.*, 2017). In the paper by Easson and Fearnehough (2000) they trialled two different film types on varying cultivars which were planted a couple of weeks apart in April. Their conclusions discovered that the use of 'floating plastic' was more effective then punched holes plastic. There was also a difference between cultivars and the earlier sowing, which allowed the plants to receive higher OU at the crucial time of development. This information is not currently reliable for the new *Miscanthus* hybrid cultivars due to the aforementioned infancy in using the crop as a staple agricultural venture.

1.5 Manipulating growth

Plant interactions with the environment can have strong effects both positively and negatively on the growth and vigour of individuals or populations. In addition to accelerating and improving *Miscanthus* yield by way of extending the growth season and achieving the most effective thermal cycle, other options are available to enhance the viability of the plants. Much progress has already occurred within the *Miscanthus* programme to select the best yielding genotypes from thousands of experimental crosses. The focus now will be on enhancing the establishment phase of the hybrids of interest, by utilising the potential of the environment around them to promote strong, healthy growth.

Major issues currently facing the seed-based future of *Miscanthus* growing are the high mortality rates and small weak plants often seen developing from seeds compared to rhizomal propagation. When plants are grown from rhizome, they have access to a reservoir of nutrients already stored in the roots, to help bolster young plants and carry them through their first year. *Miscanthus* seeds contain only a very small amount of nutrient supply for a growing plantlet. As a result, this project will aim to test varying non-temporal factors for their potential to enhance plant growth, allowing for more successful plants in the field and fewer losses. The overall goal for a successful plug plant venture in *Miscanthus* is the growth of a

homogenous population of strong plugs, which continue to perform well when planted out in the field (Clifton-Brown *et al.*, 2017). More certainty in crop establishment is highly important as it reduces unwanted planting gaps, and patchiness and yield loss which will then remain for the lifetime of the crop (Hastings *et al.*, 2017a).

In order to achieve a high yielding field plantation from plug plants, it is vital to identify the traits in young plantlets which indicate strong growth and vigour later on. In this way, it is then possible to select for growth treatments which enhance this trait at the young stage. Zub *et al.* (2012) explain that throughout the fields of agronomy and plant breeding, models exist for predictions of crop yields based on meteorological data and models for assessments of growth characteristics. In *Miscanthus* models such as these include MISCANFOR and MISCANMOD (Hastings *et al.*, 2009, Clifton-Brown *et al.*, 2000) which have been used primarily on *Mxg* to model the likely yield against meteorological data. However there is currently no strong model for *Miscanthus* to characterize new seeded hybrid trait variabilities and growth dynamics (Zub *et al.*, 2012). Due to the infancy of the plug-based hybrids, it is still unclear which early traits are strongly correlated with high above-ground biomass. In previous studies using clonal reproduction, a stable trait for comparison has been canopy height, largely due to the relative ease of measurement and a strong positive correlation with yield (Zub *et al.*, 2012). There are various phenotypic traits which could be indicators of strong growth besides plant height, however. These include leaf number, stem thickness, nodal number, tiller number and crucially, root biomass. Environmental conditions that could promote large root biomass are currently a topic of much interest and are being trialled using endophytic bacteria within the rhizosphere to aid root uptake of vital nutrients. Large root systems have multiple benefits including a higher drought resilience, better anchorage, increased sequestration potential of ground water and nutrients, and ultimately encourage larger above ground growth.

When considering manipulation of environmental conditions, multiple possible variables and their interactions present themselves. They include, but are not limited to; soil nutrient content, climate temperature, water availability, beneficial bacteria, light concentration and pot size. This project will focus on many of these variables in order to produce best practice protocols for growing strong, healthy plugs. How the plants interact with these environmental

variables will allow more definitive answers to the questions of what indicates a strong plug plant, and what conditions are best to enhance this trait.

1.6 Manipulating senescence

Plant development overall is driven by cell division and cell differentiation, combining to form the obvious structures seen in plants; the vegetative growth and formation of photosynthetically active leaves and other above-ground parts. This development does not stop at the formation stage however, but continues into the degradation and death of cells, tissues and larger organs using apoptosis, or the biologically coined term 'programmed cell death' (Ay *et al.*, 2014). Senescence then, in the botanical sense can be described as the termination of active plant growth for that season, leading to the initiation of cell death in the plant. It is the final developmental stage of plant cells, tissues, organs, or in the case of monocarpic plants; the entire plant (Distelfeld *et al.*, 2014). Ultimately, senescence is the process which can determine the level of photosynthesis, ranging from individual photosynthetic organs to a whole plant basis. The overall function of senescence is therefore to maintain the optimal conditions for plant photosynthesis and survival by way of nutrient use efficiency (Robson *et al.*, 2012). It will achieve this by removing the nutrients given to leaves deemed no longer of photosynthetic benefit, and re-assimilating the nutrients to leaves more likely to achieve good net productivity, for example leaves higher up the canopy (Robson *et al.*, 2012). Once senescence has been initiated the next stage will be a mass remobilization of nutrients from the senescing parts to the developing sinks, such as seeds, grains or plant rhizome (Distelfeld *et al.*, 2014).

As a perennial crop plant, *Miscanthus* dries down during the winter months before regrowth occurs the following spring. The key organ for this in *Miscanthus* plants is the below ground rhizome which is essentially a *Miscanthus* plant's life source. The rhizome is primarily involved in the economic translocation of nutrients, and is the organ the plant uses to survive the winter (Clifton-Brown and Lewandowski, 2000). At the end of the growing season the plant will start to senesce, transferring all its available nutrients to the rhizome to be available for next spring (Clifton-Brown and Lewandowski, 2000). The timing of this process can have a significant effect on the quality and quantity of harvestable yield in *Miscanthus* plantations; if senescence occurs too early then there is a risk that harvestable yield is lessened, due to a reduction in the duration of canopy expansion. If senescence occurs too late the crop will not have had a chance to ripen sufficiently or remobilize enough nutrients to the rhizome,

resulting in reduced biomass quality, and crucially, a reduction in the plants ability to survive the colder winter temperatures and flourish the following spring (Robson *et al.*, 2011)

Currently *Miscanthus* has exhibited poor senescence and overwintering in the UK due to its more tropicalized and warmer climate tolerant life cycle. Field trials have shown that the first winter following planting holds the greatest survival risk for a plant due to shallow and less developed rhizome than in subsequent years (Clifton-Brown and Lewandowski, 2000). In more Northerly regions overwintering losses have translated to high financial losses, further adding to the barriers faced in upscaling the crop to economically viable levels (Clifton-Brown and Lewandowski, 2000). Understanding the triggers of senescence will allow slow senescing genotypes to be artificially induced into starting the process at an earlier date. This will ensure good re-growth in following years when plants should be hardy enough to survive winter with minimal help. Dormancy (and therefore winter survival) is of great importance in a perennial crop such as *Miscanthus,* and in areas where autumn and winter climates illicit temperatures below what the plants can cope with, it is especially prevalent.

Similar aims are evident in literature across a variety of crop plants over the last few decades, with the primary goal being to harness the timing of plant senescence, whether to encourage it or delay the process, depending on the species and the desired result. Thomas and Ougham (2014) review a more recently coined term known as the 'stay green trait', which can be used to describe heritable delayed senescence in some model and crop species. They explain that functional stay green is considered a valuable trait for improving crop stress tolerance and can be associated with cereal crop domestication. The stay green types are often a result of alterations in plant hormones and signalling , focussing particularly on the networks involving ethylene and cytokinins (Thomas and Ougham, 2014). While extending the duration of greenness has improved the productivity in many crops, for other plants a stay green trait would have a more negative impact. It stands to reason that if plant hormone and signalling pathways can be altered to extend the green season, that the same idea can be reverse engineered to reduce it where necessary.

Senescence can be controlled or triggered in a variety of ways, primarily induced by environmental conditions and the result of signalling pathways, triggered by biotic or abiotic stress. It is widely accepted that phytohormones play an incredibly important role as senescence regulators, in what is a highly complex network primarily aiming to protect the

plant from stress and to optimise development (Ay *et al.*, 2014). A particularly efficacious phytohormone involved heavily in plant regulatory networks is ethylene, probably best known for its fruit ripening characteristics. However ethylene is a gaseous plant hormone, and as such can be inconvenient to use for many plant laboratories, therefore there are several chemicals which can be used as replacements, including ACC and ethephon (Zhang and Wen, 2010). Other chemicals and phytohormones which have been proven to induce senescence in leaves or whole plant include Salicylic acid (SA), Methyl Jasmonate (MeJa), and Abscisic acid (ABA) (Sarwat *et al.*, 2013, Ananieva *et al.*, 2007, Milborrow, 1974). In addition to endogenous factors such as the phytohormones, plant age, and reproductive phase, senescence can also be initiated by environmental variables. These include the length of the photoperiod, nutrient availability, temperature, water availability and salinity (Sarwat *et al.*, 2013).

All of the factors mentioned above play a crucial role in encouraging or delaying plant senescence in varying forms and concentrations. Together they produce a network of regulatory pathways whereby senescence associated genes (SAGs) can be up- or down-regulated depending on the fine balance. Through experimentation with different chemicals and, where practical, different environmental conditions, it should be possible to aid first year *Miscanthus* plants in senescing in a timelier fashion and in doing so ensure the re-growth of strong second year plants the following Spring.

1.7 Project overview

This project will be completed in collaboration with the industrial partner Terravesta. Terravesta are a Lincoln-based company, leading the way in *Miscanthus* utilisation as a significant biomass crop in the UK. Chairman of the company William Cracroft-Eley began planting *Miscanthus* in 2006, and upon realising its potential as a viable business crop on the less favoured land, set up Terravesta in 2012 as a new market for *Miscanthus* growers after the liquidation of Bical in 2010/11. The overarching priority for the company is to ensure *Miscanthus* becomes an established homegrown biomass resource. It is hoped this project will play an important part in helping secure the future of *Miscanthus* as an agricultural staple crop. Improvements to *Miscanthus* are required across the entire supply chain and this PhD will be one part of a multidisciplinary team working to achieve this. Despite the improvements already in place, there is a need to further optimise and improve the hybrids

to best match the growing conditions and required end uses in order to get potential growers on board. Uptake of *Miscanthus* growing by farmers has been slow due to a series of barriers along the development chain (Clifton-Brown *et al.*, 2017). By focusing on the current shortfalls of the species this project has the potential to improve the establishment phase of *Miscanthus* leading to less initial loss, cheaper seeded options of propagation, earlier establishment, better overwintering and ultimately a faster return on grower's investments.

The ultimate aim of this project is to aid the progression in domestication of one of the leading crops available to utilise for biomass fuel and renewable energy. This introduction reviews recent advances in early *Miscanthus* growth, what still needs to be done and where these experiments impact wider *Miscanthus* research aimed at increasing biomass supply. This project will not include work based on the chemical composition of plants, conversion technology, bio-refining or power station end of the supply chain.

1.8 Aims and objectives

The project can be broadly broken down into three sections of research, which will ultimately link into each other for an overall improved *Miscanthus* agronomy.

1) Early season planting of plugs of seeded varieties to extend the length of the first growing season enhancing establishment growth with impacts on yield maturation
2) Enhancing seed plug growth and development by manipulating growth conditions
3) Insight and understanding of the signals and triggers of senescence and improvement of overwintering ability in the first winter following planting.

2 Shared Methods

2.1 Germination scores

Germination scoring can be difficult to accurately assess due to the small size of *Miscanthus* seed, and occasional loss of the seed into soil gaps in the growth medium. Typically, germination was scored when at least 2mm of radicle emergence could be seen protruding from the seed husk, and more confidently so, when any amount of green shoot could be seen breaking through. Where germination score was a measurement factor, seeds were sown individually into each plug cell well. Germination percentage could then be scored by counting the number of cells at set amount of times post sowing, that contained any amount of green shoot.

2.2 Seeded hybrids used

The seeded hybrids used most commonly in this project are GNT 3, 5, & 14 as key hybrids with the best performance of key traits chosen from thousands of crosses in previous funded project 'GiantLINK', concluding in 2016. The majority of individual trials reported used GNT14 as the main seed of choice, due to high commercial interest in the hybrid at the time. Year produced varied as earlier experiments used 2015 threshed material, and later on used 2016 threshed material. Due to issues surrounding germination percentage, crossing compatibility and seed collection issues, and mould in the resulting seed lots, GNT14 is of less interest now, than at the start of the project, however as trials began using that hybrid, it was practical to keep using it throughout the timeframe. Multihybrid trials reported here, typically used all three of the key seeded hybrids (GNT 3, 5 & 14). For assessments of plug variation within one genotype, it was necessary to find a high performing hybrid, with good germination, homogenous field growth and excess available seed, due to the quantities needed. The seed batch of choice was GNT 27, a hybrid used in chapter 6 only. Senescence assessments in 2017 used hybrid GNT 13, a cross that had failed senescence testing in multi hybrid trials due to a tendency to stay green over the first winter. Later senescence testing used GNT14 as this had also exhibited reluctance to senesce under temperate climates.

Table 2.1 Genetic information of hybrids used throughout this study

Common seed name	Mx number	Cross type	Female parent	Male parent	Location produced	Chapter used
GNT5	Mx 3539	Interspecific hybrid	*Miscanthus sacchariflorus*	*Miscanthus sinensis*	Ceres Inc. Texas	3 & 4
GNT 3	Mx 3536	Interspecific hybrid	*Miscanthus sacchariflorus*	*Miscanthus sinensis*	Ceres Inc. Texas	4
GNT14	Mx 2779	Interspecific hybrid	*Miscanthus sinensis*	*Miscanthus sacchariflorus*	Catania	4,5 & 7
GNT 27	Mx 3522	Interspecific hybrid	*Miscanthus sinensis*	*Miscanthus lutarioriparius*	Ceres Inc. Texas	6
GNT13	Mx 2468	Interspecific hybrid	*Miscanthus floridulus*	*Miscanthus sinensis*	Ceres Inc. Texas	7

2.3 Seedling measurements

2.3a Extension

Seedling extension also known as shoot or stem height, typically was undertaken on the tallest stem or main stem of a seedling, or when necessary, all stems. This is a measurement of length between the base of the plant at soil level and the ligule of the newest fully expanded leaf. This is the most standard measure of height in a plant, as measurements of height based on to the end of the longest leaf can be confounded by differences in the leaf area and growth, which is a separate assessment to plant height.

2.3b Leaf number

Leaf number was typically scored as the amount of green leaves present on either a single stem, or more commonly the whole seedling. A green leaf was chosen as any leaf that still had >10% green pigment remaining. Brown senesced leaves are difficult to count and distinguish from each other and the stem, so were typically removed from analysis. Any emerging leaf over the length of approximately 4mm was counted in the analysis.

2.3c Stem number

The number of stems, also described in some places as tiller number, was a measure of the total number of stems over the length of approximately 1cm, and forming a leaf, on a single plant. Tiller number was done here on an individual plant basis, not as a sward or cluster.

2.3d Above ground biomass

Total above ground biomass of a seedling was the overall total of the individual leaf, and stem biomass from an individual plant. Leaves were stripped from the stem with scissors, at the point where the ligule meets the stem. For brown senesced leaves it was difficult to assess a starting point as leaves were typically dead, flaccid and had no obvious ligule. For these leaves, the easiest method of removal was to peel them away from the stem. All leaf material was weighed collectively. Stems were cut away from the roots just above the main root mass and weighed collectively. Results could then be added together to produce total fresh above ground biomass. Samples were then placed in labelled paper bags to be oven dried, at between 60 – 105°C depending on the oven available, and other material inside. Once a constant weight was reached, dry biomass was assessed for stems, and for leaves.

2.3e Root biomass

Root biomass analysis was undertaken by removing the below ground biomass from the above ground, cutting at the base of the stem just above the bulk of the root and young rhizome. Roots were washed carefully, ensuring as little loss of fine root structure as possible. After washing, roots were patted with blue roll to remove excess moisture, before being weighed on a 4-figure balance, due to low weights of many samples. Samples were then placed in labelled paper bags to be oven dried, at between 60 – 105°C depending on the oven available, and other material inside. Once a constant weight was reached, dry biomass was assessed for root mass.

2.4 Field planting

Once soil was prepared, site perimeter, based on assessment of planting density and trial area, was measured out and lines staked using a measuring tape, strong stakes and strong twine. Plug spacing was measured out using one line of twine, a measuring tape, and cable ties tied at individual plug spacing. This line was then moved across the whole plot during planting for accurate hand planting at the required spacing.

2.4a Planting

Planting took place in April or May for most assessments, with the exception of one June planting in chapter (X). Plugs were removed from trays, placed in a pre – dug small indent in the soil, covered back over and all watered at the end. Plugs were also sprayed with a pre-emergence herbicide to attempt to control weed growth, before being covered with a layer of porous Samco mulch film (Samco reference). A starch-based product, the film typically breaks down under UV exposure, but often leaves plastic residue in the soil. As such, new trials are being undertaken using other film types, but for this project the Samco film was still used.

2.5 Field assessments

2.5a Establishment

Establishment assessment, also known as survival measurements, are typically (unless stated otherwise) undertaken in the autumn following planting, and again at harvest following the first winter, to give two measurements of a) growth season survival and b) over wintering survival. Survival counts involve a complete count of all surviving plants in a plot.

2.6 Autumn phenotyping

2.6a Stem number

Assessment of stem number in field conditions usually involves a complete count of all growing stems on an individual plant. The height at which a new base node becomes a stem is subject to the individual researcher and the size of the plant but generally any stem that has formed its own leaf will be counted. Where *Miscanthus* plant basal diameter is wide or contains creepers, with a large number of stems, a half stem count will often be taken and then doubled, to give a good estimate of the total.

2.6b Canopy height

Canopy height assessment can be variable between individuals taking the measurements, as it is not an exact measurement. The height of canopy is the approximate region of the leaf canopy where maximum light interception can occur, and typically, when the leaves begin to bend downwards.

2.6c Shoot height

Shoot height, or often known as stem height, and is an exact measurement of the length of the tallest stem from the base of the plant, until the ligule of the newest fully expanded leaf. Where the tallest stem is difficult to determine, an approximately tallest stem is sufficient.

2.6d Die off height

The assessment of die off height is difficult to standardize but is generally measured as the height at which senescent brown plant material has reached from the base up to the canopy. Die off height can give a good early indication of senescent speed in an individual plant or in a sward of *Miscanthus*.

2.7 Post-winter harvesting

Total plant biomass assessment is typically done the spring following winter die back to allow as much time as possible for nutrient remobilization and drying down of the senesced above ground material. In an experimental plot design the plants in the middle of the plot will generally be harvested, the total of which is dependent on the plot size and will be described for individual field trials in more detail in chapter methods. An assessment of the total number of plants within the selected area will be taken, followed by an actual plant count, and assessment of quadrat area, for later assessment. The selected plants are cut from the base, with either hedge strimmer or secateurs, weighed collectively and representative subsamples of leaf and stem taken for fresh and dry weights, to gauge average moisture content of the entire plot. In earlier years, typically a single subsample was taken from each plot, however in later years methods have improved to include three separate subsamples, to allow an average for moisture content. Subsamples are oven dried at varying temperatures depending on the assessment requirements following drying. For simple dry weight, subsamples are usually dried at 105°C. If compositional and carbohydrate analysis is required after drying, then temperature is generally reduced to 60°C. Once dry weight and moisture content of the

entire plot is known, it is then possible to use potential and actual plant count of a known area to predict the total biomass that could be gained from a hectare of plants grown under that specific experimental condition.

3 Effects of application of mulch film covering on germinating *Miscanthus* seedlings, on subsequent glasshouse growth and field performance

Figure 3.1 The lifecycle of the first year of a *Miscanthus* seedling trial. The red highlighted area indicates where the key period of treatment and intervention took place, for the experimental treatments being presented in this chapter.

3.1 Introduction

The innovation of *Miscanthus* produced from seeds, as opposed to rhizome propagated is one of the most important advances in the *Miscanthus* programme at Aberystwyth University. Propagating *Miscanthus* from seeds, as opposed to the standard rhizome splitting method, has the benefits of producing progeny at a much higher rate of multiplication, reducing the risk of disease and total crop loss associated with clonal plantations. Additionally, this method would have the benefit of reduced labour and transport costs (Ashman *et al.*, 2018).

For the new seeded varieties currently undergoing testing, it is important that they meet a list of growth and performance criteria and perform equal to, or better than, the current commercial standard *Mxg* (Clifton-Brown *et al.*, 2017). For this reason, the current hybrids at Aberystwyth University have been selected for high quality performance across a range of tests. Of thousands of paired crosses undertaken in previous projects, a handful of best performing hybrids have been chosen to partake in Innovate UK project called *Miscanthus* Upscaling Technology (MUST). This project was designed specifically for commercial partners and researchers to improve the seeded *Miscanthus* supply chain from seed crossing and collection, to germination, growth and yield, and finally potential end uses.

While seeded varieties have much potential and benefit, there are also drawbacks to using this system. Clonal propagation relies on the splitting of rhizome into individual pieces which can then facilitate the growth of a new plant from a reservoir of essential nutrients including N, P, K and available sugars. In contrast, seeded plants do not have this substantial resource to draw from (Atkinson, 2009). *Miscanthus* seeds are small (2-3mm) and easily damaged or compromised (Awty-Carroll *et al.*, 2018, Lewandowski *et al.*, 2000). Germination is highly variable between hybrids and years; a trait of considerable interest within the testing criteria. Once germinated, seedlings are highly sensitive with little reserves, and can easily be lost to desiccation, frost damage, flood or low seed to soil contact. In addition to this, the non-clonal nature of the seeds reduces the homogeneity in the progeny, making heterogeneous establishment more likely during the first year (Christian *et al.*, 2005a). First year growth is a crucial time for determining the growth and yield of plantations in subsequent years (Robson *et al.*, 2013a). First year yields are typically low due to plant immaturity, but if plants are able to grow substantially, from as early in the season as possible, and senesce effectively over the

winter months then economically-harvestable yields are more likely to be achieved in the second and third year (Clifton-Brown and Lewandowski, 2000).

Seeds cannot be effectively direct sown at present, without considerable gaps within the field plots due to non-emergence and/or death of seedlings (Ashman *et al.*, 2018). As such the current widely accepted method for planting seeded *Miscanthus* hybrids, is based on a system of multisowing seeds into small cells of plug trays and growing the plantlets under glasshouse conditions (Clifton-Brown *et al.*, 2017). After 8-12 weeks growth under more controlled conditions, the resulting plants are more likely to survive field conditions. This can be further improved by covering field-planted seedlings with a layer of mulch film, designed to retain heat and moisture (Ashman *et al.*, 2018). Mulch films addition at planting have been proved to improve establishment rate, average height, stem number and yield in the first year, with some improvements still visible after the third growth year, making this a highly economical agronomic practice (O'Loughlin *et al.*, 2017).

While genetic differences account for a large percentage of plant characteristics and yield potential, plant treatment and the growth environment cannot be underestimated in terms of overall establishment, growth, and crucially yield (Boyer, 1982, Lewandowski *et al.*, 2000). The aim of this experiment was to attempt to apply simple agronomic methods to glasshouse grown plants to try to maximise growth before planting in the field.

The time seedlings spend in the glasshouse can be utilized to affect establishment and improve above and below ground growth (Ghosh *et al.*, 2018). Glasshouse time is costly, financially and energetically, and reducing costs and energy expenditure where possible is important to the net financial and carbon balance. Experimentation to determine the ideal establishment growth conditions and optimum time spent growing for sowing and planting was a large part of this PhD project and the MUST project.

The first assessment undertaken during the project was experimentation on the use of clear film in the emergence phase under glasshouse conditions. The aim of the experiment was to assess seed germination and establishment for potential improved yield and homogeneity in the field, in the new *Miscanthus* hybrid 'GNT5'. Growth conditions were altered using the standard agronomic practice of using clear film over the plug trays after sowing for approximately 1 month. This was hypothesised to provide a more optimal condition without

requiring significant energy input to the environment surrounding the emerging seed. This assessment was at Aberystwyth University in a “Venlo”-type glasshouse.

The hypotheses tested in the experiment were as follows:

1) Increased temperature and humidity under film will lead to higher rates of germination
2) Higher rates of germination under film will produce more homogenous growth at the seedling stage
3) Plants germinated under film will perform better, with higher survival and growth rates after transfer to field conditions

3.2 Methodology

3.2a Glasshouse phase – plant material and experimental set up

Four commercial *Miscanthus* '126' plug trays of approximately 25cm^3 soil volume per module were filled with approximately 8.5g of John Innes compost No.2. Seeds of *Miscanthus* hybrid GNT5 were sown in early January 2016. One seed per plug was added to each tray and finally the whole tray given a light sprinkling of vermiculite to cover approximately 50% of soil surface, and then watered. Once sown, all trays were covered with silver foil and placed in a glasshouse compartment. The compartment was set to a 16 h day length with supplemental light provided by SonT lights and temperature set to 18/25°C (night/day respectively), with some variation dependent on outside conditions.

Seeds were monitored daily for signs of radicle emergence through the seed coat. Once radicles had emerged all silver foil was removed, and the trays split randomly between two treatments. Treatment 1 was to cover trays with clear film after germination (Trays A & B) and treatment 2 was left uncovered (Trays C & D). Emergence data was collected every two days over the first 3 weeks, beginning on day 7 post-sowing, by counting the number of plugs that had chlorophyllous shoots.

After germination, 15 plants per tray were selected at random. In the selected plants extension growth and leaf number were measured on a weekly basis for two months (Figure 3.2). Extension growth was the height of the tallest stem from soil level up to the youngest leaf ligule. Leaf number was the total number of green leaves visible on the plant . The film was suspended using plant labels to produce a mini greenhouse, and the film was removed from the trays after one month. Trays were watered daily, and regularly moved around the glasshouse to minimize the impact of environmental heterogeneity.

Three weeks before planting, all trays were removed from the glasshouse and placed in a polytunnel to begin the process of acclimatisation to outside temperatures and light fluctuations. Plants were not measured during this time.

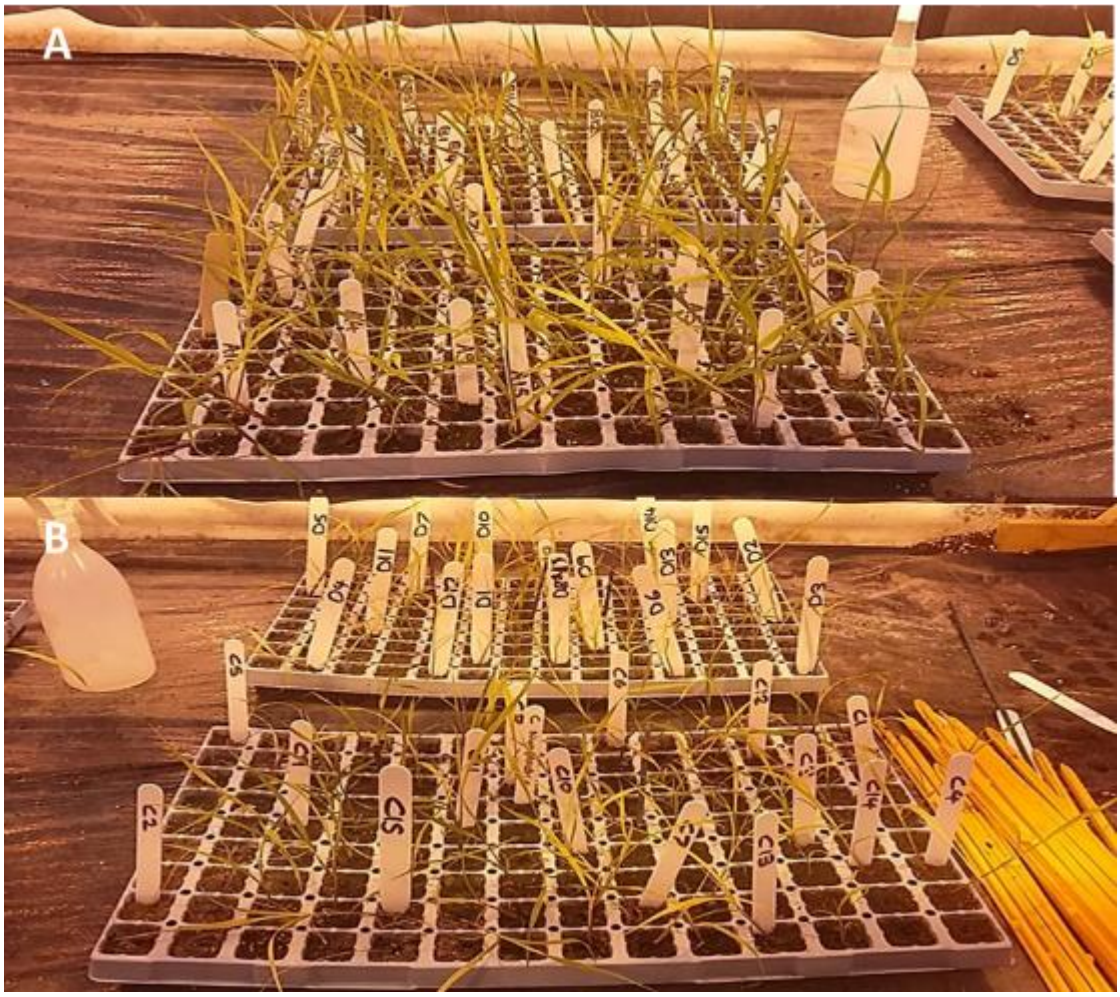


Figure 3.2 Treated trays A & B (photo A) and control trays C & D (photo B) at approximately 7 weeks old, under SonT supplemental lighting in February 2016. Labels show the randomly selected plants measured in each tray.

3.2b Field planting of all seedlings into experimental plots in Hackthorn, Lincolnshire

All plants from the four treatment trays were hand planted in a field site close to Hackthorn (Lincoln) at the end of April 2016 (see appendix 1 & 2). Treated trays A and B were planted together, treatment block 1, and control trays C and D on treatment block 2. All surviving plants had a Unique identifier (UID) number and selected plants were positioned randomly within the blocks and labelled on a field plan for subsequent measurement (

Figure 3.3). Immediately before being planted the selected plants were measured for extension growth, leaf number and stem count. Plants were spaced approximately 68cm between columns, and 75cm between rows (Figure 3.4A). To help establish the plugs all plants

were watered, sprayed with a pre-emergent herbicide and covered in Mulch film (Figure 3.4B). Plants were then left to grow through the mulch film and were hand weeded if necessary.

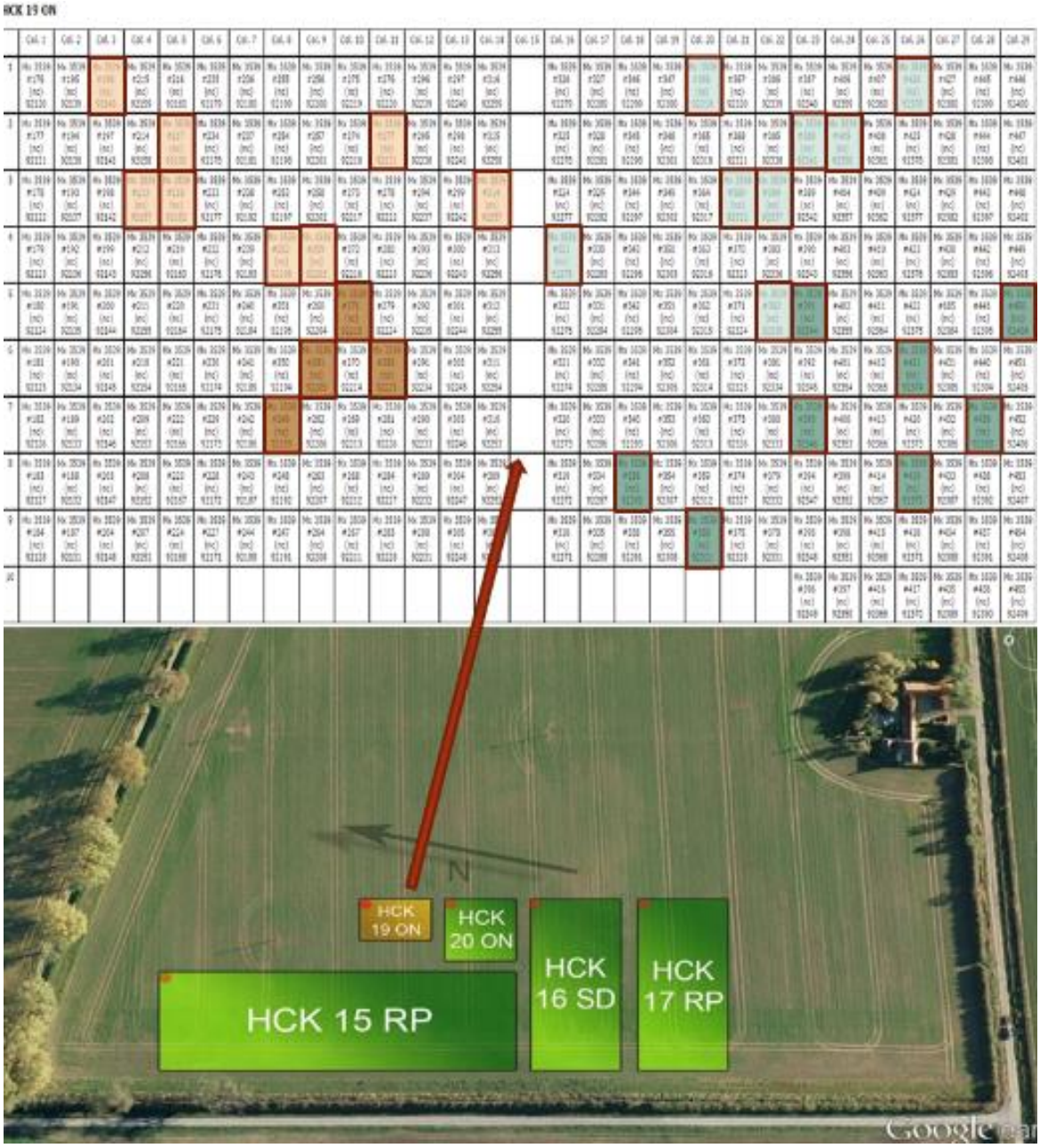


Figure 3.3 Field trial location in the Hackthorn *Miscanthus* plots, and field plan. Left side contains control plants from trays C & D, the selected plants shaded light orange for tray C, and dark orange for tray D. Right side contains the treated plants from trays A & B, the selected plants from tray A shaded light green and from tray B shaded dark green.

Figure 3.4 Seedlings from HCK 19 ON were hand planted along rows (A) and sprayed with herbicide before application of Mulch film (B).

3.2c Phenotyping in Autumn 2016 & 2017

In October 2016 and November 2017 all plants were assessed for survival rate per block, canopy height, shoot height and stem number. Canopy height was measured as the height from the soil surface to the point at which approximately the most light interception can be seen in the leaf canopy. Shoot height was measured as the height of the tallest stem, from the base of the plant to the ligule of the youngest fully expanded leaf. Stem count was the total number of stems over a height of approximately 10cm.

3.2d Spring harvest in February 2017

Spring harvest of all above ground biomass was undertaken on 25 plants randomly selected from both the treatment block and the control block. The entire plant was trimmed from the base by hedge trimmer and weighed on a tripod and scales. The plant was then shredded, and the entire plant contents placed in a labelled paper bag. Samples were dried at 105°C until a constant dry weight was reached to determine total dry biomass and moisture content.

3.2e Data and statistical analysis

Data was gathered using Microsoft Excel on an iPad. Statistical analysis was using IBM SPSS statistics, version 21. Tray means were compared using ANOVA and Tukeys *post hoc* test examined any significant effects. Phenotyping and harvest data were assessed between blocks using Students T test.

3.3 Results

3.3a Emergence and germination rate

The presence of film increased germination speed in both treated trays, with 39% emergence in tray B and 55% emergence in tray A at day 7, compared with 17% and 20% in control trays C & D respectively (Figure 3.5). Emergence remained higher in treated trays than control trays until day 22 when control tray germination reached a similar percentage (79% in both trays), to treated trays (86% in tray A, 81% in tray B).

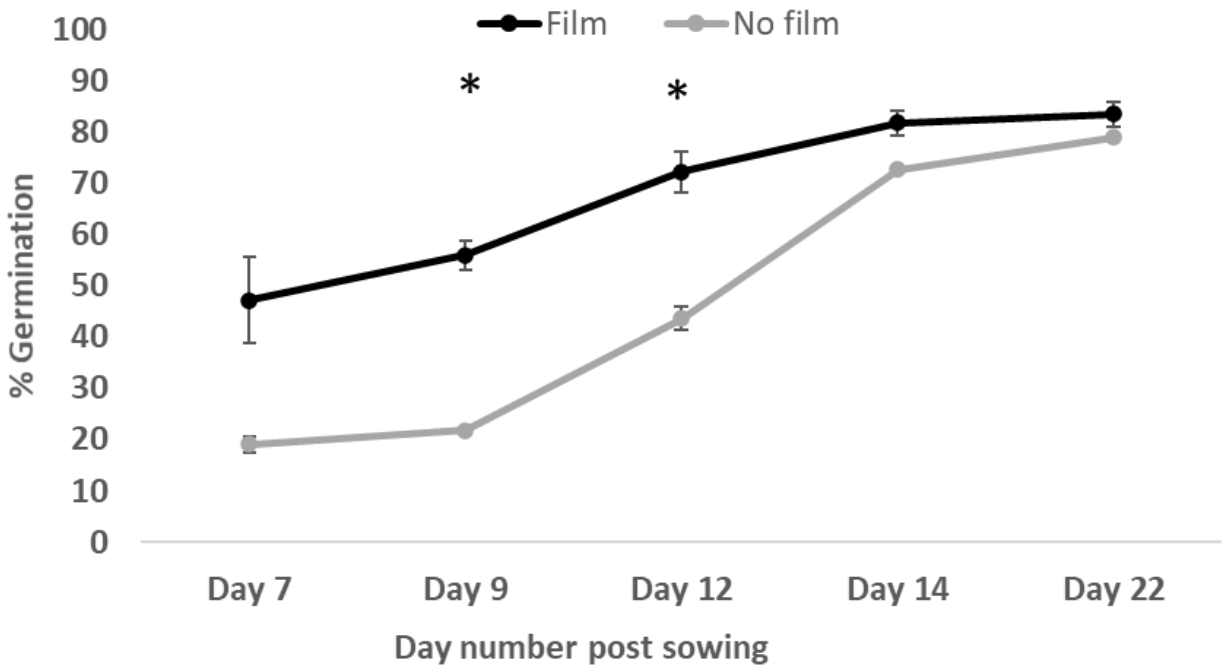


Figure 3.5 Germination percentage over 22 days from sowing, for two treatments. Black line indicates trays covered with film from germination. Grey line shows trays not treated with film at all. For both lines n = 2. Percentage shows the proportion of the 126 plug cells per tray that produced a green shoot. Asterisks indicate where comparison was significant (p = >0.05) using students T test.

3.3b Glasshouse growth and development

From 1 – 4 weeks post sowing there was no significant difference in seedling height between treated plants and control plants (Figure 3.6A). After 5 weeks, film-treated plants were significantly longer than the control treatment lacking film (p = 0.000). This trend continued over the next 6 weeks as treated plants grew taller at a faster rate than control plants. By weeks 8 - 9, exponential growth in treated plants slowed down, but the film-treated plants remained significantly taller than control plants (p = 0.000 at all time points). Green leaf number was also significantly increased by treatment from 3 weeks (p = 0.027) to 8 weeks (p = 0.010) post sowing (Figure 3.6B). After 8 weeks green leaf number was no longer significantly higher in treated plants.

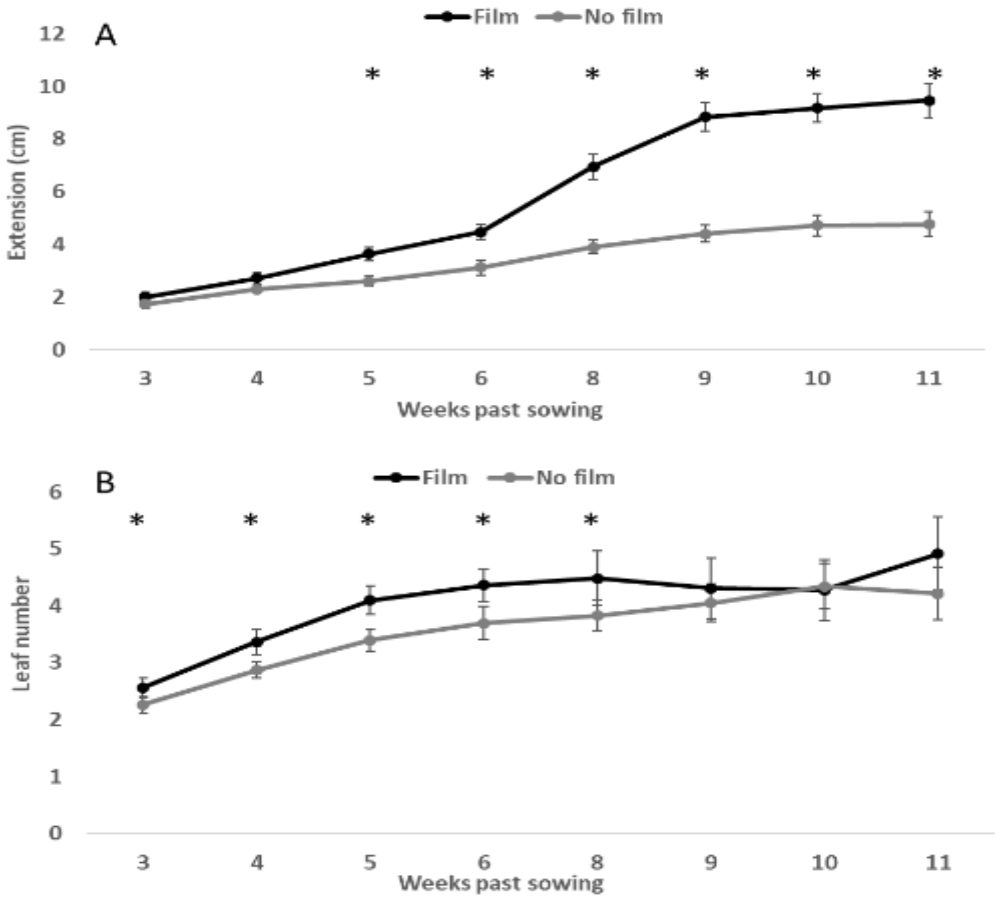


Figure 3.6 Extension growth (A) and green leaf number (B) of plants covered with mulch film (black line), and control plants lacking film cover (grey line) over 11 weeks in glasshouse conditions. Bars are standard error, n = 2 replicate trays, within which were 12- 15 individually selected plants measured, depending on tray mortality rates. Asterisks indicate where comparison was significant (p = >0.05) using students T test.

Analysis of selected plants the day before field planting is shown in Figure 3.7. Plants from treated trays A & B were taller than control plants on average, but there was no significant difference in heights across all selected plants ($p = 0.268$). Tiller number was lowest in control tray D, but no significant difference was found ($p = 0.638$). Leaf number was higher on average in plants from treated tray B but this was not significant ($p = 0.286$).

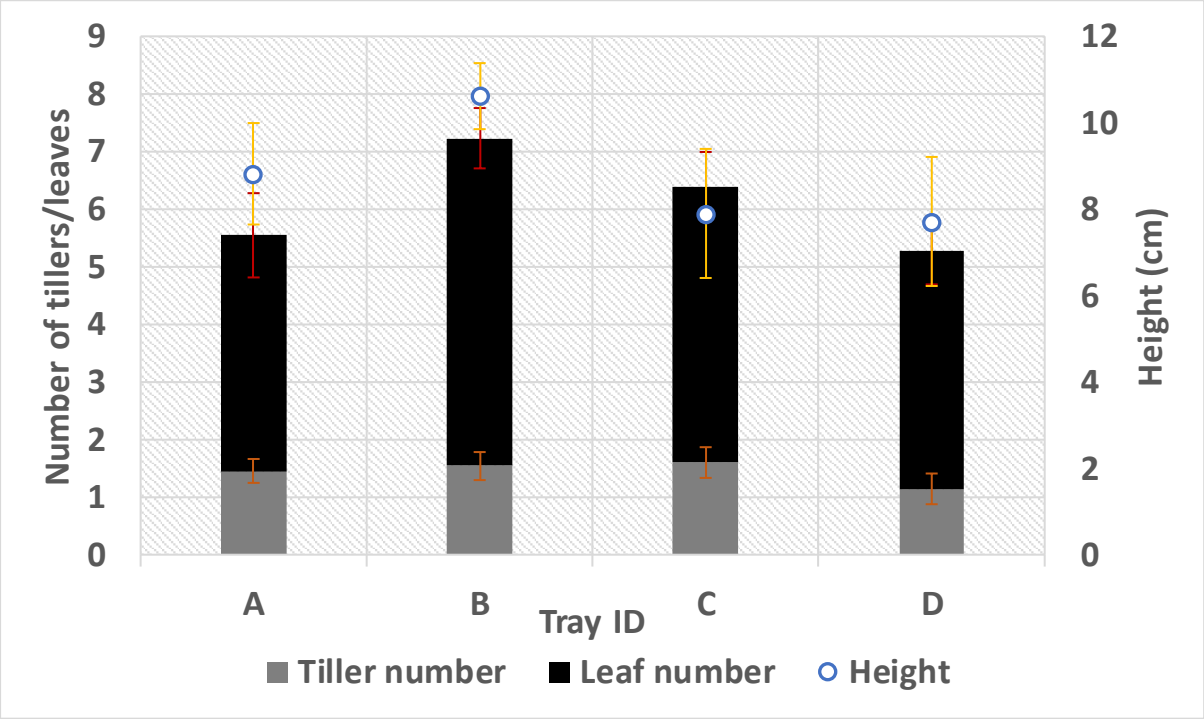


Figure 3.7 Final assessment of selected plants from tray A and B (film covered) and tray C and D (control plants) the day before field planting on the 26th April 2016 when plants were approximately 14 weeks old. Tiller number (grey bars), leaf number (black bars) and plant height (scatter points and second axis) were measured on each plant. N = 11,13,10 & 7 of original 15 in trays A,B,C & D respectively due to plant mortality. Error bars are Std E. Statistical assessment was done using ANOVA.

3.3c Environmental conditions over the first 16 months

Weather assessments indicated consistent rainfall in the weeks immediately following planting, and no obvious frost events. Temperatures were lowest at the week of planting but rose quickly following the planting week, and remained between 12 – 20°C on average over the growth season (Figure 3.8), until the end of September. Average weekly air temperature did not dip below freezing during the winter period.

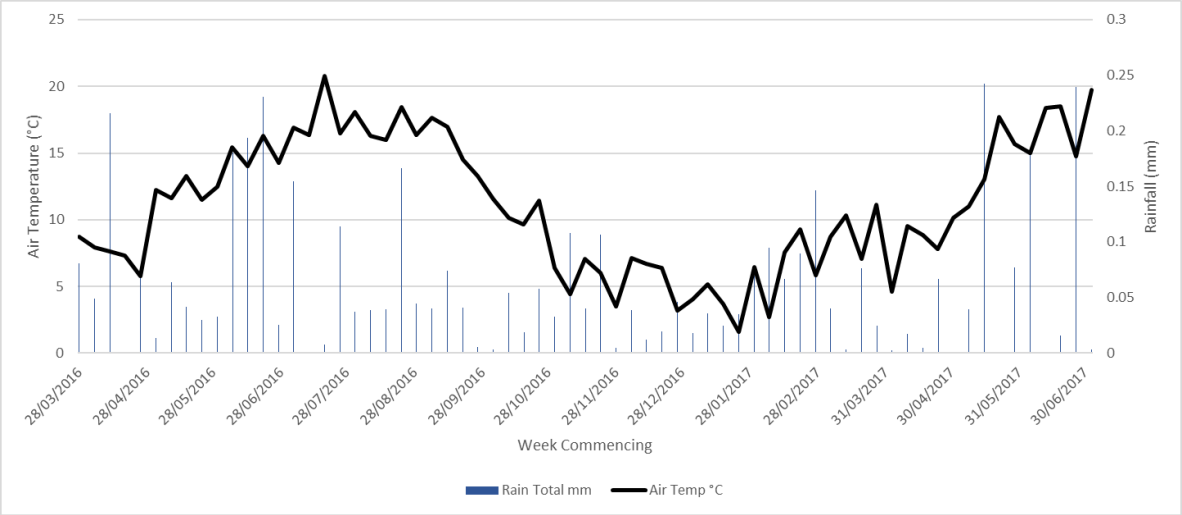


Figure 3.8 Meteorological data from Hackthorn site in the UK between the end of March 2016 and June 2017. The black line indicates average air temperature for that week, and the blue bars indicate the average rainfall (mm) for the same week (secondary axis).

3.3d October 2016 phenotyping under field conditions

There was no significant difference in canopy and shoot heights of the selected plants ($p = 0.154$ & 0.174 respectively) in October after one growth season (in the field April-October 2016) (Figure 3.9). Plants from tray D had lowest survival with 4 of the original 15 selected plants remaining, for trays A, B and C, 8 of the original selected plants remained. Plant heights of selected plants measured in either April or October were highly variable with in treatments particularly control treatments (C and D) (Figure 3.10). For heat maps of location of largest plants see appendix 3 & 4.

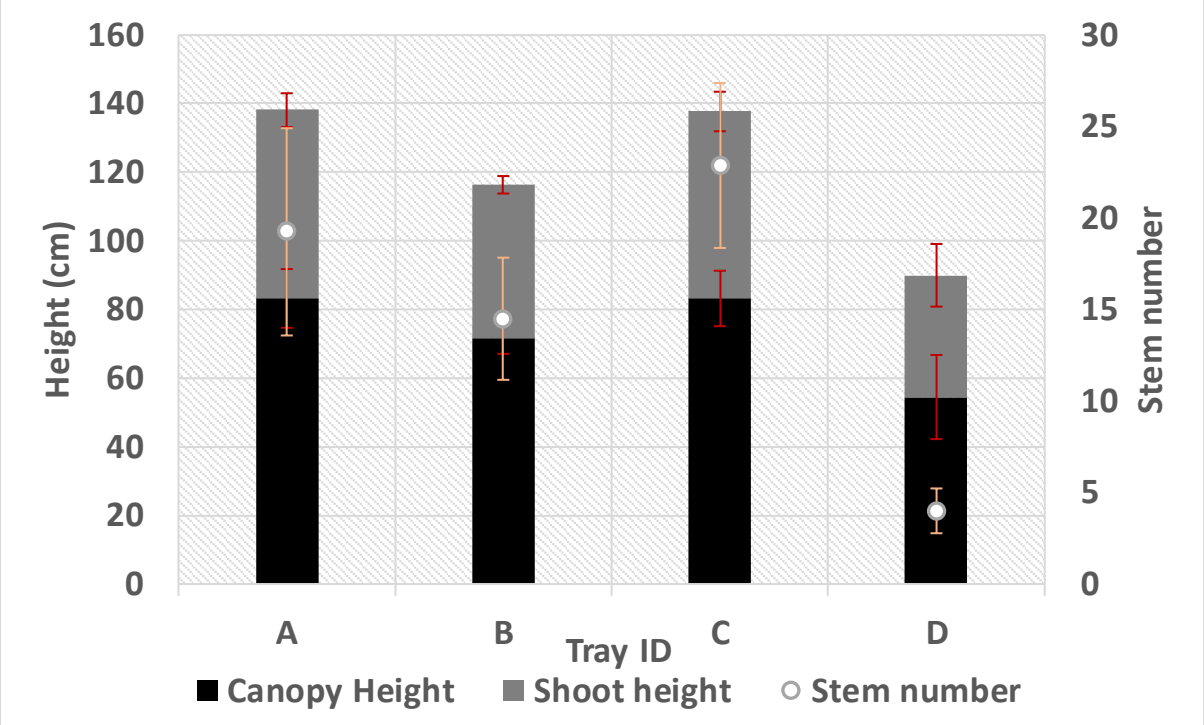


Figure 3.9 Phenotypes of selected plants in October 2016 after 5 months in field, following early growth in the glasshouse with (A and B) and without (C and D) mulch film. Canopy height (black bars), shoot height (grey bars) and stem number (scatter points). Error bars are SE; n = 8 for all except D where n = 4.

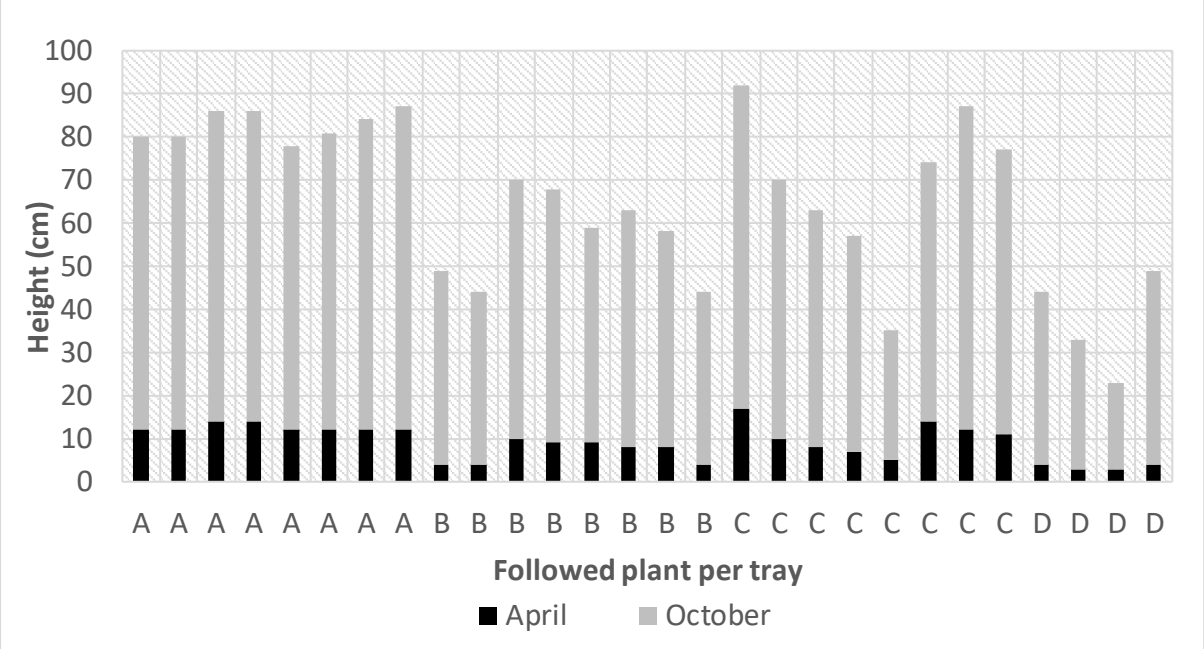


Figure 3.10 Extension of each remaining selected plant in April 2016 prior to planting (black) and the same plant's height in October 2016 (grey). Line included for ease of analysis of trends. A & B are plants from treated film covered trays. D & C are control trays that lacked mulch film.

In addition to selected plants that could be directly compared across the growth season, all plants were measured to more accurately estimate treatments differences in the field blocks. Assessments of significance as a result of row and column revealed a significant difference in shoot and canopy height for the first row of plants over the whole trial, so these were omitted from analysis. There was no significant effect of column. Autumn survival overall was higher in the film covered block by approximately 10% (

Table 3.1). Results of canopy height for all plants in each block showed a significantly higher average canopy height of 77cm in treated plants, compared with 71cm in control plants ($p = 0.042$). Results of shoot height showed a similar trend but this was not significantly different. Stem number was significantly higher in treated plants, with an average of 16 stems per plant, compared with 12.6 in controls ($p = 0.031$).

Table 3.1 Phenotypes from all plants 6 months post planting, in a trial of *Miscanthus* grown with and without mulch film in the glasshouse before transfer to the field in the trial. [1]

Block	Survival rate (%)	Canopy Height (cm)	Stem height (cm)	Stem number
Treated (film)	86.5	77.48 (±2.01)	50.52 (±1.47)	16.09 (±1.16)
Lowest value		17.0	7.0	1.0
Highest value		115.0	85.0	65.0
Control	71.4	70.96 (±2.53)	46.83 (±1.84)	12.56 (±1.14)
Lowest value		18.0	11.0	1.0
Highest value		121.0	92.0	40.0
Significance	n/a	p = 0.042	n.s	p = 0.031

[1] *Phenotypes were assessed in October 2016. Block 1 (germinated under mulch film for one month), Block 2 (control plants with no film). Survival percentage is based on the number of surviving plants out of an initial 126 planted per block. Average shown plus SE and range from each block are shown. Means were compared by students T test.*

3.3e Correlations in growth parameters pre- and post- field planting

Phenotypes of selected plants were combined, ignoring treatment, and measurements in April correlated with those in October to test how well phenotypes at planting predicted those after 5 months field growth. There was no significant correlation (R^2 - 0.033) between spring and autumn extension growth (Figure 3.11A). Extension growth at planting was not correlated with stem counts (R^2 – 0.0185) (Figure 3.11C). Stem number at planting did not correlate with either stem number (Figure 3.11B) or extension growth (Figure 11 D) in October (R^2 – 0.0326 and . R^2 – 0.0657 respectively).

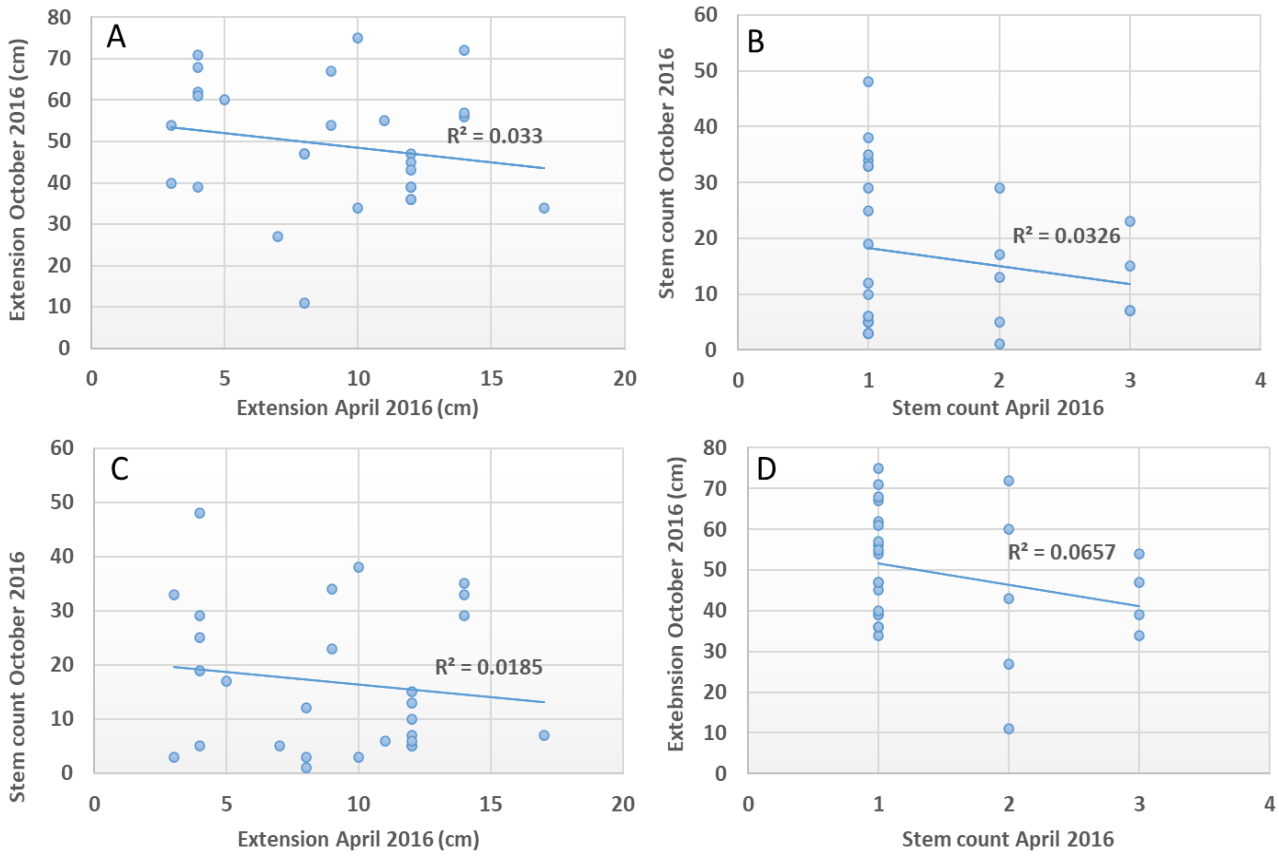


Figure 3.11 Correlation of stem extension and stem number at planting in April with the same phenotypes measured in the same plants after 5 months field growthR^2 value for each trend line is shown on each graph.

3.3f Harvesting of randomly selected 25 plants from each block in February 2017

Mulch film had no significant effect on fresh weight biomass yield at spring harvest ($p = 0.572$, F value = 0.324) (Figure 3.12A). Similarly there was also no significant effect of mulch film on dry biomass yield ($p = 0.464$, F value = 0.045) (Figure 3.12B). Moisture content of spring harvested biomass was similarly not significantly affected by mulch film treatment ($p = 0.065$, F value = 2.805) (Figure 3.12C).

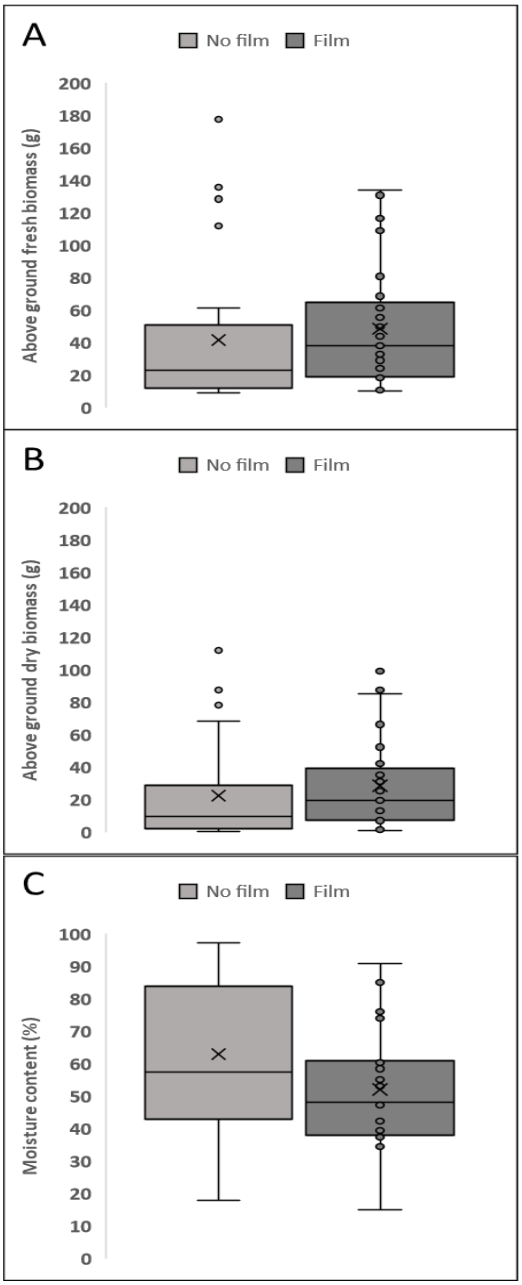


Figure 3.12 Harvest data from plants germinated under mulch film for one month (Treated, light grey), and plants lacking film (Control, dark grey). Biomass parameters measured are above ground fresh biomass (A), above ground dry biomass (B), and moisture content (C) from 25 randomly selected plants within each treatment block.

3.3g Phenotyping assessments of all plants in November 2017

The number of plants growing on the field was assessed in autumn of 2016 and 2017 after each of two growing seasons subsequent to the Mulch film treatment. After the second growth year, there was no further loss in plants compared to surviving plants in 2016 in the mulch film treated block (86% survival rate). Survival rate in the control treated block reduced from 71% survival in the autumn following planting, to 63.5% in the following autumn (Table 3.2).

Within treatment variance was high for all growth parameters measured in spring 2017 for both canopy height (Figure 3.13A), and stem count (Figure 3.13B). There was no significant difference in canopy height between mulch film treated plants and control plants in autumn 2017 ($F(1,188) = 1.289$; $p = 0.258$). Average shoot height was also not significantly different between treatments ($F(1,188) = 0.442$; $p = 0.325$). Stem number was not significantly affected by treatment at the end of the second growth year ($F(1,188) = 0.029$; $p = 0.864$), with both treatments having a comparable stem number of approximately 48 stems per plant on average.

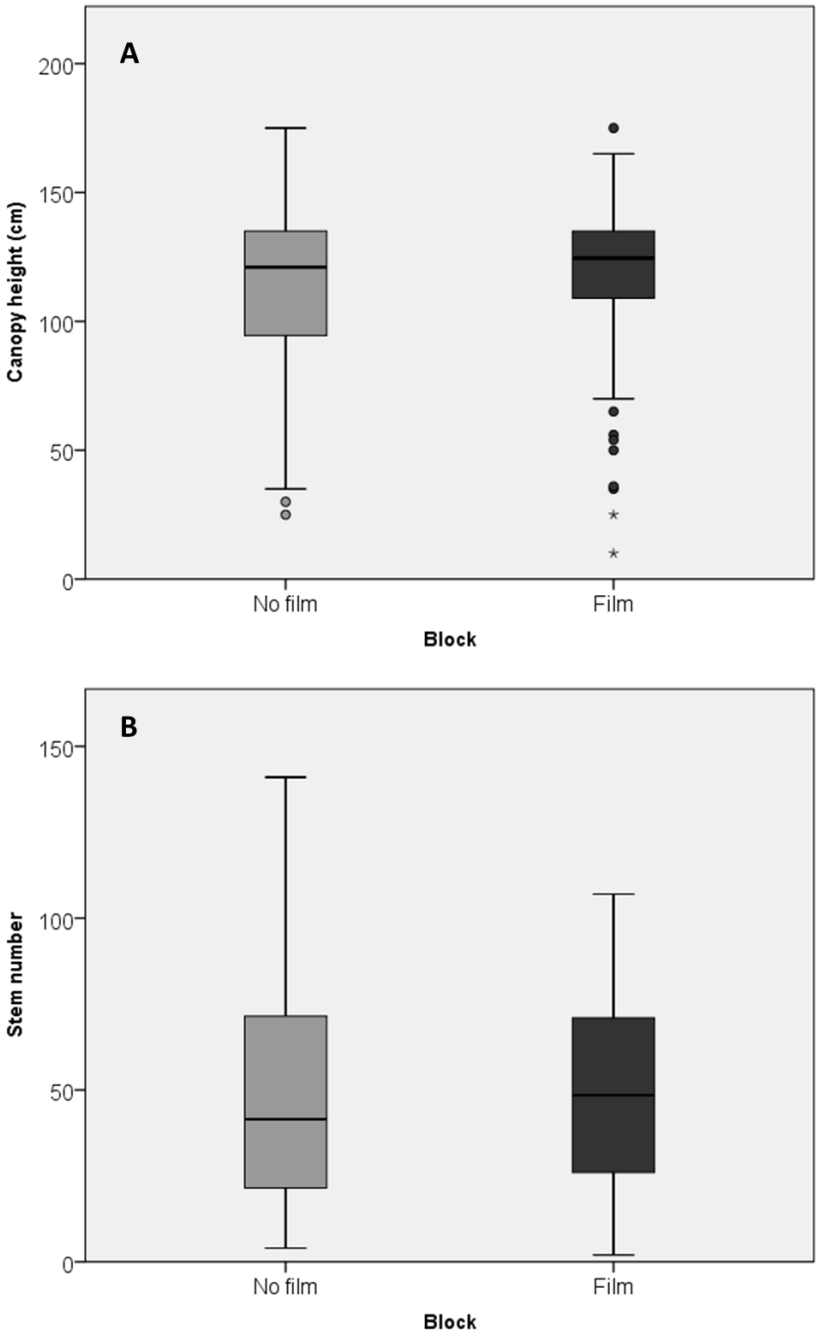


Figure 3.13 Stem growth after two growth seasons of *Miscanthus* plants germinated under film (treated) and lacking film (control). Canopy height (A) and Stem number (B) were assessed from all plants in November 2017 (treated block n = 110, control block n = 80).

Table 3.2 Phenotypes from November 2018 of all plants in a trial of *Miscanthus* grown with and without mulch film covering in the glasshouse, before being field planted. [2]

	Survival rate (%)	Canopy height (cm)	Stem height (cm)	Stem number
Treated	86.5	118 (±2.7)	100 (±2.6)	48 (±2.5)
Stdev		26.7	27	27
Control	63.5	113 (±3.7)	95 (±2.6)	48 (±3.7)
Stdev		32.9	30	33
F value	n/a	2.426	0.998	3.609
P value	n/a	0.258	0.325	0.864

[2] *Survival percentage based on number of surviving plants of initial 126 planted per block. Canopy height, stem height and stem number were taken on all plants, and the average shown plus StdE. Standard deviation is also shown. Statistical analysis was done using students t test*

3.4 Discussion

The optimisation of *Miscanthus* seedling growth in plugs under glasshouse conditions is a new but crucial area for improvement in current *Miscanthus* propagation techniques. Rhizome propagation is slower, more costly and less scalable than the new seeded variety propagation but is also much more developed and understood. The prospect of direct seeding of *Miscanthus* seeds into field plots is a long term goal, but currently exhibiting poor establishment and survival as a result of small seed size and low nutrient reserves, coupled with adverse weather conditions in spring in temperate climates (Ashman *et al.*, 2018). It is likely that direct seeding requirements for more favourable climates will differ significantly from those in more temperate regions, such as the UK. Regardless of location, however, sowing at the earliest point possible will be vital in order to maximise the growth accumulation season, and produce maximal biomass (Hastings *et al.*, 2017b). To overcome the issues associated with both of these propagation methods, a compromise has been reached, whereby *Miscanthus* seed is instead sown into controlled, high temperature glasshouses, to meet the *Miscanthus* seed high thermal requirements. Further optimisation within these environments to produce the most vigorous plug plants is one of the main themes of this PhD project. In this experiment applying a standard agronomic practice for field grown crops to glasshouse grown *Miscanthus* by germinating seedlings with and without a clear mulch film covering was tested. The effects of treatments on germination rate and plant vigour under glasshouse conditions and how any such differences related to subsequent performance in field trials was assessed.

It was hypothesized that addition of mulch film would increase germination percentage and rate. As predicted, effects of film did increase germination rate (Figure 3.5). After approximately 2 weeks, control trays caught up in terms of number of plugs with a germinating seed. The addition of mulch film resulted in faster growth rate and maturation differences such as the production of taller stems, although leaf number was seemingly unaffected (Figure 3.6). Colder soil temperatures can slow or limit seedling emergence and growth (Simon *et al.*, 1976). It is likely the application of the clear film acted as a second greenhouse on top of the seeds. Air and soil temperature under the film was likely increased, though this was not measured but has been documented in other studies. In a recent publication testing the effects of mulch films on seeds grown under direct seed sowing

methods in field trials, results showed average daily soil temperatures were much higher under mulch film than in control plots, but that volumetric moisture content was variable, based on the soil conditions prior to covering (Ashman *et al.*, 2018). Such changes in the seedling environment may better match the thermal requirements of tropicalized small seed such as *Miscanthus* seed. Under glasshouse conditions, the positive result of this additional heating agent would be variable, and subject to the ambient temperatures of the outside environment. Glasshouses easily go over set temperatures under warm conditions with direct sunlight, and can be difficult to cool, a factor of less concern under UK climates but still possible on particularly warm days. While *Miscanthus* seedlings have a tendency towards the higher temperature requirement due to their tropicalized origins (Clifton-Brown *et al.*, 2011), this requirement will differ for different genotypes and species, ensuring a need for understanding of the prerequisites of a specific species. Studies have proved that adequate soil moisture strongly influences post germination growth (Fay and Schultz, 2009). The film would likely have also increased the humidity and available water to seeds, reducing the rate of soil drying and evapotranspiration, this may be particularly useful for plug plants which grow in very small volumes of substrate and therefore are more likely to experience short bouts of drying especially once transpiration increases as large leaved plants are established. Gas exchange, oxygen, and CO_2 levels were not measured under the film, but this could also be a significant factor affecting the available resources to the seedlings. By the time mulch film was removed at approximately 4 weeks post sowing, treated seedlings were significantly taller than control seedlings (Figure 3.6), a difference that can also be seen visually in photographs in Figure 3.2. This remained the case throughout the period of growth in the glasshouse. After three weeks in a polytunnel to 'harden off' the morphological differences between film and non-film trays were less apparent, although the height and survival rates were still lower in the plants lacking establishment under film. This "catching up" process is interesting, suggesting that plants tend to reach a similar morphological stage after a period of time regardless of additional treatments, although this of course depends on the severity of the treatment.

It was hypothesized that application of film would produce more homogenous growth at the young seedling stage due to its success in the establishment and survival in field planted seedlings. This would have been easier to assess if more plants per tray had been measured

over time to reduce the impact of plant loss from the selected plant sample that were followed throughout the experiment. However, results shown in Figure 3.10 do suggest a slightly greater level of homogeneity within the plants from mulch film treated trays, with the exception of some outlying individuals, with control plants appearing more variable in terms of plant height, both pre and post planting.

Survival rate over the first growth season was considerably better within the treated plot, as opposed to the control plot containing plants which were not covered with mulch film in the glasshouse. Canopy height and stem number after the first growing season were significantly higher in mulch film treated plants. The variation within both blocks was high for all parameters measured. Canopy heights in the mulch film treated plants ranged from 17cm to 115cm and from 18cm to 121cm in the control plants. Stem number ranged from 1 to 65 in mulch film treated plants, and 1 to 40 in control plants. This indicated high levels of heterogeneity of first year growth and to an extent this is regardless of the treatment. Assessments of potential significant differences as a result of row and column were statistically analysed using ANOVA. The design of the single blocks for film and no film treated plants could have been improved by use of a multiple blocking, replicated design, to reduce the risk of gradient effects, but was not implemented at the time. There was no difference as a result of column, however the first row of plants did have significant differences, but only in terms of plant height. Stem count was unaffected. This could be a result of the high heterogeneity seen within blocks, or more likely the effects of prevailing winds reducing front row plant height, but having little effect on the amount of stems, suggesting an effect of thigmomorphogenesis (Jaffe, 1973). As a result, the front row of plants was removed from further analyses.

Crucially there seems to be little correlation between the height of plants at planting, and the height of the same plant in October. Correlation graphs confirmed this lack of an expected association between measurements at different times (Figure 3.11).

Harvest results of the whole plant after the first winter produced variable results within treatments, and no significant differences between treatments. First year *Miscanthus* harvests are typically variable, and are not yet economically viable as full yield potential is not reached until approximately year 3 (Jeżowski, 2008). Most screening programmes agree that biomass and yield quality of a genotype or sward cannot be reliably predicted based on the

first year assessments (Clifton-Brown and Lewandowski, 2002); however, being able to produce a homogenous, strong field crop in the first year would likely be hugely beneficial for subsequent years growth, winter survival and economic yield. Assessments of moisture content at harvest indicated a trend towards lower moisture contents in film germinated plants, although it was not deemed statistically significant. Moisture content at harvest is closely associated with the timing of autumn senescence (chapter 7), with reduction in moisture content strongly affecting crop quality and yield, with particular emphasis on moisture content at harvest (Robson *et al.*, 2012). High moisture content is not ideal at harvest, due to its impact on drying time, post-harvest spoiling, combustion efficiency, transport weight and end use quality (Mos *et al.*, 2013). Developmental advances as a result of establishment conditions could be a key factor in the acceleration and efficiency of senescence in autumn, and resultantly, the quality of the harvest offtake.

At the end of the second growth year in 2017, all plants were assessed again for the same growth parameters as the previous year. The survival rate in the control block declined by approximately 10%, whereas no further reduction was seen in the mulch film treated block. No significant differences were observed in the phenotypic data in the second year, although the variability remained high within both blocks. Stem number counts in control plants ranged from 4 to 141 stems, and in treated plants was between 2 and 107. Canopy heights ranged between 25cm and 175cm for control plants and 10cm to 175cm for treated plants. This level of heterogeneity is not optimal for a second growth season, potentially affecting biomass yield and economic return hugely. Physical morphological characteristics are not always a reliable indicator of the internal physiology of a plant; for example, greener leaves do not necessarily reflect higher rates of photosynthesis (Fleischer, 1935). The unmeasured impacts on physiology may have significant implications for subsequent growth, which are masking the impact of any morphological differences measured.

3.4a Concluding remarks

It is vitally important that the factors affecting plant size from the plug phase through to harvest be understood, if seeded hybrids are to become a competitive propagation method for the desired uptake of *Miscanthus* within the agricultural community. This assessment suggests that while encouraging good germination and growth under glasshouse conditions is an achievable and practical goal when utilising growth under film, the logistics of

maintaining competitive viability under field conditions is much less well understood. Using mulch film to improve temperature and moisture conditions for small seeds such as *Miscanthus* can be used to great effect under field conditions, although direct sowing is still too unreliable to be commercially viable. Using it under glasshouse conditions in the currently more feasible plug planting technique, had the effect of developmentally advancing the resulting progeny, when compared to those grown without film, which took longer to germinate and establish. These differences could still be seen under field conditions, although the gap in growth between the two treatments appeared to narrow over time. Developmentally, plants grown under film appeared marginally, but not significantly, ahead of control plants, based on the observed slight reduction of average moisture content in harvest biomass. This developmental advance could have positive effects further down the supply chain, and cannot be underestimated, even with the lack of a statistical significance. Overall, use of additional film at the germination stage, as well as at field planting should be considered as a potential, and relatively inexpensive form of germination and establishment advancement. If used, growers should be mindful of the potential large increases of temperature noticed under film, and use should be carefully monitored under bright sunshine and high temperature environments.

Further work should be undertaken in this area, covering in more depth, the phenology of an optimal seedling, and the environmental conditions that could alter seedling morphology from young seedling, to more mature field grown plant. To account for environmental heterogeneity, it would be best practice in future studies of this kind to apply a more gradient centred approach, using more block designs or randomisation in both the glasshouse and field phase of assessment.

4 Beneficial bacteria – experimentation on the effects of endophytic additions of *Herbaspirillum frisingense*, and *Pseudomonas fluorescens* on the growth and vigour of seeded *Miscanthus* hybrids and clonal *Miscanthus giganteus*

4.1 Introduction

One important characteristic of next generation energy crops such as *Miscanthus*, is the ability to thrive on lesser-valued (marginal) agricultural land, thus reducing competition for land with food production (Valentine *et al.*, 2012). A report by McCalmont *et al.* (2017) assessed the potential UK land availability for growing *Miscanthus* in the UK, and noted that the 2012 UK Bioenergy Strategy (DECC, 2012) suggested a total growing area for *Miscanthus* alone to be in the region of 0.72 – 2.8 Mha. They also stressed the importance of utilizing energy crops on areas of land where it 'makes most sense'. Therefore, McCalmont *et al.* (2017) suggest that it would be good practice for farmers to assess where on their land yields of the more standard crops are the poorest, and the effort, labour and chemical input required to make the land profitable the highest. This land could then be considered as

detracting effort and labour force from other areas of the farm and given over to at least one type of low input perennial energy plant. This type of less-favourable land can be typically characterized by poor-quality soil with a stony tilth, steep incline, and/or subject to abiotic stresses such as drought, flood and low nutrient availability. As a result, it is often necessary, especially during the plant establishment phase, to add fertilizer to the soil until the soil nutrient status begins to improve over time. However, use of fertilizer can detract from the sustainability of the crop due to the high embedded energy requirement, and the overall economics of biomass production (McCalmont *et al.*, 2017). Desirable energy crops will predominantly harbour certain traits such as efficient, low-cost establishment and rapid growth and ideally this would be achieved in the absence of chemical inputs (Farrar *et al.*, 2014). Rothballer *et al.* (2008) agree that a key element of cultivation of energy crops such as *Miscanthus*, is that it is only sustainable, and hence worth doing, if costly agricultural procedures, agrochemicals and fertilization can be minimized. *Miscanthus* species in general have been seen to have a low requirement for nitrogen additions (Christian *et al.*, 2008). However, on particularly low-grade soil, fertilization may be required. Using chemical inputs to boost crop yields has been a standard method within agriculture and horticulture for decades; however, with an increasing focus on a green revolution across the western world, more sustainable and natural methods are now becoming the preferred choice where practically possible (Vessey, 2003).

One way to potentially improve yields on low-grade land, without the use of chemicals and artificial fertilizers is to harness and manipulate already naturally occurring plant growth promotion techniques, using plant – rhizosphere interactions using a technique known as biofertilization'. Biofertilization is an umbrella term referring to the use of natural inputs to improve soil health, crop productivity and reduction of chemical fertilizer. These inputs can include decaying remains of organic matter, domestic sewage, animal manure and microorganisms (Carvajal-Muñoz and Carmona-Garcia, 2012). Soil microorganisms as a means to improve availability and uptake of vital nutrients for plants are an increasingly popular method within agriculture for replacing synthetic, chemical inputs (Vessey, 2003). Implementation of these sorts of solutions will focus on the manipulation of known beneficial plant – microbe interactions, which have potentially been reduced by the uses of the artificial fertilizers (Farrar *et al.*, 2014).

4.1a The Rhizosphere

The area of soil closest to the root is the most crucial part for governing plant – microbial interactions. This small region of soil is known as the rhizosphere (Figure 1), and in comparison to soils such as composts, it is extremely rich in nutrients as a result of root exudations and deposits (Spaepen *et al.*, 2009). The result of this is that the numbers and species of bacteria surrounding the plant roots within these systems will be significantly higher than those grown in other types of soil. These bacteria are called 'rhizobacteria' and based on observations of their effects on the plant hosts, can be classed as beneficial, deleterious or neutral rhizobacteria (Spaepen *et al.*, 2009). Relationships between the plant and the microbe exist for both fungi and bacteria, and can be further classified in a variety of ways, mainly based on location and relationship to the plant. Soil science and the importance of the soil and soil micro-organisms has been known to a certain degree, since ancient times (Bhattacharyya and Jha, 2012). The study of soil microbiology only dates back to the 19th century; however, land workers were aware of some relationships between crop and soil long before. In the current, more modern times, uses of bacteria to improve plant growth include a range of applications within agriculture, forestry and environmental restoration (Lucy *et al.*, 2004).

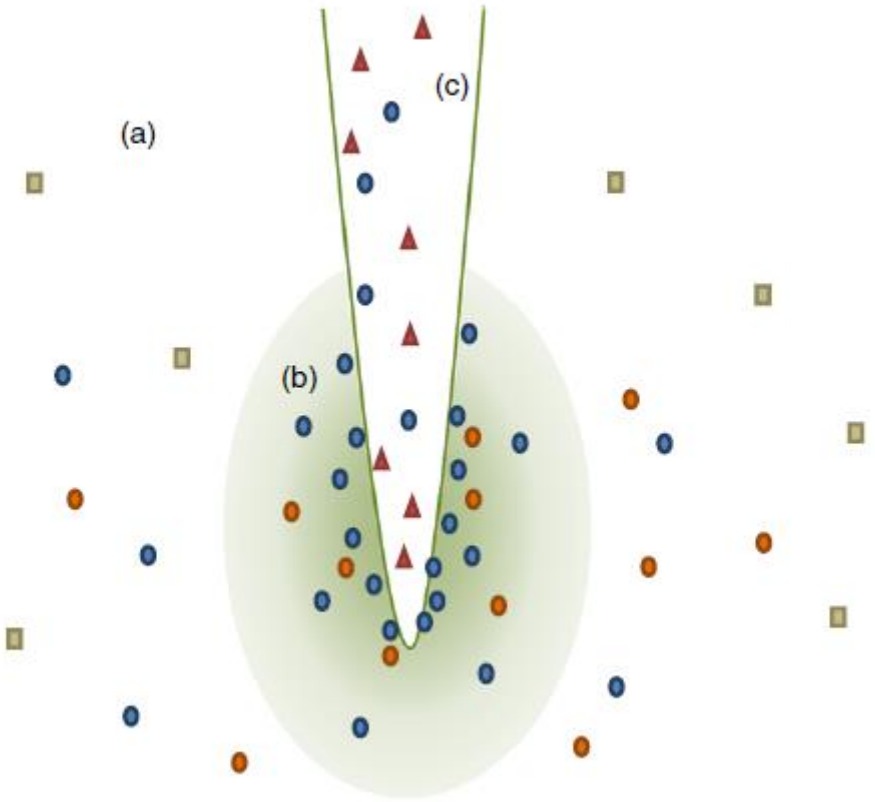


Figure 4.1 Diagrammatic representation of root, rhizosphere and soil system of fungi and endophytic colonization. Area (a) depicts free living bacteria within the soil. Area (b) depicts the root rhizosphere and the bacteria populating it. Area (c) depicts colonies within the root itself. Blue circles are bacteria able to enter the host. Red circles are bacteria attracted to the rhizosphere but unable to enter the plant. Red triangles signify more specialized microbes, and free-living squares and circles signify more generalist species. Picture courtesy of Farrar et al (2014).

4.1b Benefits and Mechanisms of Plant Growth Promoting Bacteria (PGPB)

Under most conditions, it is likely that plants are in constant interaction with a huge range of soil microbes, bacteria and fungi within the soil, both benign and parasitic. Those that are seen to have beneficial effects can also be named 'plant growth promoting bacteria' (or PGPB). These free-living plant growth promoters are known to be beneficial to plants in a number of ways, some more understood than others. Known direct benefits include the provision of bio-available phosphorous for plant uptake, the fixation of nitrogen, sequestration of iron, and production of plant hormones such as auxins, cytokinins and gibberellins. Indirect benefits include increased pathogen resistance within the plant roots by way of antibiotic resistance against harmful bacteria, reduction of available iron to phytopathogens, and synthesis of fungal cell wall lysing enzymes (Lucy *et al.*, 2004).

These interactions are incredibly complex, dynamic, and difficult to categorize based on the constantly changing nature of the relationships (Farrar *et al.*, 2014). Plant roots in particular are colonized by a huge variety of micro-organisms including those that can be classed as either mycorrhizal fungi or endophytic microorganisms; however, there are some that span both of these classes (Mayerhofer *et al.*, 2013). There is a vast array of microbes living within plant tissues which do not cause any signs of disease, and these are broadly named 'endophytes'. These types of bacteria reside within specific plant tissues such as within the cells or intracellular fluids, typically entering the plant through spaces between root cells or junctions between root hairs and root structure (Compant *et al.,* 2010).

This study is most concerned with the endophytic bacteria branch of microorganisms. Endophytic colonization is considered an extremely important trait of an effective PGPB because endophytes have a more intimate and stable relationship with their plant hosts (Rothballer *et al.*, 2008). It is generally accepted that the endophyte relationship lacks three key features in comparison to mycorrhizal symbioses. Firstly the lack of a cellular interface, where one can see the occurrence of specialized structures such as hyphae or arbuscules; secondly the lack of synchronized development between the plant and fungi in question; and thirdly the lack of a significant benefit for both partners (Brundrett, 2006). The term "endophyte" has been used to describe types of microorganisms that exist within plant tissues, both in roots and systemically throughout the plant, without causing a negative response from the host, or eliciting any harm (Mayerhofer *et al.*, 2013). However, this is controversial, as influences of fungal root endophyte colonization on the host plant has given rise to both negative (Tellenbach *et al.*, 2011) and positive (Bhattacharyya and Jha, 2012) results across the plant world. Compant *et al.* (2010) describe endophytes as a subset of soil bacteria, which effectively colonise the plant without triggering a defence response. They must also be able to exist in a free-living state in order to transition from soil media to the plant host. Plants with a positive bacterial relationship will be observed to gain multiple benefits from this co-existence including increased germination rates, root growth, yield, grain yield, leaf area, chlorophyll content, magnesium content, nitrogen content, protein content, hydraulic activity, tolerance to drought, shoot and root weights and delayed leaf senescence (Lucy *et al.*, 2004). The added benefit as mentioned earlier is improved disease resistance that is often observed alongside other numerous benefits. For the most part, a

plants ability to survive and thrive is due to plant genome and adaptation. This however can be a limitation as it is slow to evolve and adapt in comparison to the potentially rich microbiome associated with the species. This reservoir of dynamic interactions and species can provide additional functionality to that already seen from the genome, aiding the plant when growth circumstances change or become less than favourable (Cope-Selby *et al.*, 2017).

While this may sound like the ultimate dream for crop production, the reality can often be incredibly complicated in terms of best practice and species matching. The artificial uses of PGPB to increase crop yields can be highly variable and inconsistent, particularly when comparing between laboratory, greenhouse and field grown plants (Mishustin and Naumova, 1962). It is suggested that best practice for the use of PGPB in growth promotion is matching the beneficial bacteria to their preferred crop. This is true especially when different plants are cropped in soils with the same bacterial composition, therefore ensuring the importance of identifying bacteria that have similar growth effects on plants that share the same soil (Schlemper *et al.*, 2018). This highlights the importance of careful selection when choosing bacterial strains for plant inoculation. Schlemper *et al.* (2018) indicated that endophytes isolated from sugarcane were seen to have a positive impact on biomass and plant nitrogen content when the endophyte was inoculated onto plantlets of sugarcane. This specificity cannot be underestimated when researching bacterial interactions and their benefits on agricultural crops. In seeking to apply the benefits of endophytic interactions to *Miscanthus,* endophytic fungi from *Miscanthus* have rarely been characterized for stress resistance or yield improvements. There are more studies using species of closely related genus *Saccarum* which includes sugarcane (Beekwilder *et al.*, 2019) which may identify possible compatible endophytes that are suitable for *Miscanthus*.

4.1c Endophytes used

There are two main endophytes used in the assessments documented here. The first is *Herbaspirillium frisingense*, a diazotrophic betaproteobacterium which has been isolated from C4-energy plants such as *Miscanthus*, Sugarcane and Sorghum (Rothballer *et al.*, 2008, Straub *et al.*, 2013). This endophyte is primarily known for nitrogen fixation. Results of genome sequencing showed that *H. frisingense* has all the genomic requirements to fix nitrogen, while lacking several factors that may contribute to pathogenic characteristics (Straub *et al.*, 2013). Rothballer *et al.* (2008) describes *H. frisingense* as a microaerobic

diazotroph, which invades the intercellular spaces of C4 grass roots, without causing any apparent damage to the host. It is as yet unclear how molecular mechanisms of these bacteria suppress the plant immune system and invade the host; however, there are several reports documenting biofertilization of *Miscanthus* genotypes using *H. frisingense* (Straub *et al.,* 2013, Rothballer *et al.,* 2008). Raaijmakers and Weller (2001) suggest that matching a beneficial bacteria species with the preferred crop should improve root colonisation and biocontrol. Therefore, a preferred PGPB for *Miscanthus* genotypes should ideally be a strain already naturally found in the rhizosphere and soil of *Miscanthus* plants.

The second endophyte used in the assessment is *Pseudomonas fluorescens*, a strain of PGPB most commonly found colonizing roots of potato, sugarbeet and radish (Kloepper *et al.*, 1980). This group of PGPB is utilized for its added defence against pathogens and anti-fungal properties (Kumar *et al.*, 2002). It is thought these strains add to growth promotion of the host by the use of siderophores, molecules which bind to available iron in the rhizosphere (Kloepper *et al.*, 1980) (Gull and Hafeez, 2012).

4.1d Nutriss

The endophyte media used in this experiment came from Nutriss ltd, a company specializing in delivery systems of key endophyte combinations to plants. The Nutriss brand aims to efficiently deliver microorganisms to enhance plant growth, yield and resistance to diseases, pests and drought. There are typically three main ways to deliver endophytes exogenously to a plant as can be seen in Figure 4.2. The foliar sprays and seed treatments predominantly only deliver one microorganism at a time, while the third method, utilising a physical carrier, can deliver numerous species at the same time. Nutriss have developed a novel carrier to deliver microorganisms to the plant that is called the 'Simulated Rhizosphere' or SRS, which can be inoculated with a range of synergistic microorganisms specific to the crop in question and its environment. The material is a micro-porous sponge like structure (patented design), which can then be inoculated with the endophyte medium in a sterile environment and cut into small granules before being added to the growth medium. Once growing, the fine root structures grow through the micro-pores of the structure, allowing direct contact with the available microorganisms (Figure 4.3). This method has been used to great effect in enhancing growth and resistance in numerous species including bananas in Indonesia, cocoa and oil palm (company-supplied information).

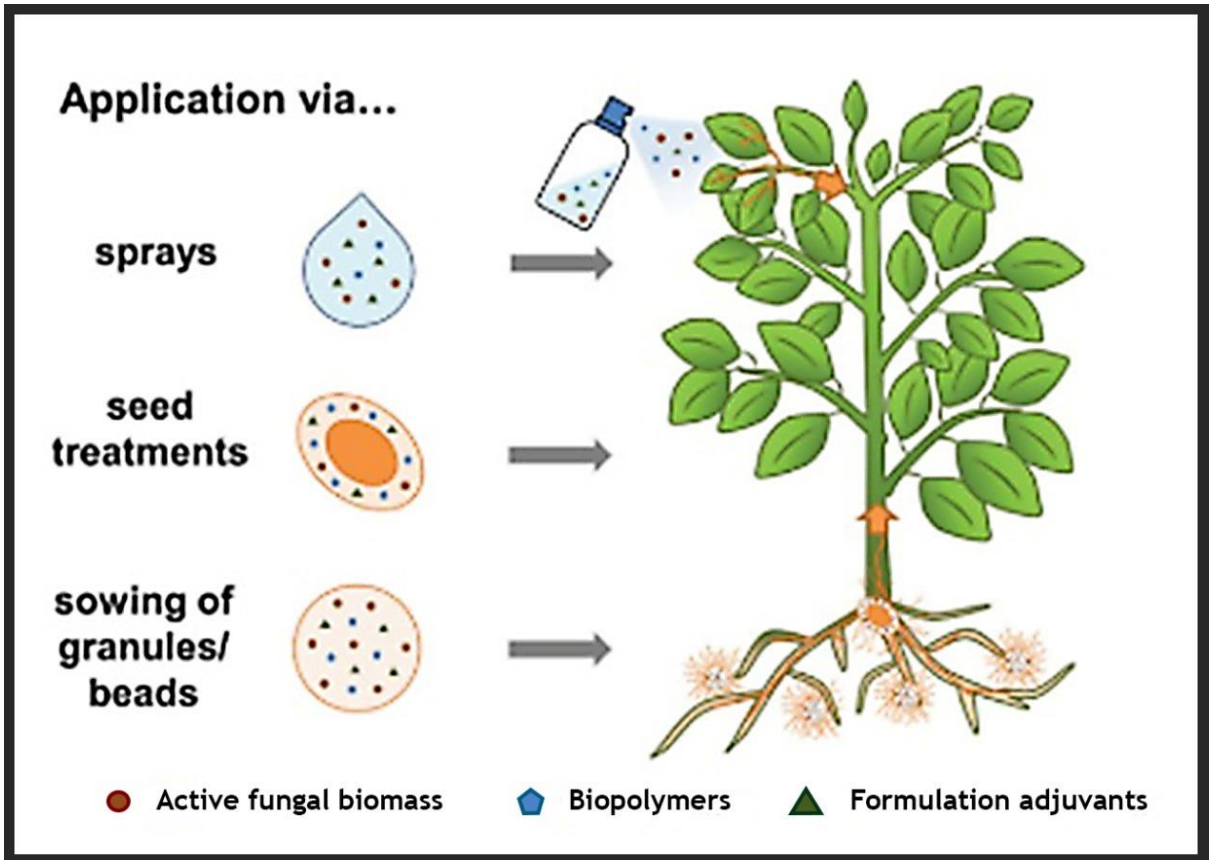


Figure 4.2 Three most common methods of application for endophytes. Image from Beekwilder et al., (2019)

Figure 4.3 Young *Miscanthus* roots growing through SRS polymer. Polymer and roots have been washed free of surrounding soil.

The company Nutriss ltd is one of the commercial partners in the *Miscanthus* Upscaling Technology 'MUST' project funded by the UK government that includes Aberystwyth University. The general aim of the MUST project was to improve on and develop innovations for the seed-based propagation of *Miscanthus* through a plug grown plant production line. The use of microorganisms via the Nutriss method to improve establishment and yield was assessed both in smaller glasshouse trials, and at a larger scale in field trials in Lincolnshire over the course of approximately 3 years. Experiment 1 was begun in 2015, as a glasshouse and field-based assessment, trialling the effects of two different microbial growth treatments, and two non-bacteria treatments on plant performance by inoculating SRS with endophyte culture at two different growth stages. The second experiment was started in 2016 and used the optimal endophyte growth stage chosen by the company as a result of experiment 1, to test seedlings of hybrid GNT14 under glasshouse conditions to assess in depth, the seedling growth characteristics and root hair changes. This was repeated the next year for further assessment in experiment 3, and for a subsample of seedlings to be flash frozen for molecular analysis. The fourth experiment was started in 2017 to test the product on multiple seeded hybrids and also on *Miscanthus giganteus* clonal plants under field conditions. The experiments included examining the effects of endophytes on both glasshouse growth of seedlings and mature plant within the field subsequent to SRS treatment.

4.2 Assessment 1

4.2a Methods – Field trial planted in 2015 studying the effects of two bacteria treatments and two control treatments on seedlings of *Miscanthus* seeded hybrid GNT3

Growth Conditions in Glasshouse

Seeds of *Miscanthus* commercial hybrid GNT3 (see chapter 2) were sown on 3/2/2015 in a commercial nursery (Boston, Lincs. UK) at around three seeds per cell, into the then commercial standard 126-well trays with 25 cm^3 soil volume for each seedling. The seedlings were sown into nitrogen-free compost with added granules of the SRS inoculated with endophyte cultures that had been growing for either 48 hours (treatment A) or 24 hours (treatment B) under lab conditions in the company premises. This was a preliminary assessment of concentration requirements best suited to *Miscanthus* plantlets. The optical density of both *H. frisingense* and *P. fluorescens* used in the two concentrations can be seen in Figure 4.4. Two control treatments were added to the design, one in the same nitrogen-free compost (N-) as used in the treated trays, and the second in typical commercial compost with nitrogen (N+) to show any differences because of the poor-quality growth medium. Plants were grown under controlled conditions in Bell Brother's commercial plant nursery in Boston, Lincs for approximately 2 months (Figure 4.5) before being planted by machine into trial fields at Hackthorn UK (Latitude = 53°19'N, Longitude = 0°28'W) (see Figure 4.6).

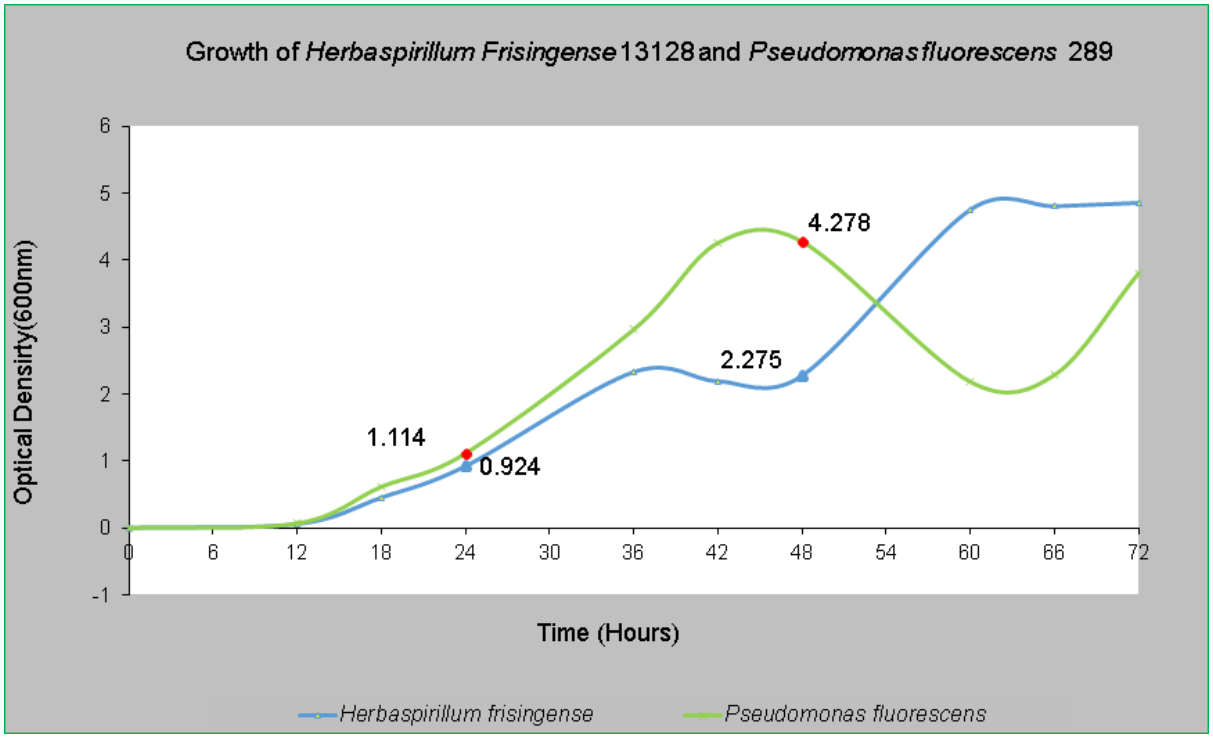


Figure 4.4 Growth of endophyte cultures over 72 hours. 24-hour and 48-hour cultures were added to SRS to test the effect of different inoculation sizes on the growth of *Miscanthus* plants (data provided by Nutriss ltd)

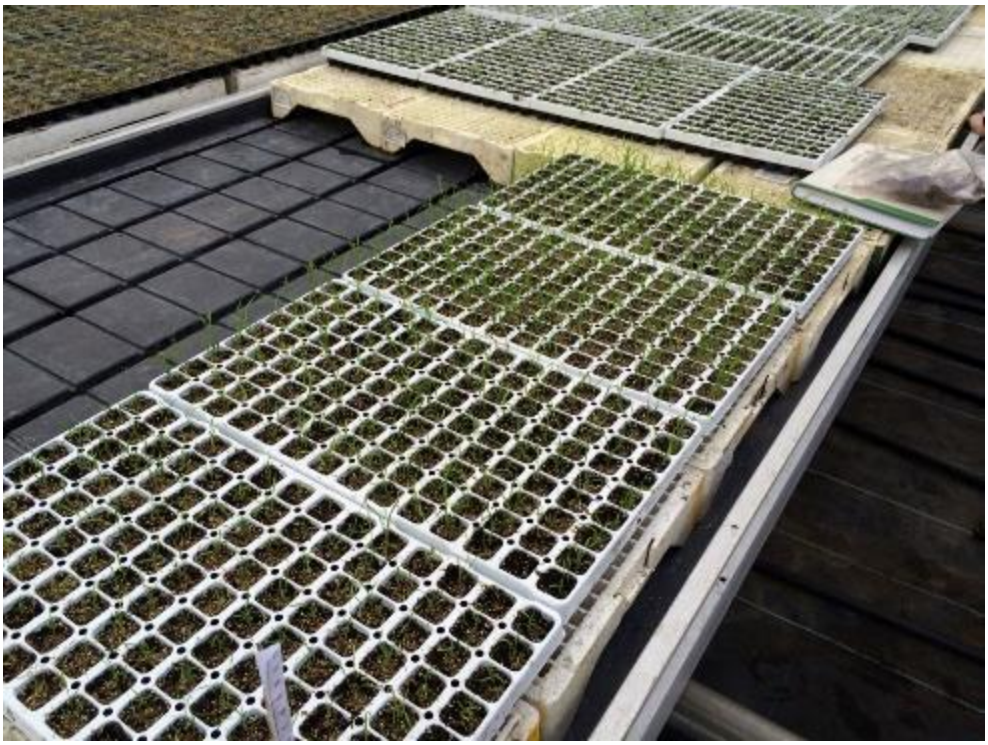


Figure 4.5 plug plants of young *Miscanthus* plants growing in 126 module trays in the commercial nursery in February 2015. Plants were grown for 2 months before being planted in the field; photo shows plants after approximately 2 weeks. Each plug contains approximately 25 cm^3 of soil

Field Conditions

Two weeks prior to planting, plug trays were moved outside in preparation for field weather conditions and temperatures, a process called 'hardening off'. Plugs were hand planted in April of 2015. The four plant treatments were placed into a replicated plot field trial with three plots for each variable, resulting in a 3x4 grid (Figure 4.6). Each replicate contained 4 rows and 5 columns of plugs at 1 metre spacing between rows, and 75 cm spacing within rows. Once planted, plugs were sprayed with a pre-emergent herbicide, watered, and covered with a layer of biodegradable mulch film, to help retain heat and moisture. Plants were allowed to grow through the film, which degrades under sunlight over time, and were weeded when necessary.

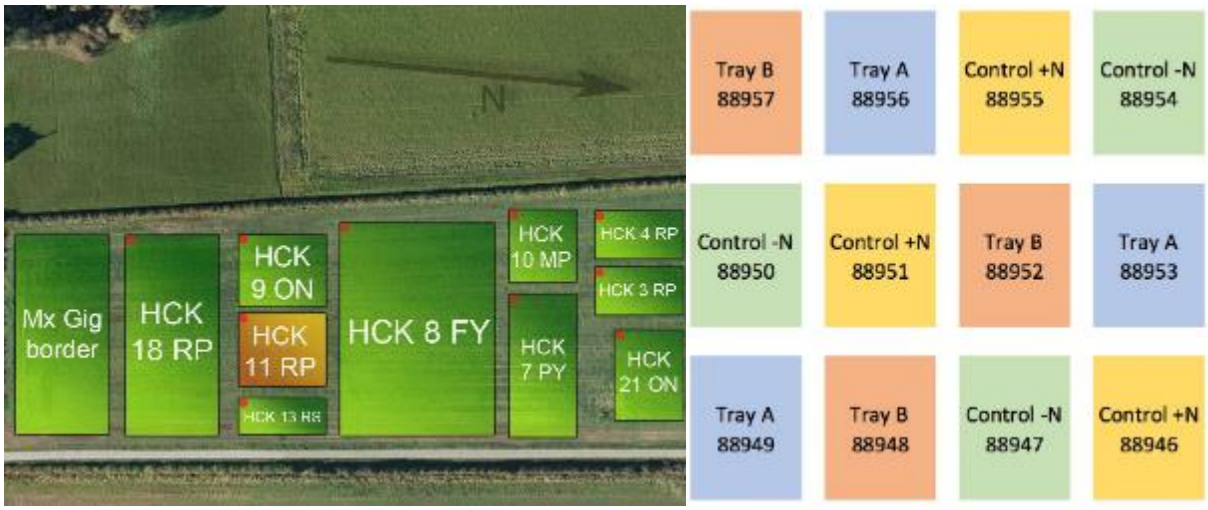


Figure 4.6 Left image shows the trial placement amongst others in the experimental plot fields (approximately 53°19N, 0°28W). Right image shows replicated design of HCK 11 field trial. A is plants grown in 48-hour growth inoculum. B were grown in 24-hour growth inoculum. Each replicate plot contains 20 plants in a 4 x 5-plant design.

Autumn phenotyping

Autumn (end of growth season) phenotype was assessed from 2015 to 2018. Phenotyping involved assessing the same plant characteristics, and on the same plants, although there were some differences in what was measured between years. The first autumn phenotyping was undertaken on ten plants within each of the individual treatment replicates shown in Figure 4.6. Plants chosen came from the central two rows of 5 plants, a practice improved on in later protocols. Parameters measured included die off height, shoot height, canopy height and stem counts (see shared methods chapter 2). Die off height was measured in all years

except 2016. Shoot height was measured in all years, as was stem number. Canopy height was not taken in 2015 but was in 2016, 17 & 18.

Surviving plant counts

A visual count of all surviving plants within a treatment plot, as a percentage of the total planted. Establishment was assessed during the second autumn phenotyping to assess overwintering survival rates of the planting year

Die off height

Taken as a measurement of distance from ground level to the point where stems begin to exhibit traits of early senescence, and starts to transition from green to brown in colour.

Shoot height and Canopy height

Shoot height is measured as the distance in cm from the ground to the top ligule of the longest stem. An assessment of canopy height is measured as the distance in cm from the ground to the thickest part of the leaf canopy at the top of the plant, where the leaves are obviously bending and is approximately the point in the canopy where maximal light interception occurs.

Stem counts

A count of the total number of stems present for each plant. If plants were particularly large, or had produced high numbers of stems, approximately half stem count was used, and doubled for the total stem count.

Spring harvesting

A first-year harvest (Spring 2016) was not undertaken on this plot; however above ground biomass was harvested in the spring of 2017, 2018, and 2019 all subsequent years were harvested for yield. Second year harvest was undertaken on the 9th February 2017. During the harvest all plants were cut from the base, at a height of about 10 cm, with a hedge strimmer and total fresh weight of above ground biomass determined with a tripod and hanging scales. Moisture content was assessed by taking a representative subsample of leaf and stem material, placing it in a labelled paper bag, and weighing the fresh weight of the subsample and bag. Subsamples were then dried at 105°C in drying ovens until a constant dry weight was reached, and the dry weight recorded. In this way the moisture content of the whole

plant was estimated, and the result applied to the fresh weight of the whole plant or quadrat. The maximum plants that were planted in the quadrat area and actual number of plants available for measuring were noted, allowing for an estimation of the biomass of the quadrat if all plants had survived, a method called gap correction. In this way it is then possible to model the expected tonnes of aboveground biomass yield per hectare for each treatment with a reasonable degree of accuracy. Protocols improved over the course of the project and the results for the 2018 and 2019 Spring harvests were based on the inner 6 plants per plot (Figure 8), as opposed to all plants in 2017. Harvesting only the inner 6 plants reduced variability due to edge effects, including weather conditions, and the effects seen from more space available for plants around the edge of the trial, potentially affecting growth and development. Methods for calculating the total wet and dry weight yield of the plot were the same as the first harvest, with an adjusted quadrat area to allow for a smaller number of plants. In later harvests, moisture content analysis was taken from three subsamples per plot, as opposed to one in the second and third harvest year (2017 & 2018). This was to ensure any variability in drying, for example samples placed near the back of the oven being dryer than those at the front, is accounted for. The moisture contents can then be averaged, and the final result used as the correct moisture content overall.

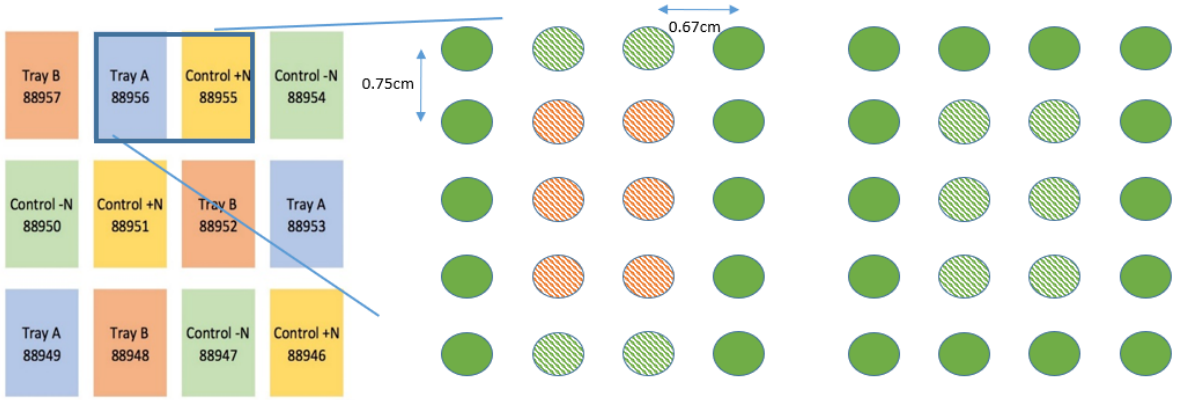


Figure 4.7 Sampling regime of HCK 11 plots. Blue circles represent a plant. In the left plot hashed circles show plants measured during phenotyping in year 1. In year 2, all plants were measured. In year 3&4 only plants coloured in orange were measured. The right block shows the different plants harvested, in. 2017 all plants were harvested in subsequent years only the inner-hashed green plants were harvested.

4.2b Results – HCK 11

Glasshouse growth

Seeds were sown on Feb 3rd 2015 and Figure 4.8 displays the growth over time. Stem extension measurements in Feb showed bacteria 24hr treatment had significantly shorter extension than C+N and 48hr bacteria ($p = 0.007$ & 0.003 respectively). After 7 weeks growth plugs treated with 48hr inoculated SRS had grown to around 90mm on average and were significantly higher than all other populations ($p < 0.01$), which remained around 40 – 50mm. Plugs treated with SRS inoculated for 48hrs continued to be significantly taller than both control treatments throughout the nursery phase. Figure 4.9 consists of photographs taken at the 3-4 week old point, and the 7-week-old point, allowing visual comparison of the differences between treatments at the seedling level.

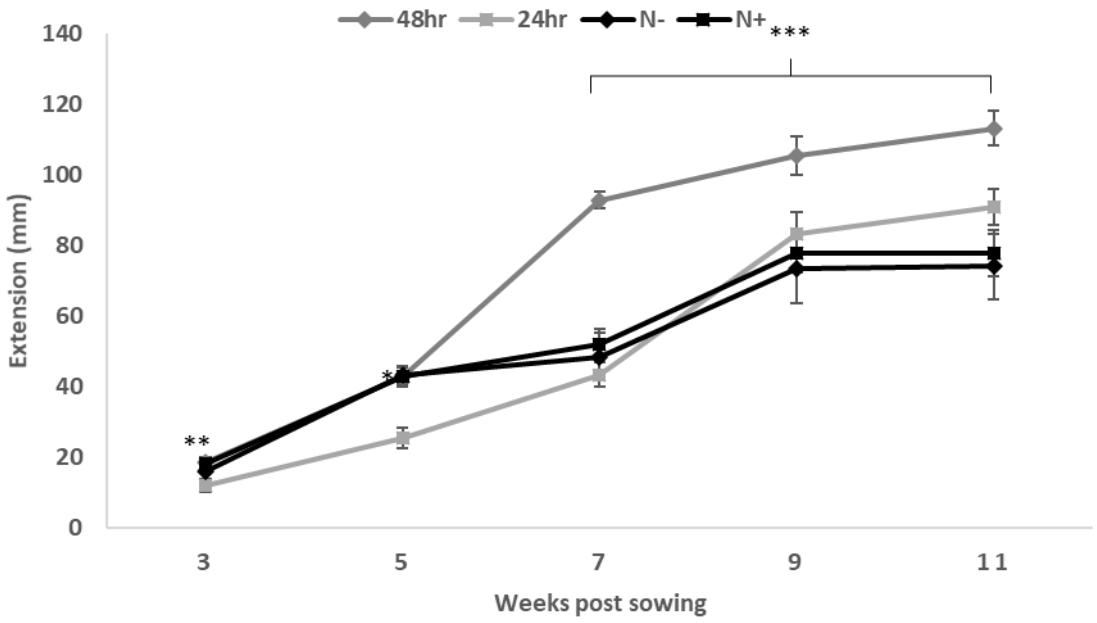


Figure 4.8. Extension growth over 8 weeks under glasshouse conditions. Extension is measured from the base of the stem to the newest ligule. Each line of data is taken from a single tray. SRS treated seedlings are shown in grey (48hr inoculum shown with diamond points, and 24hr inoculum shown with squares) control plants are shown in black (with nutrients are square data points, without nutrients are diamond data points) n = 10 per data point. Error bars show ±1 se

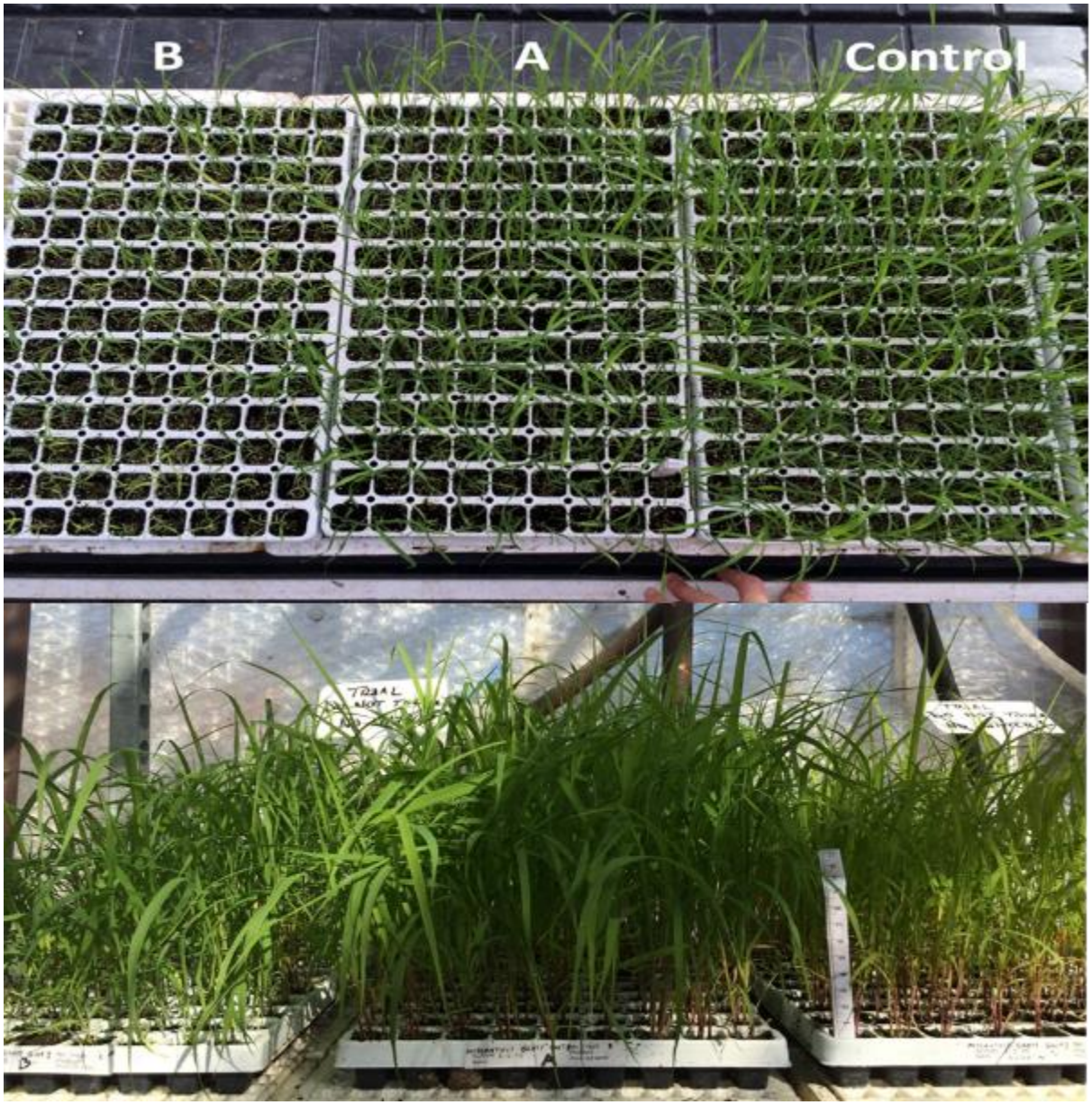


Figure 4.9. Photographs taken of both bacteria trays of seedlings at 3-4 week old stage (above), and later on around 7 weeks old including the control (from L-R, treatments B -24hr, A – 48hr, & Control with zero N)

Phenotyping results

Results of all phenotyping for each year can be seen in Figure 4.10. Not all parameters were measured at each phenotyping but all are there for at least three out of four years.

Shoot height results for the first year revealed a difference between groups of $p < 0.01$. Tukeys analysis revealed that treatment B (24hr growth inoculum) had significantly higher average shoot height of 40.5cm than all other treatments ($p = 0.013$, 0.008, 0.001 for A, C+N & C-N respectively). Second year and third year shoot height was not significantly different between groups. The most recent results (2018) produced a significant difference of $p = 0.006$ between plots. Further assessment with Tukeys test suggests that the C without N group had a significantly lower shoot height (147cm) than A and B bacteria treatments ($p = 0.023$ & 0.006 respectively) with shoot height averages of 173 & 177cm respectively.

Stem counts in the first year showed no significant difference between groups. Number of stems continued to produce non significant results, throughout all successive assessments.

Die off height displayed no significant difference between treatments in 2015 and was not measured in the second-year phenotyping (2016). In 2017 die off height measurements were resumed, but no significant differences were found. In 2018 die off heights were significantly different between groups ($p = 0.002$). Further analysis indicated that the control without N treatment had significantly lower rates of die off (99cm) than A and B treatments ($p = 0.003$, 0.009 respectively at 136cm & 133cm).

Canopy height was not measured in 2015, but was in subsequent years. 2016 & 2017 assessment of canopy height indicated no significant differences between groups. 2018 canopy height had a difference between groups of $p = 0.004$. The differences were found in the control without N treatment, showing significantly lower canopy height of 184cm in comparison to both A & B (213 & 222cm respectively with a significance value of $p = 0.034$ and $p = 0.003$).

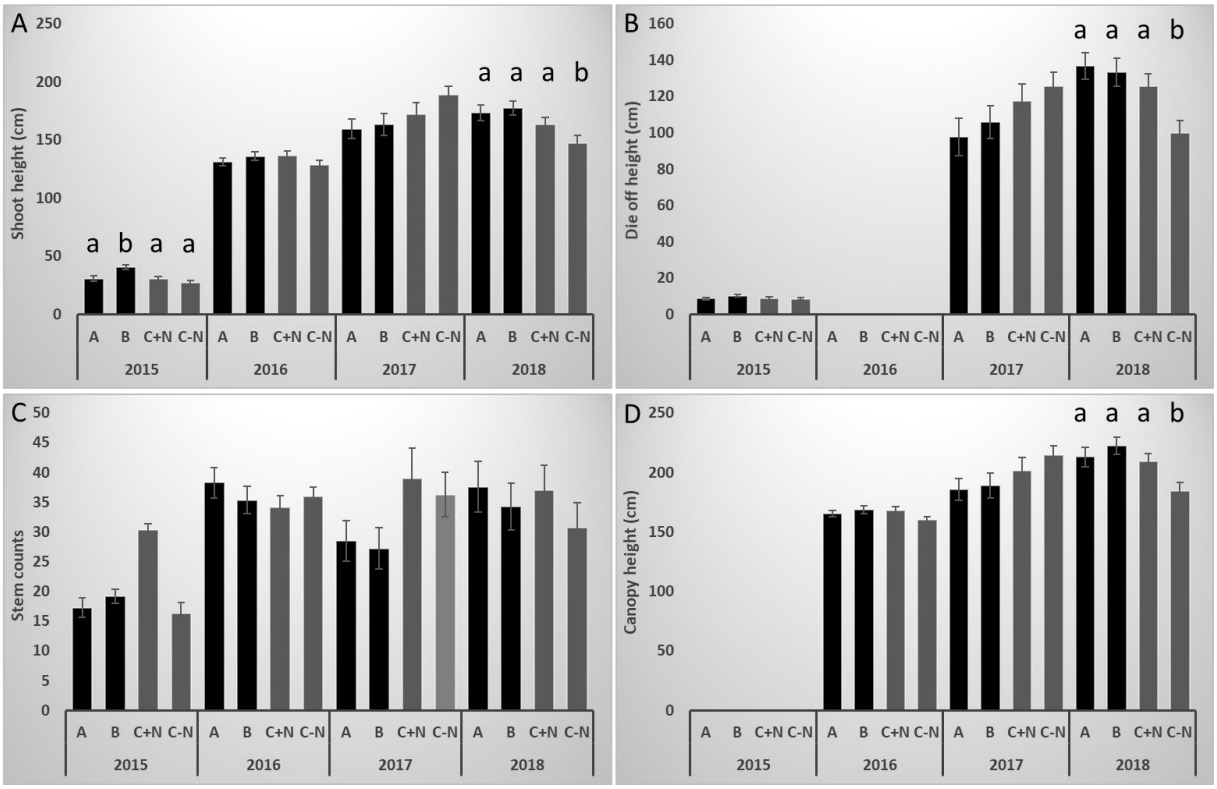


Figure 4.10. Results of Autumn phenotyping over four years. A = 48hr inoculum, B = 24hr inoculum, C+N = control with nitrogen, C-N = control without nitrogen. Chart A – Shoot height. Chart B – Die off height. Chart C – Stem number. Chart D – canopy height. Where data is missing, the parameter was not measured in that year. Each bar is an average of three replicate blocks, with an n of between 8-10 plants in 2015, and 9-19 plants in 2016, depending on plot survival. In 2017 and 2018 n = 6 plants per treatment plot. Error bars show ± 1 se

Survival and establishment

Survival was not significantly different between groups. Lowest survival rates were seen in the 48hr inoculum treatment blocks with an average of 12 out of 20 plants surviving, or 62% (Figure 4.11). The highest survival rates were seen in the commercial standard compost treated blocks, with an average of 17 surviving plants out of 20 (83% survival).

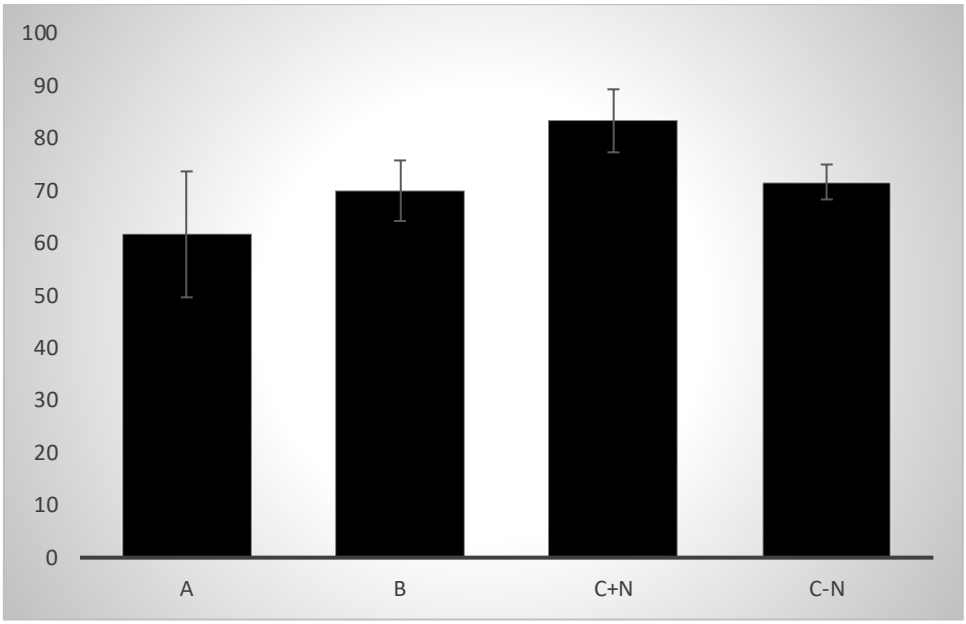


Figure 4.11 Surviving plant percentage for each treatment at the end of the second growth year, Autumn 2016 Error bars show ± 1 se n = 3.

Harvest biomass

Average biomass yields at the first harvest after the second growth year (2017) was not significantly different. Gap correction values on the first year harvest were also not significant (Figure 4.12).

Third year (2018) yield was significantly higher than second year yield for all groups ($p < 0.01$), with the exception of the control without N treatment. SRS treated plots had a slightly lower yield on average (14 and 15T/ha) than the control standard compost plots (16T/ha), but there was no significant difference. Control without N plots produced much lower yield average of approx. 9T a hectare, which was significantly lower than all other treatments ($p < 0.05$). Fourth year harvest (2019) produced a small increase in yield from the year three, with trends remaining the same. The largest increase in yield was seen within the controls without N plots,

gaining on average 2T/ha since the 2018 harvest., although the yield was still significantly less than treatment A and controls with standard compost ($p < 0.05$).

Moisture content was measured for each treatment plot and averaged for each treatment over the three years (Figure 4.13). First year moisture content was high across all treatments at between 61- 64%. This drops year on year from 49-51% in year two, to around 40% for each treatment in the third year. No treatment was significantly different from another.

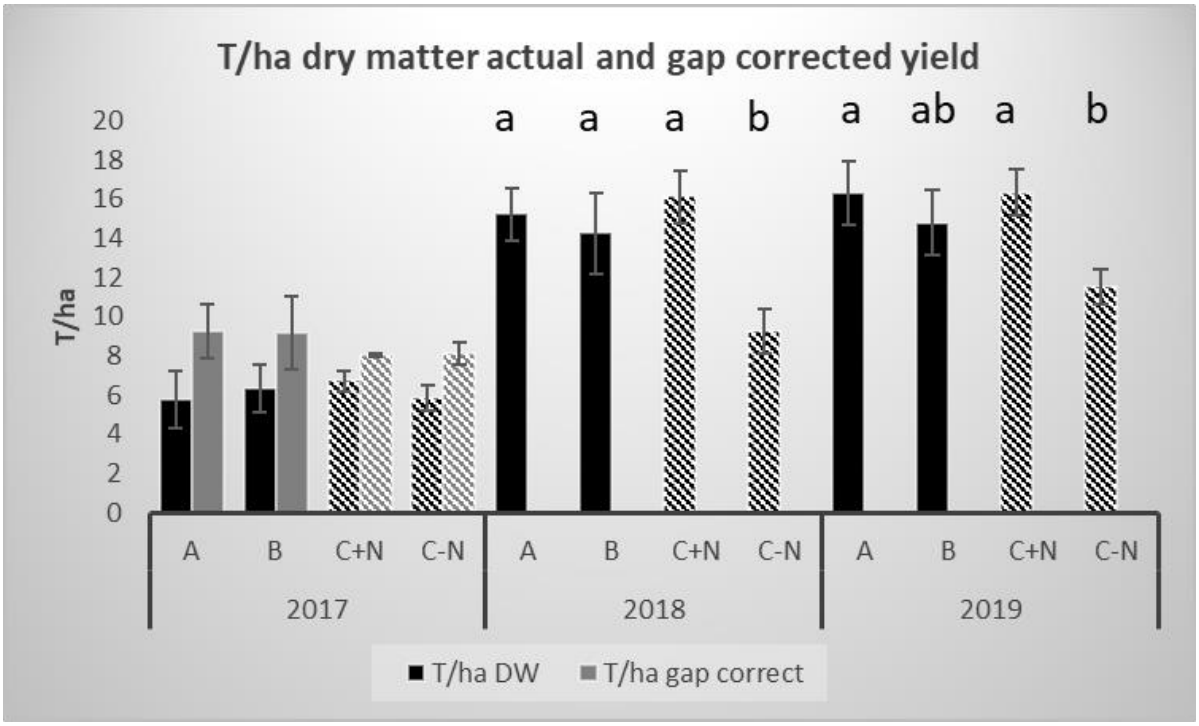


Figure 4.12 Avg yield (T dm $ha^{-1}y^{-1}$) in 2017, 2018 and 2019 (yr 2,yr 3 and yr 4) for GNT 3 grown with and without addition of inoculated SRS media to nutrient free sowing compost. A) 48hr inoculated SRS, B) 24 hr inoculated SRS, C+N) standard potting compost and C-N) Nutrient free compost. Error bars show ± 1 se, n=3. All surviving plants were harvested in 2017 (between 9 – 19). All other years 6 plants from the inner rows were harvested. Gap correction is shown for first year yield due to variable n within treatment plots

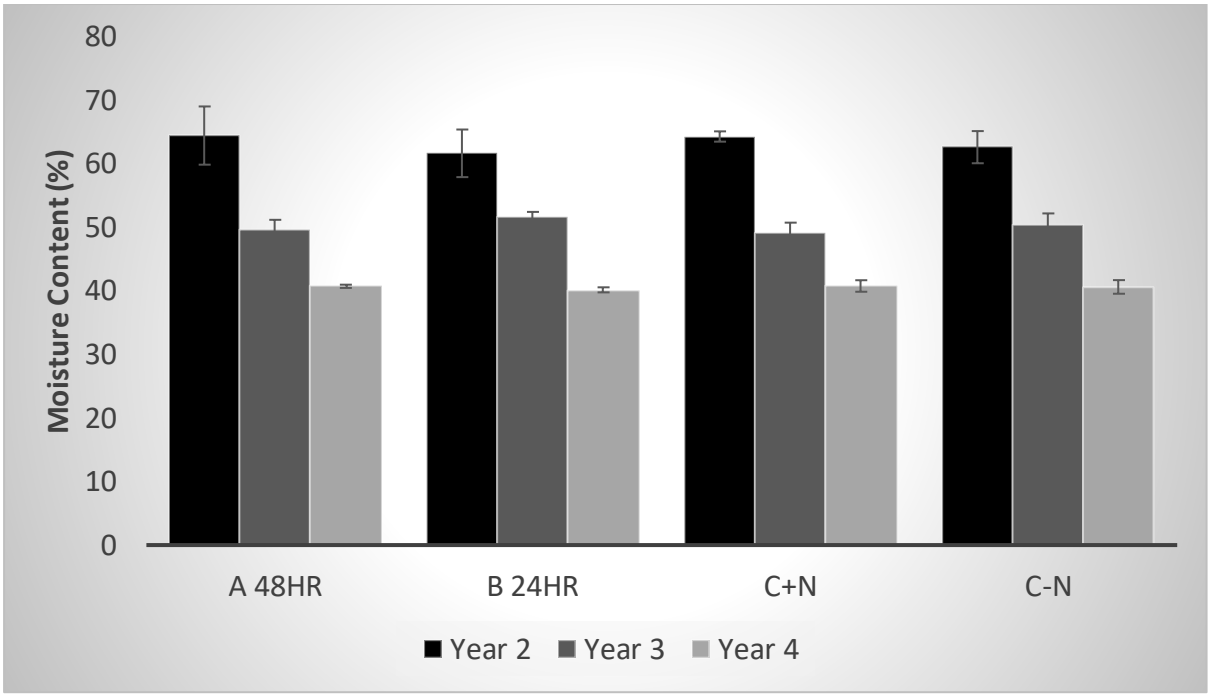


Figure 4.13 Moisture content averages for each treatment (48-hour growth inoculum, 24 hour growth inoculum, control with nitrogen, and control without nitrogen) over three harvest years 2017,2018 & 2019. Error bars show ± 1 se, n=3.

4.3 Experiment 2 - Endophyte glasshouse assessment at Aberystwyth - 2016

Experiment 2 was to study the impact of endophyte delivered via simulated rhizosphere treatment to *Miscanthus* seedlings of genotype GNT14, on biomass and root development in spring 2016

4.3a Methods – Assessment 1

Growth conditions and design of glasshouse trial

The first experiment using the Nutriss polymer in the glasshouse was undertaken during the spring of 2016. The company used the then standard commercial *Miscanthus* plug trays of 126 cells, of approximately 25 cm^3 soil volume. Soil used was nitrogen free compost and SRS granules were added to one tray, leaving the other as untreated control. *Miscanthus* hybrid seed from GNT14 (See Chapter 2 for genotype details) were sown in the Nutriss labs via the method of multi-seeding (usually between 3-5 seed per cell) to ensure each plug contained at least one *Miscanthus* seedling. There were two trays; one with endophyte and one control, ideally producing 126 individuals per treatment. After sowing, trays were carefully packaged to keep in moisture, and sent to Aberystwyth University Gogerddan campus where they were unpacked and placed in a 25°C glasshouse under 16 hour Son T lighting with a night-day temperature range of 18 - 25°C. The endophyte treated tray was placed in watertight container to ensure no leaching or contamination could occur to nearby controls, which were also placed in a watertight tray. Trays were watered daily or as required and regularly moved to reduce the impact of possible environmental variability within the glasshouse growth space. Once seedlings were established, they were thinned out to one plant per cell. Twenty seedlings from each tray were randomly selected to be measured weekly for stem extension (Figure 4.14), and leaf number. The measurements started when the seedlings were 20 days old and continued until they were approximately 10 weeks old.

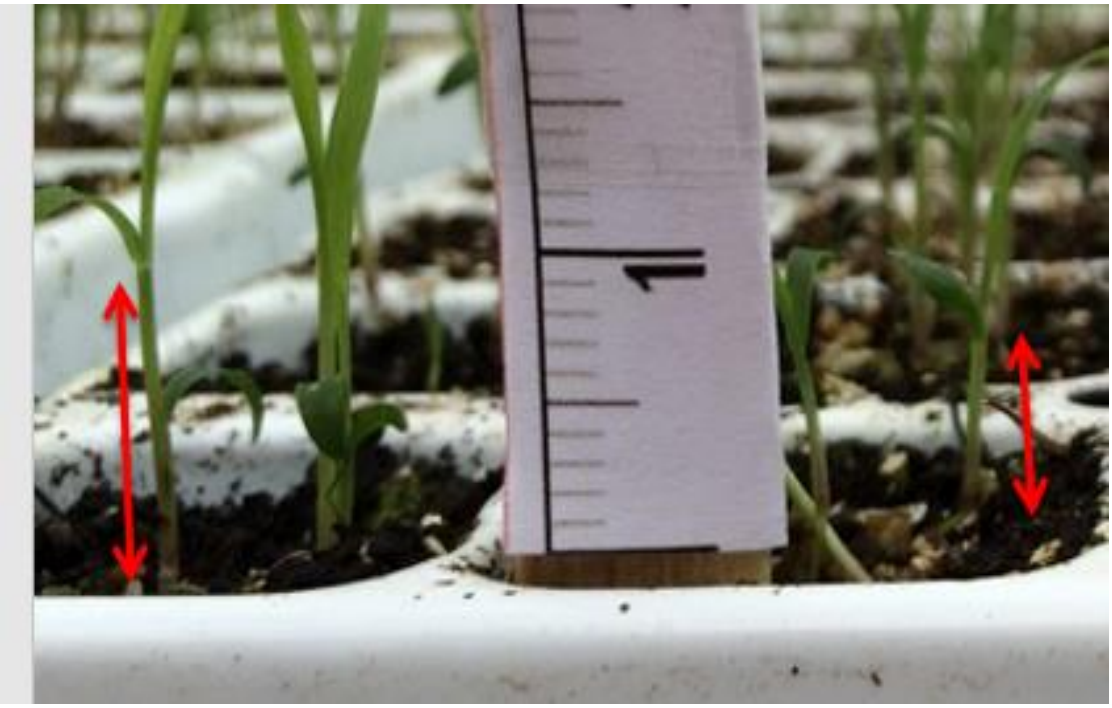


Figure 4.14 Young *Miscanthus* seedlings growing in SRS treated, nitrogen-free compost. Illustrating the measurement of seedling stem growth from the base of the stem at the soil surface to the newest ligule.

Harvesting Biomass

Three consecutive harvests were completed at 2, 3 and 5 months to assess the effects of endophyte polymer on biomass accumulation. This is a longer period of time than is typically suggested for growth of plants in such small modules; however, it was useful as an assessment of the ability of endophyte treatment to aid plants affected by the stress of growth in restricted soil volumes and low nutrient content. At each harvest, ten plants were randomly selected and carefully removed from the plug tray. Roots were gently washed in a bowl of water to remove as much soil as possible while retaining fine root structure and any adhering granules of polymer. The roots and above ground parts were separated into root, leaf and stem, weighed and dried at 80^{o}C until a constant dry weight was reached. Before drying, photographs were taken of the entire plant, and of the root section alone. Following this, a 5mm section of root was carefully cut away from the tip of the roots (from herein described as the distal region), and another 5mm section, from just below the base of the plant at the source of the root (from herein described as the proximal region). The two samples from differing places on the root allowed a comparison between newer material and the oldest

material nearest the base of the plant. Root samples were placed in a 50% ethanol solution and stored in labelled centrifuge tubes at 4°C in the fridge until required.

Root Hair Analysis

To assess the effects of endophytes on the number of root hairs, the 5mm root samples were analysed using a Leica DM6000 B microscope that was part of a Lecia LMD6000 Laser microdissection system. Root samples were placed individually on microscope slides, covered in a layer of deionized water, and a cover slip placed over the top. Each sample was viewed with the UVI 5X/0.12 objective lens and the root segment centred within the viewing window. The focus was adjusted until root hairs could be seen clearly. Once the root image was clearly aligned and in focus, a picture was taken using the microscope Hitachi HV-D20 3CCD camera, to be subsequently assessed using ImageJ. In the ImageJ software, each photograph was individually measured for root area, and the number of visible root hairs counted. From this data the average number of root hairs per mm^2 was estimated.

Statistical analysis

Statistical analysis was undertaken using IBM SPSS statistics 21 version 21.0.0.0. Analysis was undertaken on the raw data to test for significance between each growth parameter at each harvest. Statistical analysis of all growth parameters, per time point and treatment were undertaken using a student's T test. Where equal variances were not assumed, the significance value for this was accepted. Data were tested beforehand for normality using SPSS Skewness and kurtosis values, Shapiro-Wilk test and visual inspection of Q-Q plots and box plot outputs.

4.3b Results – Experiment 2 – glasshouse assessment on GNT14 in 2016

Growth curves

Measurements of extension began when seedlings were 20 days old, and there was no significant difference ($p>0.05$) at any time points between treated and control for the first 5 weeks after sowing (Figure 4.15), with both measured populations averaging at approximately 2.5cm tall by 5 weeks old . After this time point endophyte treated plants significantly surpassed the growth rate of control plants, showing a height difference of approximately twice that of control plants by the 9-week-old stage with an average height of 7.98cm, compared with 3.95cm in the control plants. Plant survival was assessed at week 5, with 60.3% of the 126 SRS treated plugs containing a growing plant, compared with 76% in control trays. Analysis of number of green leaves showed no significant difference over the same period.

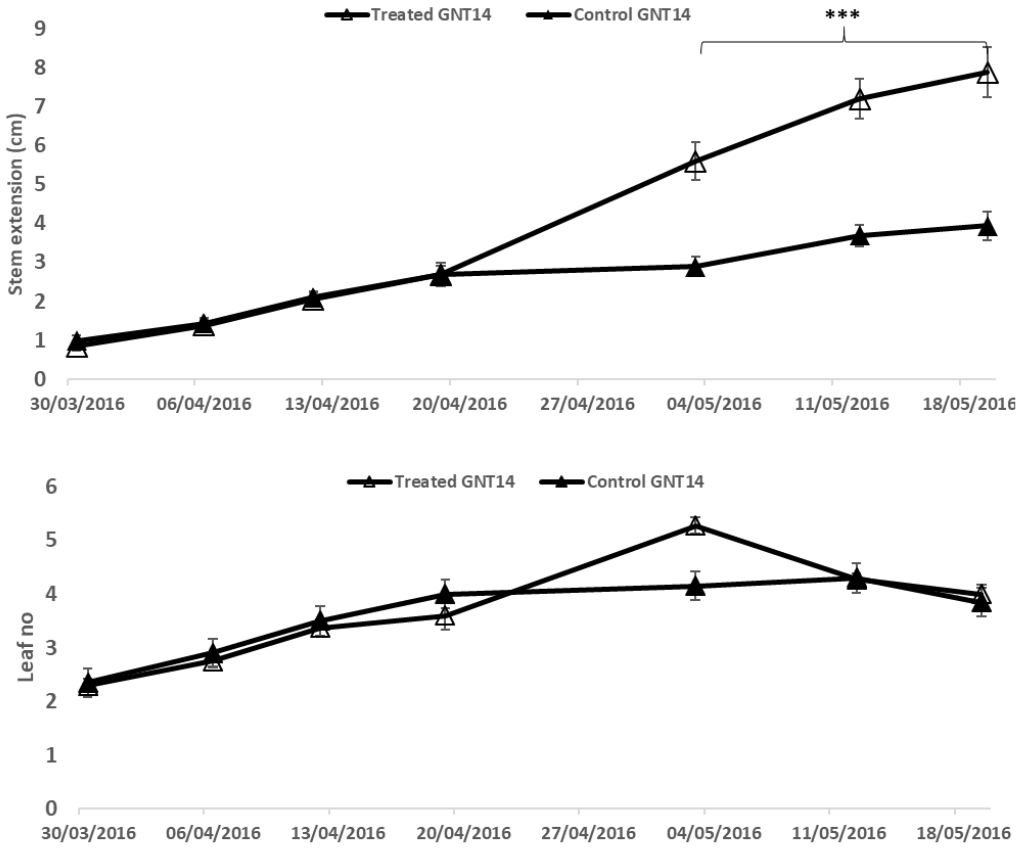


Figure 4.15. Growth curves of *Miscanthus* seedlings germinated in soil media with (clear triangles) and without (black triangles) endophyte SRS treatment. Top chart shows extension over first 6 weeks of growth of assessment, and the below the amount of green leaves for the same growth period 1. N= 20, at each time point, per treatment

Harvest analysis – growth parameters in 2016 trial

The additions of endophytes to the growth media using the SRS bacteria resulted in a significant increase on growth parameters of *Miscanthus* seedlings of GNT14 from the first harvest on day 76 to the third harvest on day 147, results which can be seen visually in Figure 4.16 and Figure 4.17. Whole plant average dry biomass (Figure 4.18A), for treated plants on day 76 was on average 0.16g and for controls 0.068g (F statistic 13.24, $p = 0.002$). On day 110 average whole plant biomass for an SRS, treated seedling was 0.42g and an average biomass of 0.1639g for control plants (F statistic – 21.419, $p < 0.05$). On day 147 whole plant average dry biomass was 1.73g for SRS treated plants and 0.2372 for control plants (F value 19.971, $p < 0.05$).

The average above ground dry matter of an endophyte treated *Miscanthus* seedling on day 76 was 0.15g, whereas dry matter of control treated *Miscanthus* seedlings lacking the endophyte was 0.05g (F statistic 13.767, $p = 0.002$) (Figure 4.18A). Over the next two harvests this significant trend continues. On day 110 the average above ground dry matter of an endophyte treated seedling was 0.37g and 0.13g for control seedlings (F value 17.722, $p=0.001$). The final harvest on day 147 above ground dry biomass for SRS treated seedlings was approximately 1.43g and was 0.18 on average for control seedlings (F value 23.145, $p < 0.05$).

Endophyte treatment did not have a significant effect on below ground biomass before day 76 (Figure 4.18B). Over the next two harvests (at day 110 and 147) the impact on root biomass was more visible, with endophyte roots yielding greater average biomass of 0.0541g per plant, and controls averaging 0.035g at day 110, although at this stage the result was not significant (F value 1.770, $p= 0.200$). Root biomass at harvest 3 (day 147) was; however, significantly larger in endophyte treated plants with a significant average of 0.3037g per plant, and 0.0546g in controls (F statistic; 4.478 $p = 0.049$). As was the case for above ground biomass, the variance in root biomass was far greater in endophyte treated plants, including two outliers as can be seen in Figure 4.18 chart B.

Assessments of stem length during the harvests revealed variable results. At day 76 stem length for SRS treated plants averaged at 95.1mm stem length (± 29.9), as opposed to the control seedlings with 56mm (± 13.7) average (F statistic; 3.463, $p= 0.079$). Harvest two at 110

days old revealed average stem length for SRS treated plants as 152.4mm (± 152.4). Control plants had gained approximately 17mm in height with an average of 73.4mm (± 19.2) (F statistic 7.107, p = 0.016). At 5 months old (day 147) treated plants had an extension of just over three times that of control plants with an average height of 268mm (± 74.4). Control plants stem height averaged at 74.6mm (± 17.6) (F value; 18.269, $p < 0.01$).

Figure 4.16. First harvest (10 weeks old) seedlings before biomass measurements. SRS treated plants are above the controls. Visually, plants appear a deeper green than control plants. SRS polymer can be seen adhering to the roots

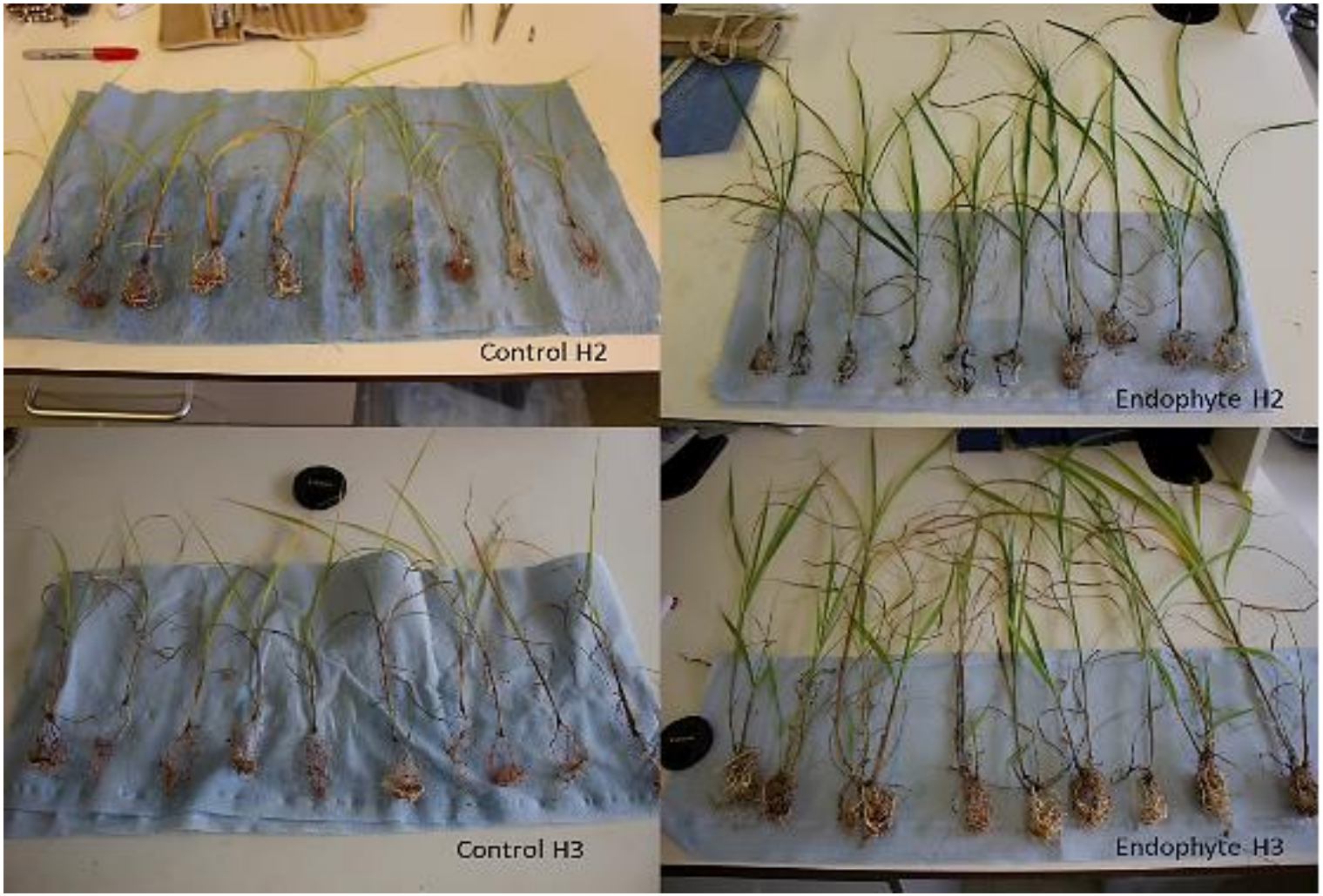


Figure 4.17 Harvest 2 (above two images - plants were approximately 14 weeks old), and harvest 3 (below two images – plants were approximately 5 months old). Endophyte treated plants are very obviously greener and larger than those grown in N free compost alone.

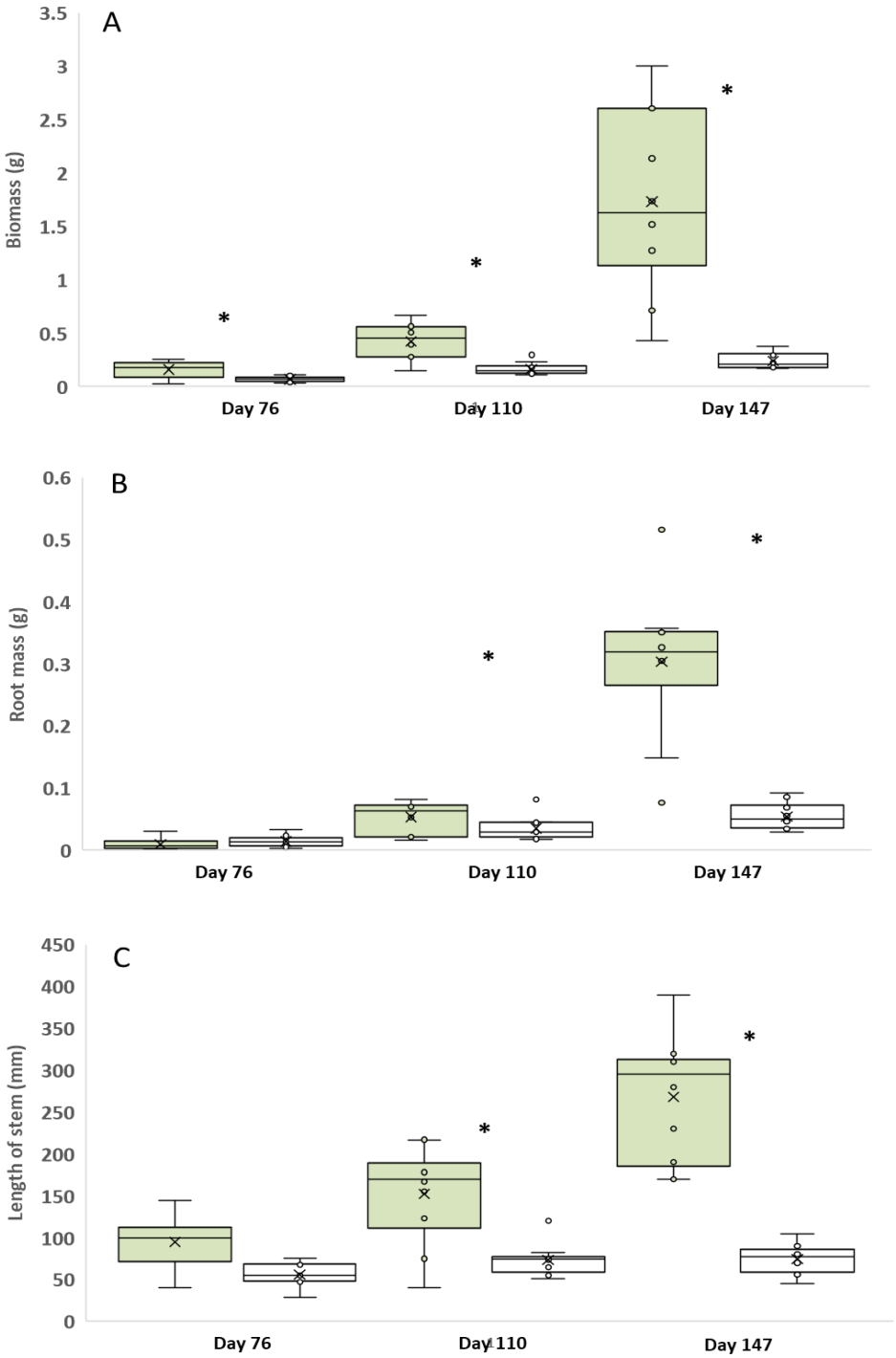


Figure 4.18 Growth parameters measured of control and SRS treated endophyte plants over three consecutive harvests, taken at day 76, 110 & 147 after sowing respectively. Chart A – Whole plant dry biomass. Chart B – Root dry biomass. Chart C – Length of tallest stem. SRS endophyte treated plants are shown in green. * Symbol indicates a significant difference between the treatments. N = 10 per bar.

Root hair assessments

Root hair density at the first harvest on day 76 at the proximal root region, was significantly higher for SRS treated seedlings (4.4 hairs per mm^2 ± 4.19) than the control seedlings (1.6 hairs per mm^2 ± 1.6) F statistic 11.923, p= 0.003. The distal root region difference was not significant. At the second harvest, (day 110) root hair density had increased for both endophyte-treated and control seedlings at both proximal and distal root regions. Plants treated with endophyte additions had higher average root hair number (12.17 hairs per mm^2 ± 11.2) at the proximal region than control plants (6.9 hairs per mm^2 ± 11.2) but this difference was not significant. At the distal region SRS treated plants had significantly higher root hair number than control plants (9.9 hairs per mm^2 (± 11.6), to 4.04 hairs per mm^2 (± 3.8) respectively (F statistic 6.995, p= 0.016). At day 147 the proximal region of the root in control seedlings had significantly more root hairs than SRS treated plants with 13 hairs per mm^2 (± 13.8), to 10 hairs per mm^2 (± 9.4) in endophyte treated plants (F statistic; 7.058, p= 0.016). At the distal region differences became less apparent, with no significant difference between SRS treated and control seedling root hair number.

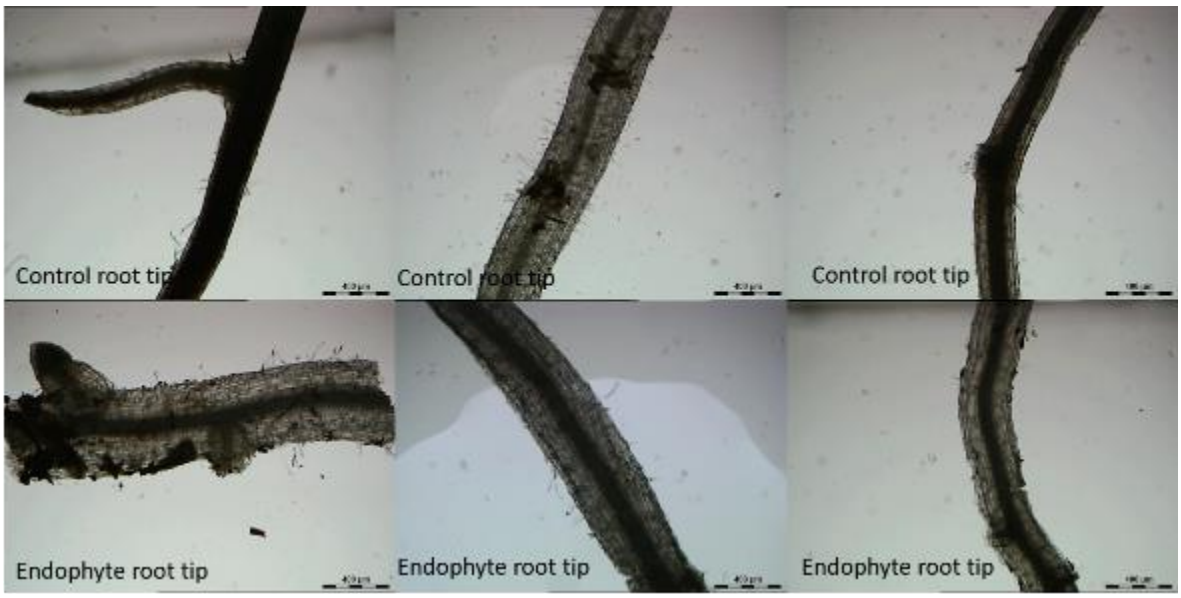


Figure 4.19 Representative images of root tip segments taken from *Miscanthus* seedlings grown in low nutrient compost without endophyte treatment (A), (B) and (C) or in low nutrient compost with endophyte (D), (E) and (F). Photographs were taken at harvest one (Day 76). Images are taken from the tip of the roots and were magnified by the UVI 5X/0.12 objective

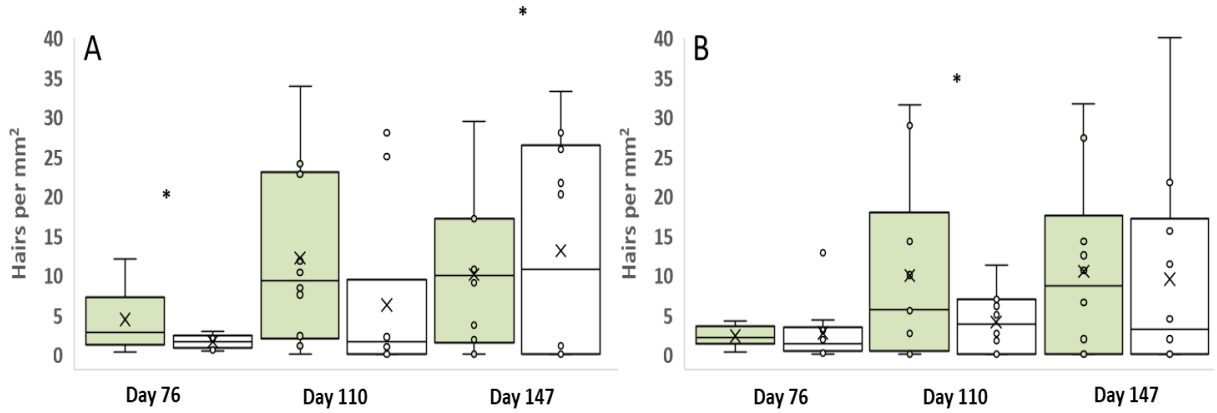


Figure 4.20. Average number of root hairs per mm² in the tips (A) (distal) and proximal (B) ends of roots of *Miscanthus* seedlings treated with endophytes in growth media (green blocks) and control treatment lacking endophyte (white blocks). Roots were analysed over three harvests from 10 - 22 weeks old. No significant differences between treatments were found.

4.4 Experiment 3 – Glasshouse assessment using GNT14 – 2017 analysis

Experiment 2 repeated the study the impact of endophyte delivered to *Miscanthus* seedlings of genotype GNT14, via simulated rhizosphere treatment on biomass and root development in spring 2017.

4.4a Methods – Experiment 3

Growth Conditions

The second plug experiment took place in spring 2017, using the same "GNT14" commercial hybrid used in the previous SRS-endophyte experiment in 2016 (See Chapter 2 for genotype details). Three plug trays of nitrogen free soil with SRS, and three trays of nitrogen free soil without SRS were sown as described in section 4.3a, at the company lab and transported to Aberystwyth two days later. The plug size used and experimental conditions were the same as described in Endophyte glasshouse experiment 1. Plants were watered regularly as needed. This trial was replicated at the commercial nursery in Boston, Lincs. to assess treatments under commercial glasshouse conditions normally used for *Miscanthus* plugs. Assessments of germination and establishment were undertaken at the nursery, and at approximately 6 weeks old, a tray of each commercially grown treated and control plants were sent to Aberystwyth, to be harvested and assessed. These trays were harvested in the same way as the Aberystwyth grown trays to allow direct comparisons of an experimental and commercial system.

Growth Measurements

Establishment, survival and plant death were assessed from plant counts per tray a month after arrival at the Aberystwyth glasshouses. Extension and leaf number were measured weekly from a sample of 10 randomly selected seedlings per tray which were labelled and followed for the duration of the experiment. Extension was measured as the height of the plant from the soil surface to the youngest ligule. Only photosynthetically active leaves were counted which were defined as being leaves with 10% or more green leaf area. Values were averaged to give the mean value per tray and standard error calculated (n = 10). Progression of growth over time was estimated for each tray.

Biomass Harvest

Five plants from each tray were harvested when the plants were approximately 6 weeks old. Plants were carefully removed from the plug tray and the root systems washed thoroughly of soil. Plants from each tray were then photographed as a group before being destructively harvested and roots and shoots separated, by cutting the stem away just above the join of the root and stem. Stem and leaf were further separated, and the main stem length measured. Fresh weight of leaves and stems were assessed individually and placed in labelled paper bags. Roots were photographed and weighed fresh before being placed in labelled bags. All plant material was then dried at 105°C until a constant dry weight was reached, before being removed from the bags and weighed individually again, on a 4-digit scale, the high accuracy was needed due to the extremely low weights being recorded.

Statistical analysis

See section 4.6

4.4b Results – Experiment 3 – glasshouse analysis on GNT14 – 2017

Growth Curves

Growth analysis of extension over time in Figure 4.21 indicates from three to six weeks old SRS treated seedlings had significantly lower height than control seedlings ($p = 0.003$). There were no significant differences in seedling height of SRS and control seedlings b etween weeks 7 – 8. After this time, the average seedling extension for treated plants began to increase and became significantly taller than control seedlings at 9 – 10 weeks post sowing. At week 10 SRS treated plants had an average height of approximately 8.6cm and controls on average 4.02cm ($p< 0.05$). At 12 weeks SRS treated plants remained significantly taller with an average extension of 9.59cm, compared to an average of 5.03cm in control plants ($p < 0.01$)) The third endophyte tray had extreme levels of plant fatality after the 7-week point (Figure 4.22), and was removed as a replicate.

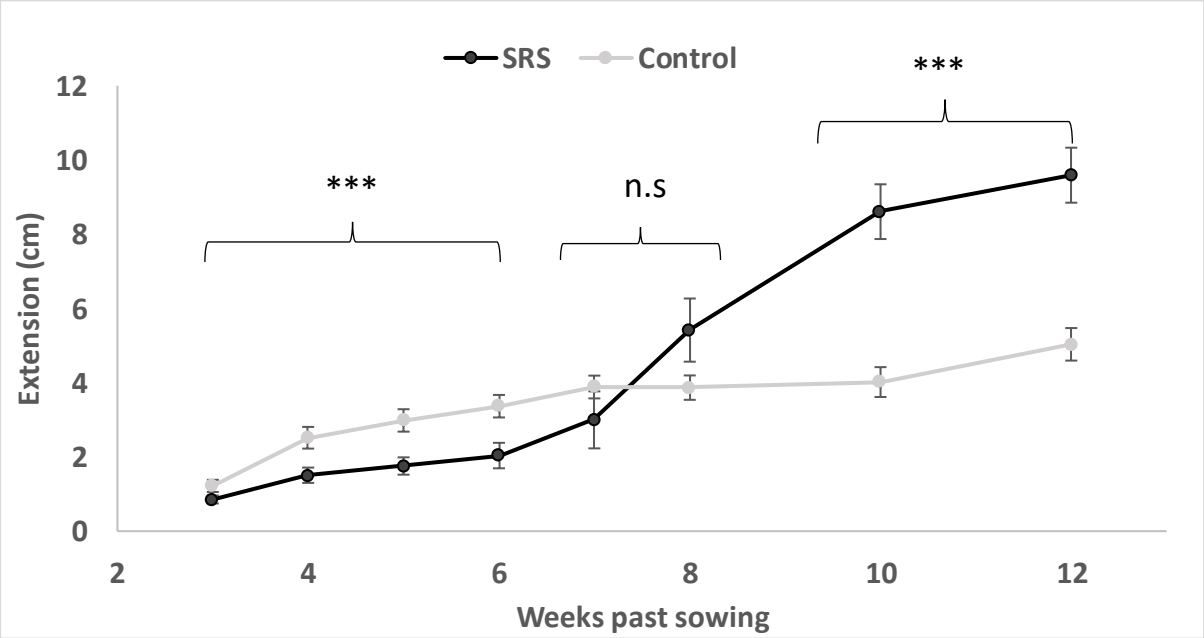


Figure 4.21 Elongation of the main stem of *Miscanthus* seedlings growing in low nutrient compost supplemented with endophyte in SRS (blue circle) and without endophyte supplementation (orange circle). Plants were measured per tray over the first 12 weeks of growth and 3 trays assessed per treatment (averages ± standard error; n = 3 except endophyte-SRS treatment at 8,10 & 12 weeks due to death where N = 2). The value of stem length from each tray comprised between 4-10 pseudoreps for SRS trays due to poor survival rates and 10 for all control trays. * signifies a significance of $p < 0.001$. n.s - non significant**

Figure 4.22 Photograph of the SRS treated tray three with the highest seedling mortality. One seedling has survived infection and is beginning to display good growth. Image taken after the harvest at approximately 7 weeks old.

Table 4.1 % Survival rate of plugs in each tray as a fraction of 126 initially sown Empty plugs signifies no germination occurring. Dead plugs signifies germination of plant but later death. SRS – bacteria treated. C – Control. Assessment taken after approximately a month post sowing.

.

Tray	Empty plugs (%)	Dead plugs (%)
SRS 1	16	27
SRS 2	13	39
SRS 3	24	53
C1	13	0
C2	16	0
C3	10	0

Harvest Results

Figure 4.23 includes images of all measured replicates before harvest, and the variation within treatment is particularly obvious visually as well as statistically. Figure 4.24 shows the results of the biomass assessment.

All measurements shown in Figure 4.24 indicate a great amount of heterogeneity both between treatment replicates and within them, and as a result, treatment replicate trays were separated and assessed independently.

Stem length assessments (Figure 4.24 Chart A) with ANOVA revealed a p = 0.005 significance between all groups (F statistic 3.668). Further assessment for testing homogeneity of variances revealed a Levene's statistic of 2.420, and a significance of p= 0.042. A non-parametric independent samples median test produced a significance of 0.014 between groups, and the null hypothesis was rejected.

Above ground biomass ANOVA (Figure 4.24 Chart B) revealed a significant difference of p = 0.025 between groups, including the trays taken from the commercially grown plants at Bells nursery. Levene's test for homogeneity of variances indicated unequal variances between groups (Levene's statistic; 2.763, p= 0.023). Nonparametric independent samples median test produced a significance value of 0.045, rejecting the null hypothesis. Further, multiple comparisons using Tukeys post hoc tests produced two homogenous subsets 'a' with the lowest above ground biomass and 'b' with the highest above ground biomass. Lowest overall biomass, placed in subset 'a' alone was endophyte replicate 2. Highest biomass was seen in control replicate 2 (subset 'b' alone). All other replicates were placed in group 'ab'.

Results of the roots analysis (Figure 4.24 Chart C) indicates a significant difference of p = 0.007 between the groups. Tukey's analysis showed that the majority of the differences occur in control rep 2 again, with roots biomass significantly more than all endophyte trays, with the exception of endophyte 1. Figure 4.24 Chart D shows the approximate root to shoot balance and average plant size for each tray. This allows a visual graphic of the extreme differences seen across all trays. Control plants produced the lowest levels of heterogeneity.

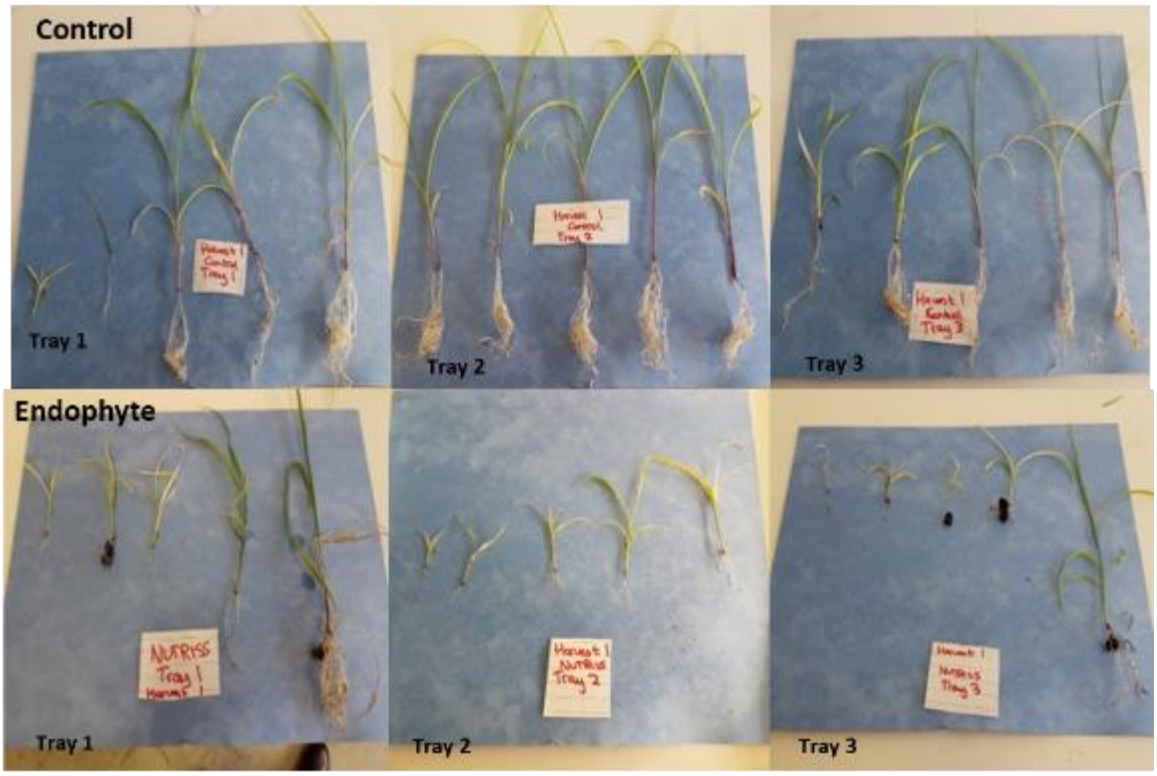


Figure 4.23 Photographs of experimental reps before sacrificial harvest. Plants are approximately 6 weeks old. Control trays 1,2 & 3 are the top three pictures from L-R. Endophyte trays 1,2 & 3 are below (L-R).

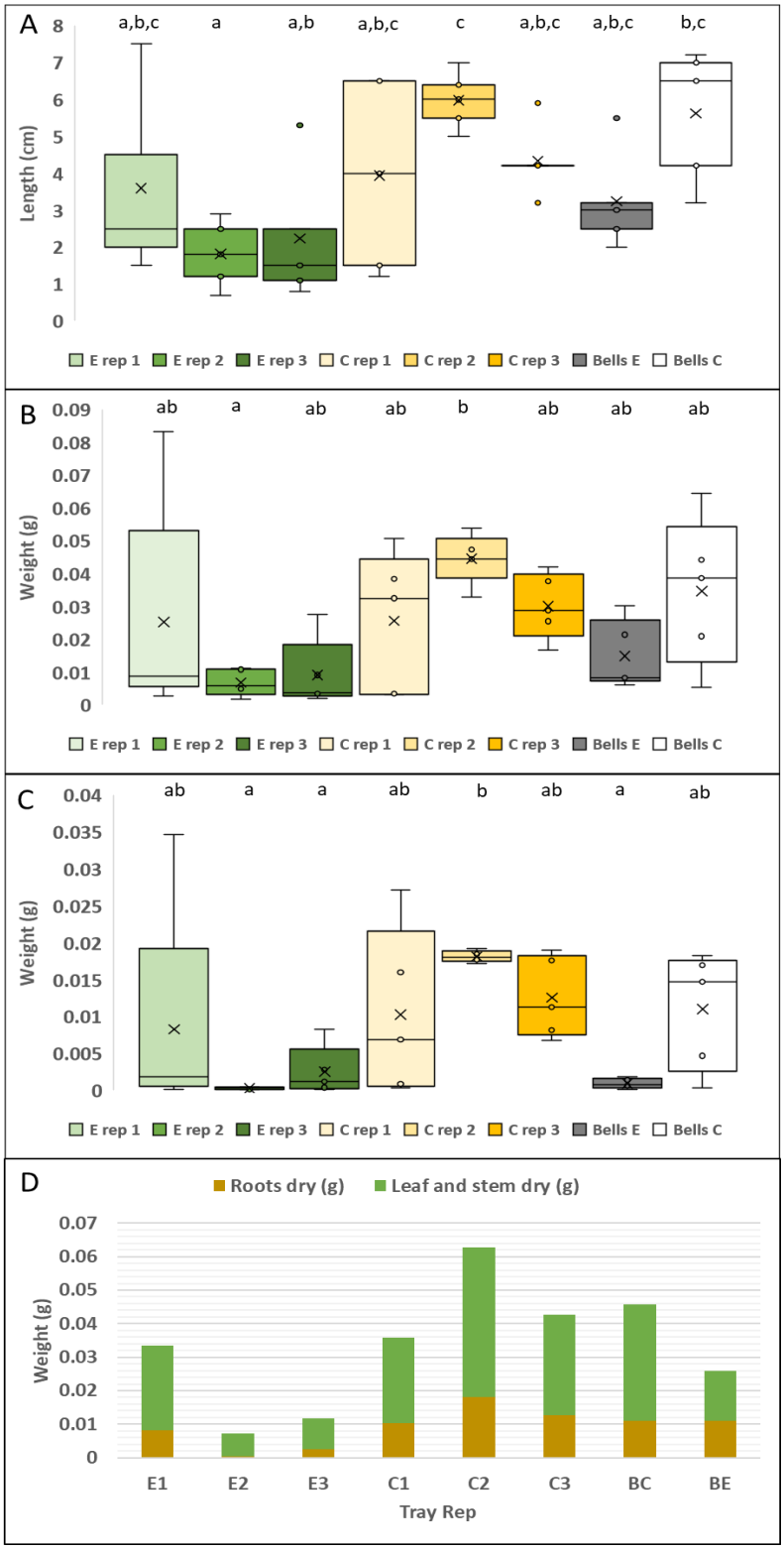


Figure 4.24. Harvest results of 2017 endophyte additions assessment on 6-week-old seedlings of *Miscanthus* genotype GNT14. Chart A – stem length, Chart B – Above ground biomass, Chart C – below ground biomass, Chart D – Root and shoot ratio. Three reps of SRS endophyte treated plants are in green shades (E rep 1/2/3), and three reps of control are in yellow shades (C rep 1/2/3), with an additional replicate of control (white bar) and SRS endophyte (grey bar) seedlings sent from commercial nursery. N = 5 for each rep. Each replicate population taken from one tray of treated or control seedlings

4.5 Experiment 4 - Multihybrid trial testing one bacterial treatment on 3 seeded hybrids and two forms of *M giganteus* establishment in a replicated plot trial in the Lincoln experimental fields

4.5a Experiment 4 – Multihybrid trial methods

Plant material and growth conditions

This field trial was set up to test for population differences in response to SRS – endophyte treatment of three commercial hybrids, and to compare growth of the new commercial hybrids treated with and without SRS – endophyte, to the current commercial standard *Mxg.* Additional assessments were done to determine the effects of SRS endophyte treatment on rhizome propagated *Mxg*, which due to its triploid nature cannot be propagated by seed. Because of concerns surrounding the 'polymer to root contact efficiency' in free rooting field plants as opposed to the closed system within a pot there were two SRS treatments applied to *M×g*. The first treatment added the same SRS to soil ratio additions given to the seedlings in plugs, to free rhizome planted in the field, along with three control plots without SRS. The second treatment involved propagated *M×g* rhizome in pots with the same SRS to soil ratio given to the seedlings in plugs, before being moved to the field.

Three seeded hybrids of commercial interest were used; GNT3, GNT5 & GNT14, which were acquired from the breeding team as fresh seed, and sown in multiple trays of 126 25cm^3 plugs. Half of all trays were inoculated with the SRS polymer into nitrogen free compost, and the other half were given nitrogen free compost alone. Seeds of each hybrid were multisown (3-5 seeds per well) to ensure full establishment and grown at Bells Nursery for between 6 – 8 weeks in February 2016. The seeded hybrid plugs were routinely assessed for growth and establishment during the glasshouse phase before being planted into the field at the Hackthorn site in July 2016. *M×g* potted plants with and without SRS additions were also planted at the same time.

Three replicate blocks of each genotype and treatment were planted in a randomized design with blocks of three columns by nine rows. The experimental plots were surrounded by a double row of *M×g* from rhizome as guard plants to reduce edge effects. The total area for the whole trial was 26.33m x 28.50m, totaling 750.40m^2. Distances between each plug was 0.75m between rows and a 0.67m within the rows and density was 2 plants m^{-2}, or 20,000

plants a hectare. Paths between were incorporated by 'missing a plant' for ease of access (see Figure 4.27) for within plot spacing and sampling regime). The outer edge of each treatment plot contained experimental plants that were discarded at harvest. The inner 3 x 8 plants were for harvest, and the innermost 3 plants in the centre were assessed for height and shoot density at the end of the growing season.

For specifics on harvest methods and phenotyping see section 2.6 and 2.7.

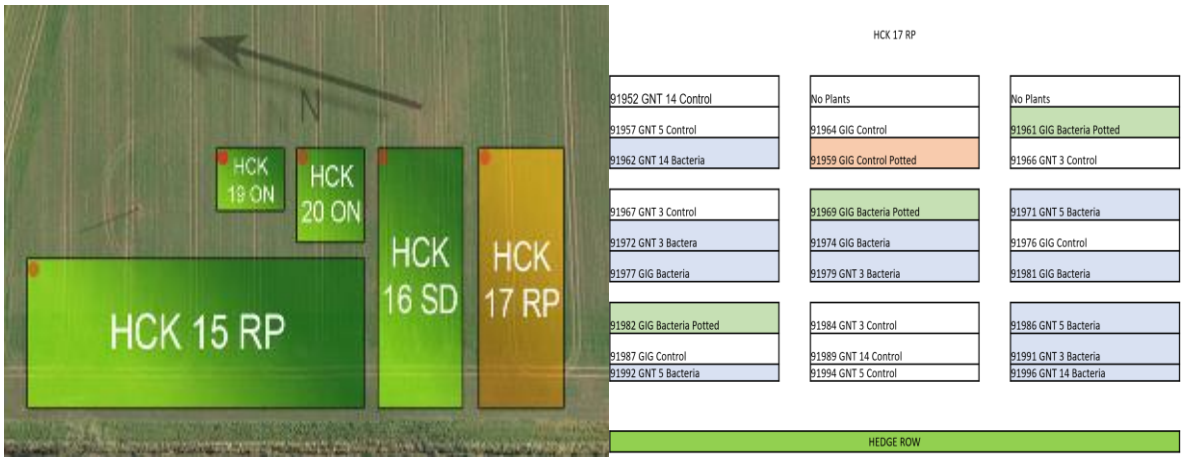


Figure 4.25. Position of the field trial in experimental field plots (right). Treatment block design within the field trial (left). Each individual replicate plot contains 50 plants in a 5 x 10 row and column matrix

Figure 4.26. Planting of HCK 17 on 6th July. A = Plugs planted at equal spacing. B = *Mxg* plant grown in a pot prior to planting. C = Pot grown plants planted in equally spaced rows in the foreground with mulch film covered plots in the background

Figure 4.27. Sampling regime for HCK 17. Each circle represents a plant within a treatment plot. The left block represents the phenotyping sampling plants from the centre of the plot. The right block shows the sampling regime. All yellow circles signify plants harvested for biomass from each experimental plot in 2018 & 2019. In 2017, 12 plants were harvested and can be seen as hashed yellow.

4.6 Statistical analysis

Data was imported from field assessments into Microsoft Excel format on iPads or android tablets during both phenotyping and harvesting. The data was then manipulated with Microsoft Excel version 1907. Statistical analysis was done using IBM SPSS statistics 21 version 21.0.0.0. Analysis was undertaken on the raw data to test for significance between each growth parameter and each treatment variable, at each phenotyping and harvest. Statistical analysis of all growth parameters, per time point and treatment were undertaken using a one-way ANOVA, and where significant differences were found within groups, a Tukeys test was performed for more in depth comparisons. Data were tested beforehand for normality using SPSS Skewness and kurtosis values, Shapiro-Wilk test and visual inspection of Q-Q plots and box plot outputs.

4.7 Results – Experiment 4 – Multihybrid trial - Glasshouse phase

Seeded hybrid germination scores 1 & 3 weeks post sowing indicate a significantly lower level of germination in both bacteria and control trays of the GNT14 hybrids with approximately 65 and 80% germination respectively, in comparison to 95 – 100% for other genotypes and treatments (Figure 4.28). Three weeks post sowing, seedlings were assessed for extension and leaf number. Control trays for each genotype showed significantly higher extension at this stage (Figure 4.29). Leaf counts were also significantly higher for control trays, except for GNT5. An example of the poorer establishment and growth of GNT14 seedlings can be seen in Figure 4.30.

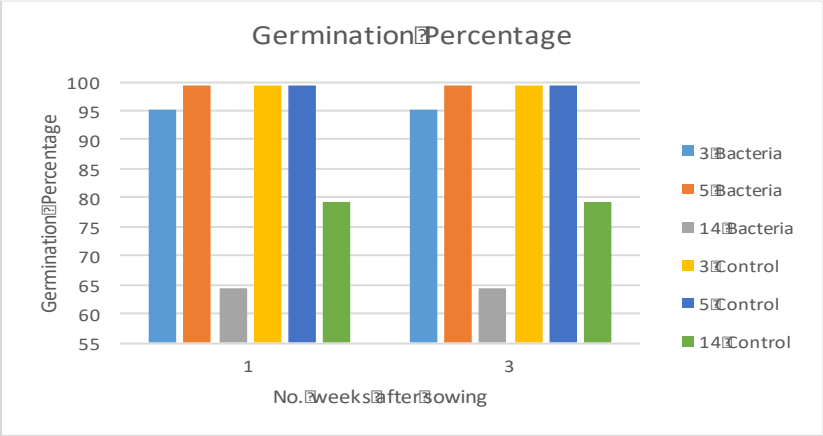


Figure 4.28 Germination assessments of seeded hybrids during first 3 weeks after sowing into SRS bacteria inoculated and control plug trays. Images courtesy of Bells nurseries Ltd

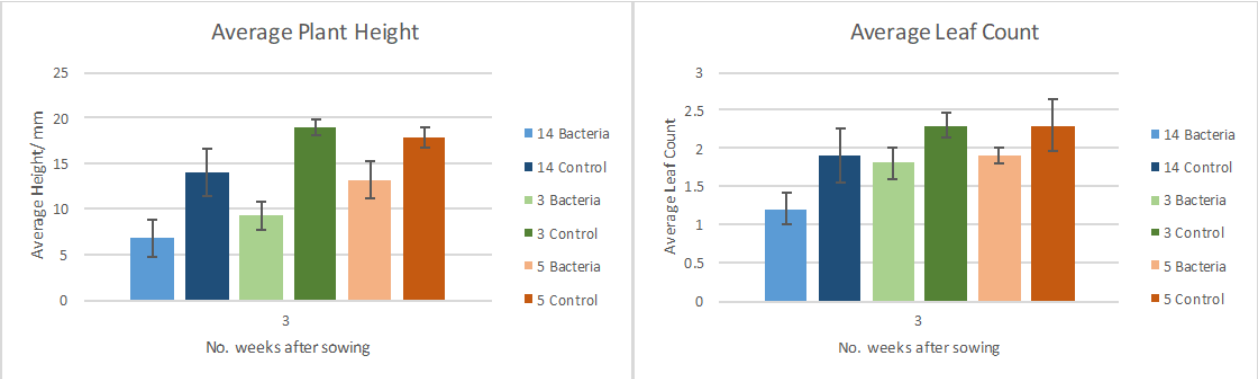


Figure 4.29. Average plant height and leaf counts for seeded hybrids, three weeks after sowing into SRS bacteria inoculated and control plug trays. Images courtesy of Bells nurseries Ltd.

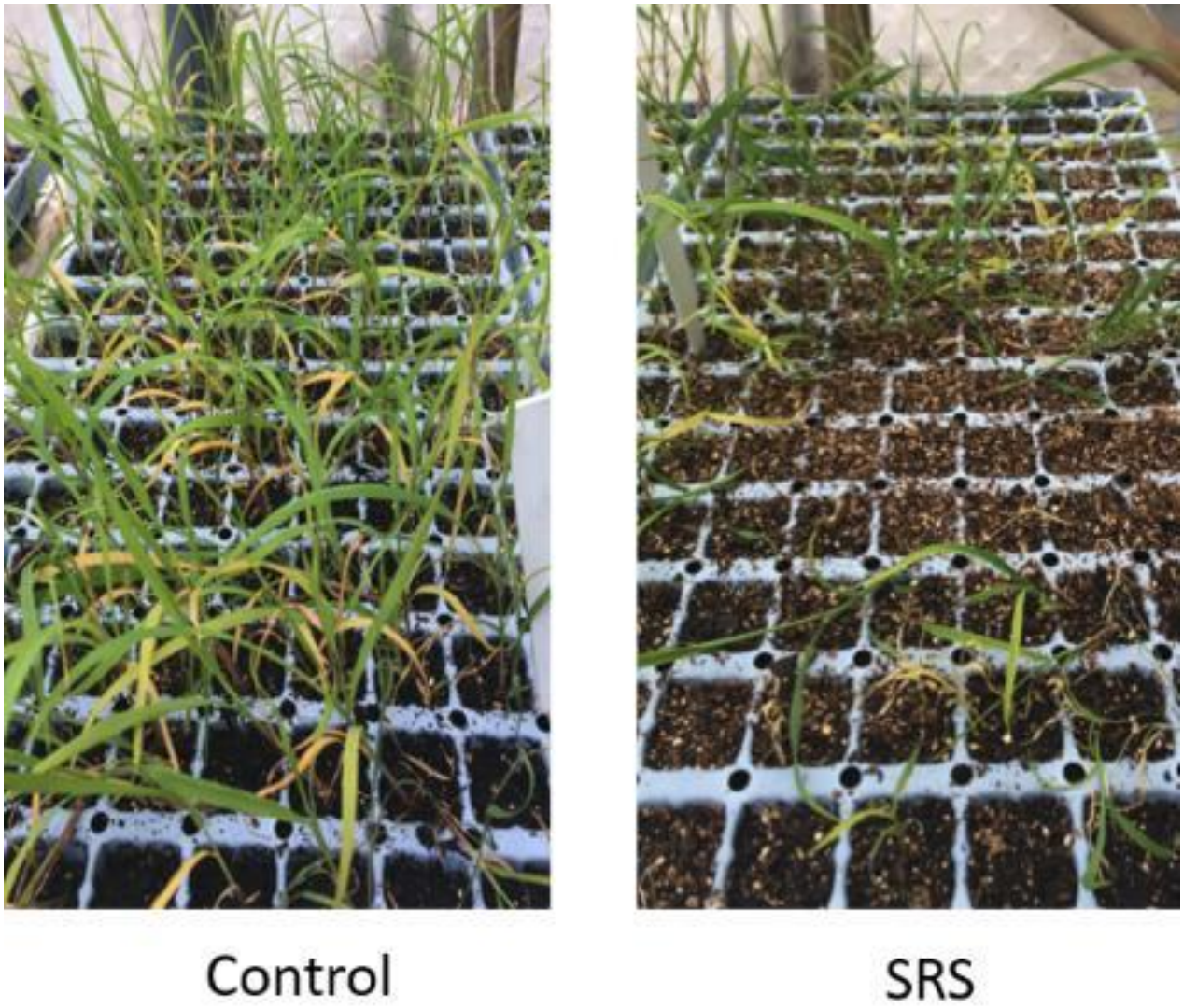


Figure 4.30. Establishment comparison in control and SRS treated GNT14 bacteria trays grown under controlled commercial conditions. Control (left) have much fuller establishment than treated SRS (right). Photo taken when plants were approximately 1 month old.

Field phase establishment

All *Mxg* plots had over 85% establishment, GNT 3 and GNT 5 had comparable establishment rates to *Mxg* plots, with establishment of between 76 & 90% regardless of treatment. SRS and control GNT14 had the lowest establishment at 60% and 71% respectively. There were no significant differences in establishment across all treatments.

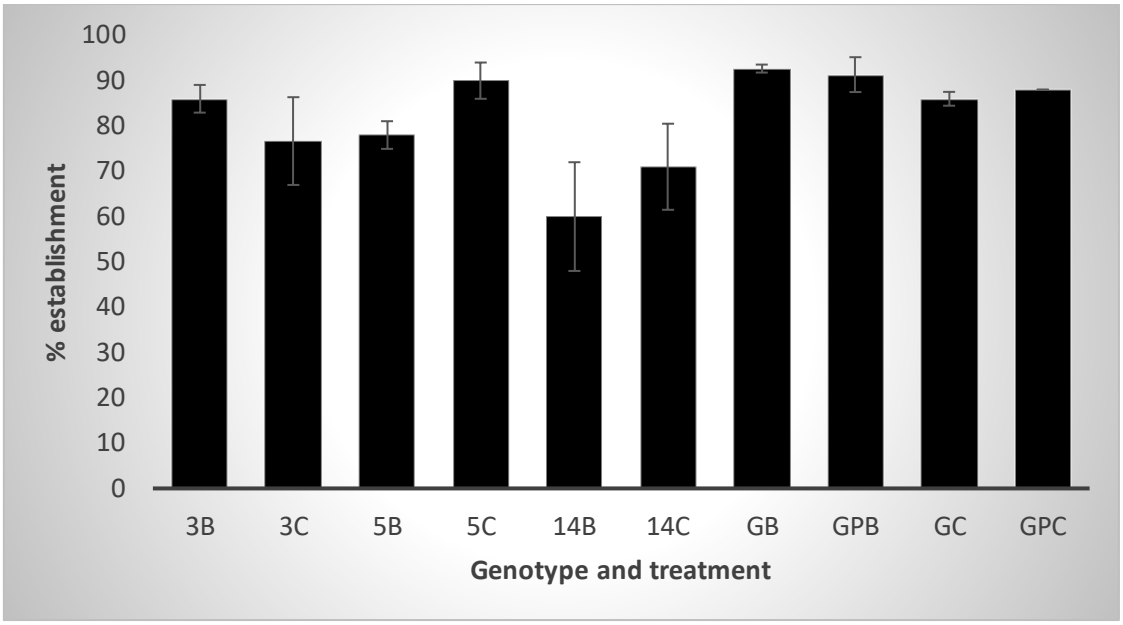


Figure 4.31 Field establishment of GNT 3, GNT 5, GNT 14 with and without Bacteria. Genotypes succeeded with B signify treatment with bacterial SRS. Those succeeded with C signify controls. G signifies *Mxg*. B – SRS bacteria, PB – Potted bacteria, GC – *Mxg* control, PC – potted control. Error bars show ± 1 se, n=3

Phenotyping – seeded hybrids

All seeded hybrid phenotyping results are reported in Table 4.2. 2016 autumn phenotyping of the seeded hybrids revealed no significant differences between SRS and control plots in GNT3 or GNT5. GNT14 treated plants had significantly lower canopy height ($p < 0.001$), of 68cm, in comparison to control plants at 111cm average. This difference remained significant within the shoot heights, ($p < 0.01$), as bacteria plants tallest shoots were approximately 47cm, and control plants 71cm. Stem number analysis confirmed significantly smaller plants, as control plants had a 25-stem average compared to treated 12 stem average ($p<0.05$).

In 2017 GNT3 bacteria treated plants had significantly lower stem number than control plants ($p< 0.01$). Canopy height and shoot height showed no significant difference in GNT3. GNT5 plants varied greatly between treatments, with bacteria treated plants having significantly

increased canopy and shoot height ($p<0.01$). Stem number was not considered significant. GNT14 plants contrasted sharply with GNT5, having significantly lower canopy and shoot heights in bacteria treated plants ($p<0.05$).

By the end of the third growth year no significant differences were seen in GNT3 growth parameters. GNT 5 bacteria treated plants had significantly higher canopy height ($p<0.05$), which was reflected in shoot height results, although this was not statistically significant. Stem number was also not significantly different. Due to highly variable die off height results, non-parametric test for independent samples was used for a significance value of $p - 0.008$, for the vastly lower die off height average seen in control plants. GNT14 also revealed a significant difference only in the die off height, with bacteria treated plants showing significantly reduced die off height of 30cm as opposed to controls, 132cm ($p < 0.01$ – Non-parametric test). GNT14 plants treated with bacteria were on average shorter, with fewer stems than control plants, although no significant difference was found in year 3.

Table 4.2. Phenotyping results of the seeded hybrids over the course of three years[3].

Year	Genotype	Treatment	Die-off Height (cm)	Canopy Height (cm)	Shoot Height (cm)	Stem Count
2016	GNT3	SRS		105.65 (±5.34)	63.04 (±3.48)	21.48 (±1.7)
		Control		104.85 (±5.04)	64.9 (±3.36)	28.2 (±2.53)
	GNT5	SRS		87.75 (±2.81)	49.35 (±3.25)	18 (±1.65)
		Control		77.93 (±4.79)	41.64 (±2.95)	16.36 (±2.25)
	GNT14	SRS		68.08 (±10.42) ***	47.42 (±7.31) **	11.64 (±2.75)
		Control		111.46 (±4.66)	71.31 (±4.89) **	24.69 (±1.69) *
2017	GNT3	SRS		166.67 (±4.79)	140.43 (±5.06)	33.83 (±2.34) **
		Control		164.3 (±3.62)	140.33 (±3.54)	49.03 (±4.48) **
	GNT5	SRS		185.37 (±7.5) **	156.63 (±8.62) ***	34.77 (±2.13)
		Control		147.55 (±6.56)	119.2 (±5.86)	42.2 (±4.35)
	GNT14	SRS		125.05 (±8.93) **	105.05 (±8.28) *	38.9 (±4.8)
		Control		159.7 (±6.39)	138.75 (±6.3)	45.05 (±4.85)
2018	GNT3	SRS	99.33 (±9.91)	193.58 (±8.57)	171.63 (±8.17)	30.75 (±2.95)
		Control	73.08 (±11.85)	177.33 (±4.68)	156.71 (±4.9)	45.25 (±4.94)
	GNT5	SRS	89 (±12.31) **	173.54 (±6.65) *	150.75 (±6.52)	35.96 (±3.82)
		Control	30.06 (±10.72)	131.75 (±14.56)	115 (±13.22)	35.06 (±5.95)
	GNT14	SRS	36.875 (±13.39) ***	139.06 (±9.72)	121.19 (±10.93)	32.88 (±3.13)
		Control	132.06 (±16.71)	178.5 (±12.38)	158.63 (±11.43)	36.88 (±5.35)

[3] . Each value is an average of between 1-3 treatments plots, within which was measured a variable number of pseudoreps, depending on year. 2016 – 5-8 plants per treatment plot. 2017 – 10 plants per plot. 2018 – 8 plants per plot. Hybrid types are differentiated by banding. SRS plots are bacteria treated; Control are not bacteria treated. Where a significant difference was found between the treated and control of a particular hybrid, the results are awarded stars (* - $p \leq 0.05$, ** - $p \leq 0.01$, * - $p \leq 0.001$)**

Phenotyping – *Miscanthus giganteus* plots

In 2016 canopy and shoot height measurements of all treatments of *Mxg* showed a significant difference of $p < 0.01$ between groups (Figure 4.32). Post hoc analysis by Tukeys provided further assessment that bacteria alone was significantly taller than bacteria potted groups ($p = 0.001$) and control potted ($p< 0.05$). Control was also significantly taller than control potted groups ($p – 0.003$). Assessment of stem number revealed a significance of $p = 0.042$ between groups, however the only near significant Tukeys result was that control had more stems than control potted treatments by a p value of 0.053 (24 and 15 stems respectively). In 2017 and 2018 no significant differences were found in any parameter measured.

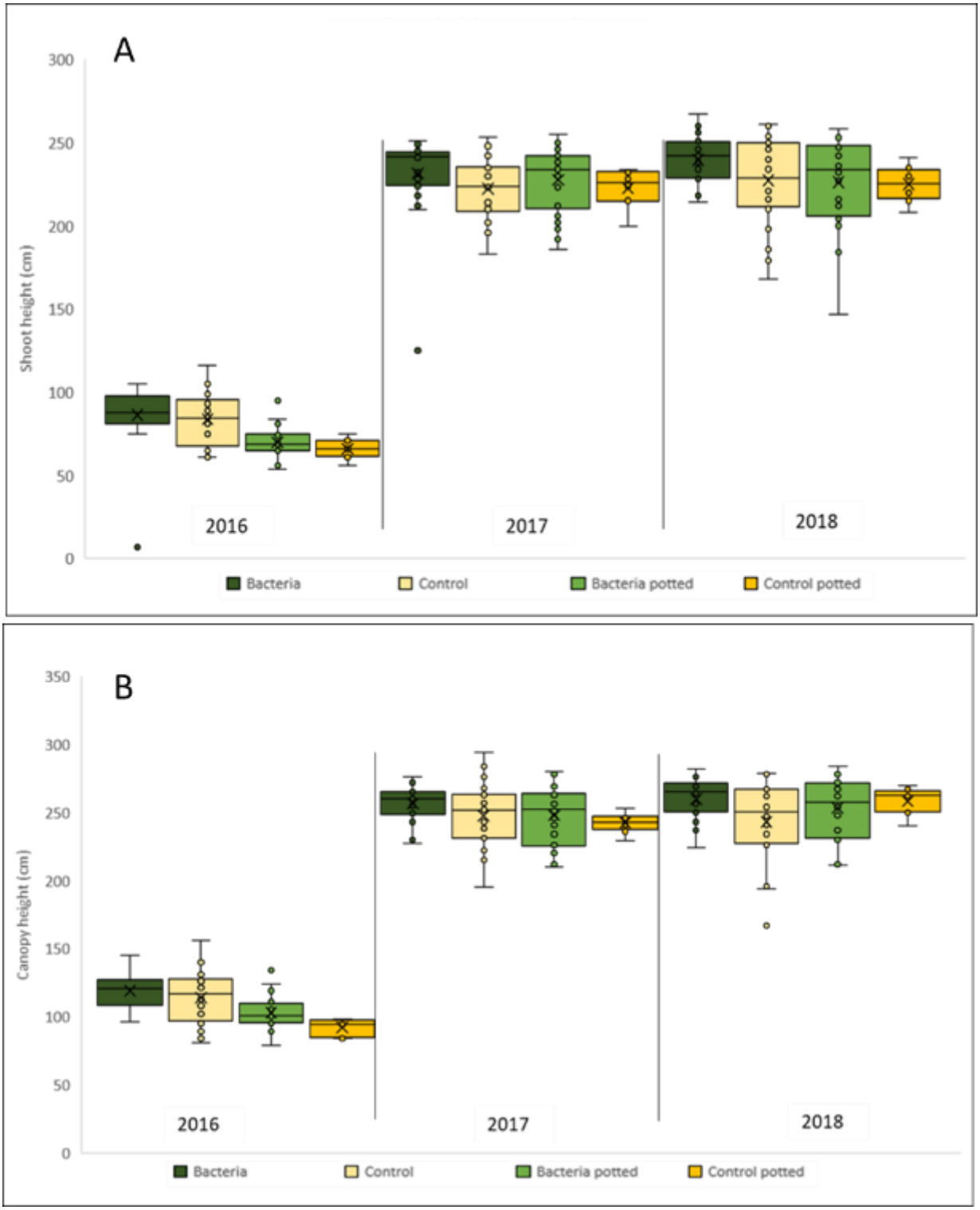


Figure 4.32. Phenotyping results from M giganteus plots shoot height (chart A) and canopy height (Chart B) parameters over the course of three years, from planting year (2016) to the most recent data at the time (2018). Four treatments are Bacteria alone (dark green), control alone (light yellow), bacteria potted plants (lighter green), and control potted plants (dark yellow). Bacteria potted, and control alone are averages of three treatment plots, while control potted had only one treatment plot. Within each plot n = 8 in 2016 & 2018. n = 10 in 2017.

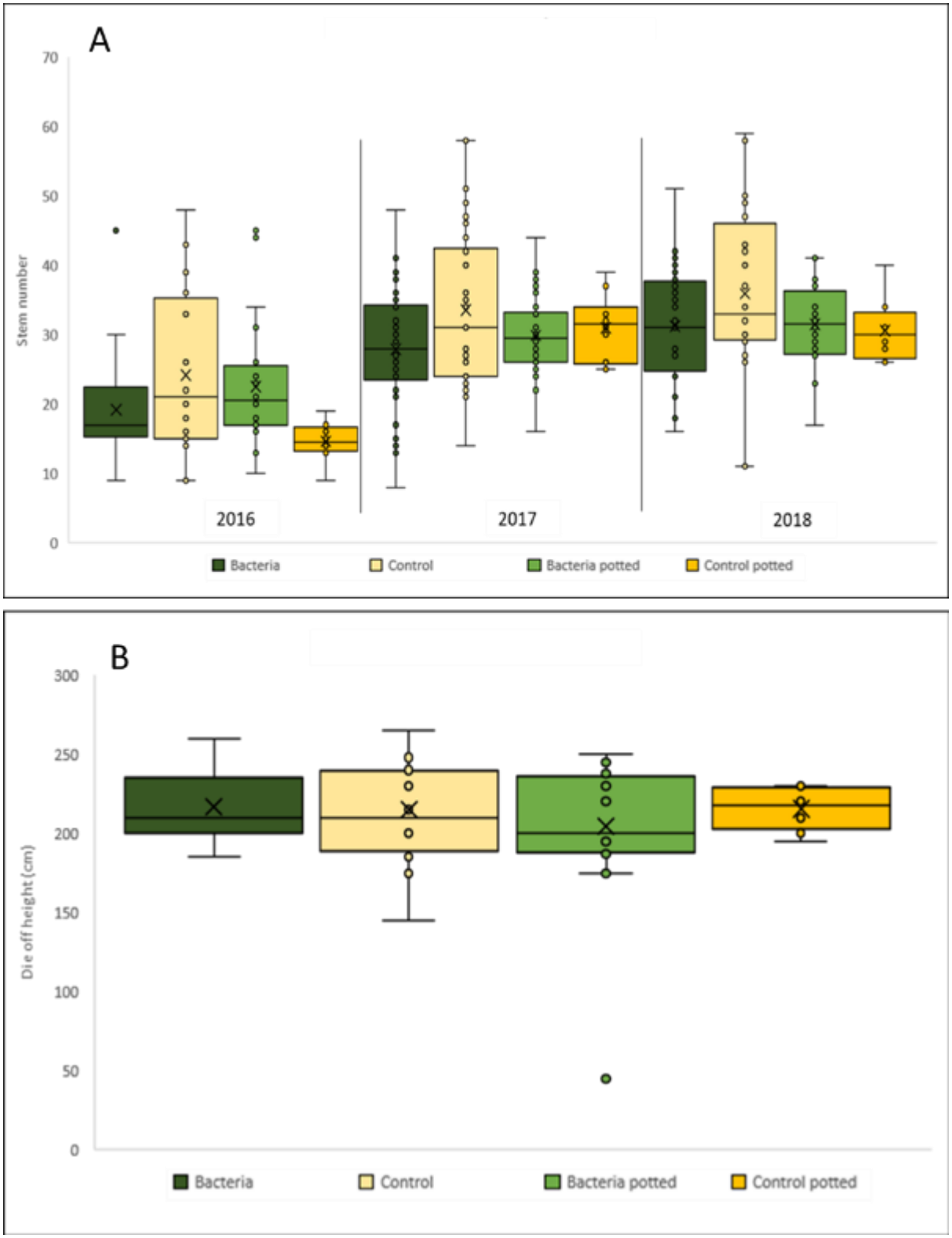


Figure 4.33. Phenotyping results from M giganteus plots, A - Stem count over the course of three years, from planting year (2016) to the most recent data at the time (2018). B - Die off height from 2018 alone. Four treatments are Bacteria alone (dark green), control alone (light yellow), bacteria potted plants (lighter green), and control potted plants (dark yellow). Bacteria potted, and control alone are averages of three treatment plots, while control potted had only one treatment plot. Within each plot n = 8 in 2016 & 2018. n = 10 in 2017.

Harvest yield

Treatment did not significantly affect yield in year 1 on any hybrid or genotype (Figure 4.34). The treatments with the largest yield in year 1 were *Mxg* with the SRS added freely to the soil, and GNT3 with SRS, with yields of approximately 2.1 T/ha. Lowest yields were seen in GNT5 control plots with average 0.9T/ha.

Harvest yields in 2018 (second year) were significantly different between genotypes ($p - 0.004$). Yield increased significantly for all *Mxg* plots and the seeded hybrids, regardless of bacteria treatment in year 2 ($p< 0.05$). Bacteria treatment had no significant increase in yield for any hybrid or genotype comparison.

There was a drop in yield in year 3 for all *Mxg* plots. Yield of GNT 3, GNT 5 and GNT 14 ranged from 6 – 12 T/ha, with SRS treated plots appearing slightly better in GNT3 and GNT5. There were no significant effects for any seeded varieties, but *Mxg* with SRS added to the soil was significantly higher than the control ($p <0.05$), in the actual yield data analysis. The gap corrected yield results were not significantly different.

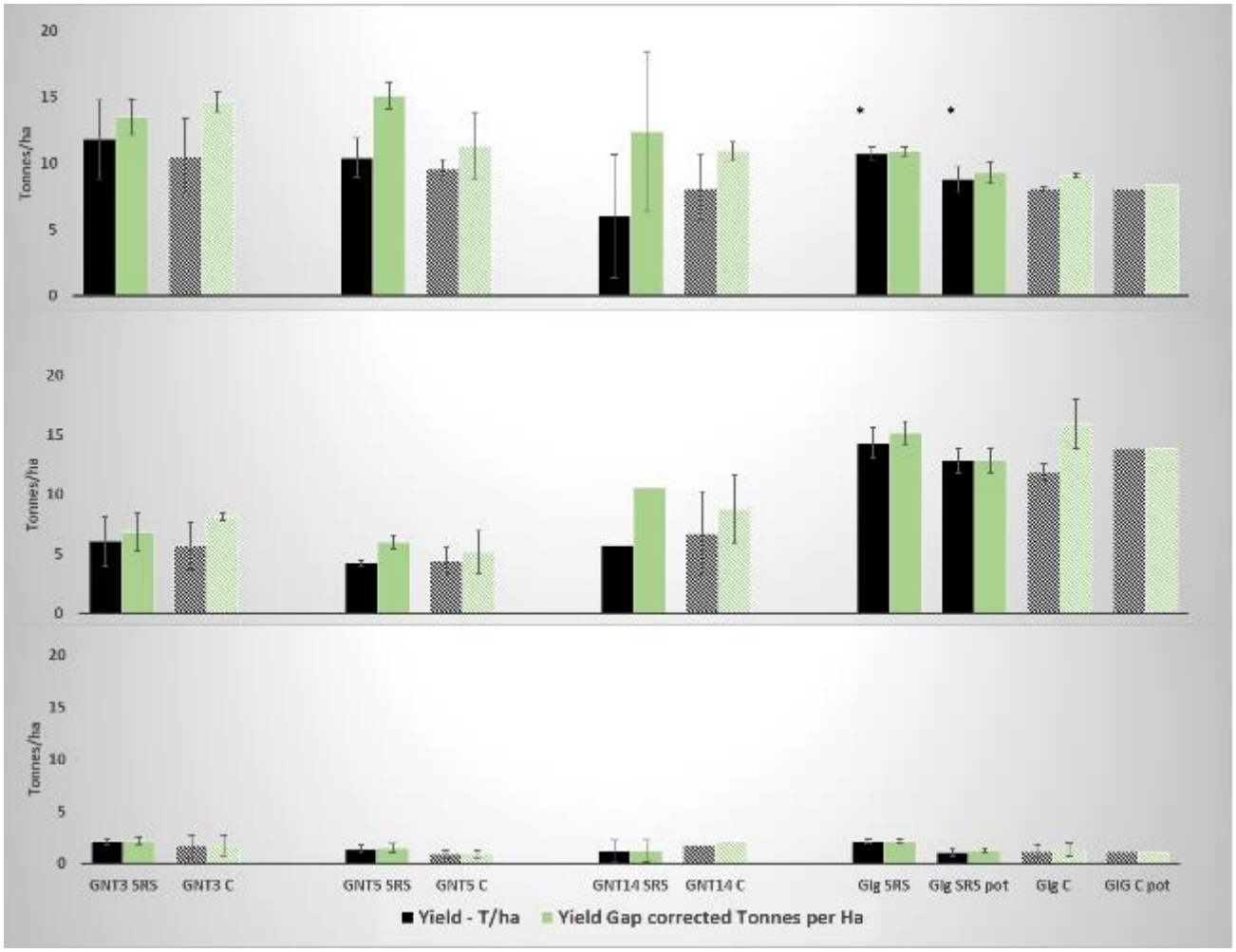


Figure 4.34. Harvest yield over three consecutive years, starting at the first harvest after the first winter (2017) until the most recent harvest (2019). Chart incorporates all genotypes and hybrids included in the trial and is measured in tonnes per hectare. Actual T/ha values are displayed in black/black hashed. Gap correction values to model full sward yield had no individual plants in each quadrat died, are displayed in light green/hashed green. Filled blocks signify treated with SRS plots, hashed blocks are the equivalent control. Error bars show ± 1 se, n=3. Where no error bar exists, only one data point is available for that treatment.

4.8 Discussion

For biomass crops such as *Miscanthus* to be carbon neutral, if not carbon negative, improvements in crop establishment need to be achieved through methods that are not to the detriment of sustainability, particularly energy balance (Rothballer *et al.*, 2008). One way to fertilise *Miscanthus* and maximise growth potential on nutritionally degraded soils, without the use of energy expensive fertiliser applications, is to exploit beneficial plant- microbe interactions to bio-fertilize plants. The use of plant growth promoting bacteria (PGPB) known as endophytes, was tested in the absence of other plant fertilizers, to assess the effects on growth and yield. Endophytes provide benefits to the host plant including enhanced nutrient uptake, growth promotion, enhanced tolerance to abiotic stress and higher resistance to plant pathogens (Muthukumarasamy *et al.*, 2002). We trialled the addition of two endophytes (*Pseudomonas fluorescens* and *Herbaspirillium frisingense*) to different hybrids of *Miscanthus*, inoculated into the plant via a novel delivery system called the Simulated Rhizosphere (SRS) developed by project partners Nutriss ltd.

4.8a Glasshouse assessments

The glasshouse trials in 2016 and 2017 produced highly variable results for the additions of SRS to plug soil medium. The data suggested positive effects of endophyte additions in SRS on the growth of GNT14 seedlings in 2016 (Figure 4.18).

Plant growth in small substrate volumes usually plateaus once plants have depleted the available nutrients. *Miscanthus* seedlings typically spend 6 to 10 weeks in plugs before being transplanted into the field. *Miscanthus* plug plants are not usually grown in nutrient deficient compost, as was tested here; however, even in germination compost, seedlings will usually exhibit signs of stress after 8-10 weeks of growth including arrested growth and leaf yellowing. The control plants in the glasshouse experiment of 2016 continued to grow throughout the 5 months that they were in small volumes, albeit at an extremely slow rate (Figure 4.18). The continuous growth may be a result of lower than usual growth rates overall, due to the low nutrient conditions, so plants took a much longer time to reach maximum potential root and above ground growth. Endophyte treated plants also continued to grow, at a much higher rate of biomass accumulation until the final harvest (Figure 4.18). The enhanced growth may be due to endophytes fixing nitrogen and potentially other essential nutrients such as phosphorous and potassium to overcome nutrient limitation in the

substrate. By the third harvest date it was noted that endophyte treated plants had begun to lose a degree of greenness and this can be seen in the bottom right hand photograph of Figure 4.17. This could potentially signal that available nutrients within the small plug volume had been sequestered. The significant increase above ground growth was not matched by a significant increase in root biomass until treated plants were substantially larger than controls. Endophytic colonisation from the SRS may increase uptake efficiency of water and nutrients, to an extent that plant requirements could be provided by small root systems. Control plants lacking the specific endophytes are likely to require larger root systems, filling the small available space, in search of nutrients. As aboveground biomass increased, the treated plant roots also significantly increased over time, but control plant root systems remained smaller, in parallel with the aboveground material by maintaining a near 1:1 root:shoot ratio. There is a wealth of literature surrounding the benefits of endophytes associated with host plants, and many papers conclude that endophytes can enhance plant nutrient uptake, and plant growth (Muthukumarasamy *et al.*, 2002, Farrar *et al.*, 2014).

One of the endophytes used (*Herbaspirillium frisingense*) has been associated with *M. sinensis* plants in southern Germany, and genome sequencing has shown that it has the genomic requirements needed to fix nitrogen (Straub *et al.*, 2013). Endophytes such as this enhance growth through a multitude of ways, including regulation of production and activity of plant hormones including auxins, gibberelins, cytokinins and ethylene, the latter is generally considered a plant growth inhibitor (Mei and Flinn, 2010). Straub *et al.* (2013) tested the efficacy of this endophyte on young *M. sinensis* seedlings, and found that the growth promoting potential of this endophyte was dependent on the nitrogen supply, as a greater growth promotion effect was observed under low nitrogen treatments. The typical phenotype observed in a strongly positively responsive cultivar of most inoculated crops in vitro included a larger, more branched, root system, and a more developmentally mature individual after 3 – 4 weeks than controls, with sturdier stems, and more root hairs (Nowak *et al.*, 1998). Kim *et al.* (2012) noted similar results in switch grass, but also identified unresponsive cultivars that lacked notable improvements.

The growth promotion from endophyte-SRS treatment here was up to 40% compared to controls, especially in the amounts of fine root structures, laterals and root length. The impact of endophyte treatment was similar to published studies except the effects appeared to occur

over a longer period. Fine root structure analysis within the plants measured here was confined to amount of root hairs per mm^2 and was not conclusive.

In 2017, the experiment was repeated, to include a higher number of replicates and in order to harvest plants for bacterial sequencing. The same genotype and growth conditions were used as the first experiment but the treated seedlings grew less well. In control trays an average 13% of plugs failed to produce a plant, compared with 17% in treated trays. Once established there were no further losses of seedlings in control trays; however, there was an average 40% mortality rate in the treated trays. In the third replicate tray 24% of plugs did not emerge, and 53% of the ones that did failed to survive the first month, resulting in a severely low number of surviving plants within this replicate (Table 4.1).

Plant growth was low in all trays, endophyte treated plants had significantly lower rates of growth, and poor development until approximately 6 weeks old. At the 6-week-old stage, the mortality rate in tray 3 of treated plants was so high, that it was removed from further growth measurements and the remaining plants taken for harvest only. Growth of the remaining two trays of endophyte-treated plants began to improve and plant height surpassed control plants by 8 weeks, and this difference was significant by week 10. Plants from the treated trays were highly heterogeneous compared with control plants (Figure 4.23).

The growth patterns are suggestive of growth inhibition or pathogenic responses to the endophyte inoculations, which is highly unusual as most papers agree that bacterial and fungal endophytes cause no ill effects, (Souza *et al.*, 2015, Mei and Flinn, 2010, Rothballer *et al.*, 2008). The negative impacts on seedling growth of endophyte-SRS treatment occurred as early as 2 weeks after sowing. Previous work with *H. frisingense* in *Mxg* seedlings using immunological labelling techniques showed that cells of the bacteria were present inside root cortex after just three days, and after 7 days were colonizing the vascular tissue in the central cylinder (Rothballer *et al.*, 2008). After the endophyte-SRS treatment many of the seedlings died but some overcame the inhibitory effects and were much like the previous year's results, surpassing control plant growth and expressing higher levels of chlorophyll. It is tempting to suggest that the inoculation had a 'what doesn't kill them makes them stronger' effect. A similar effect was seen in the plants received from the commercial nursery, and was also reported in the HCK 17 field trial seedlings across all hybrids, although GNT14 was the most badly affected, and had the poorest germination across the trials (Figure 4.31).

The cause of this phenomenon is likely to be multifaceted. The seed used for the GNT14 progeny for that year had consistently poor germination and establishment, with high mould growth across every experiment in which it was used, including the endophyte trials. It was concluded this likely resulted from use of a new threshing machine which damaged the seed coat. This goes someway to explaining the poor response to inoculation in GNT14; however, reductions in growth over the first 2-4 weeks of bacteria inoculation were seen in other hybrids also in that growth year. Tellenbach *et al.* (2011) suggest that bacteria may slow down plant growth by allocating the resources that are produced or taken up by the plant, or penetrating living host cells and killing them. As *H. frisingense* is a known nitrogen fixing bacterium the allocation of resources may be a significant issue initially. For atmospheric nitrogen (N_2) to be available to plants it must be reduced to ammonia (NH_3), which is the nitrogen fixation process catalysed by bacteria such as *H. frisingense*. This conversion is complex with multiple steps described below:

$N_2 + 8e^- + 8\ H^+ + 16\ ATP \rightarrow 2NH_3 + H_2 + 16\ ADP + 16 + 16$ inorganic phosphate (Campbell *et al.*, 2012)

This multistep process is catalysed by the enzyme nitrogenase. The process of nitrogen fixation requires eight ATP molecules for each NH_3 synthesized, therefore nitrogen fixing bacteria require a rich supply of carbohydrates from root secretions or decaying plant material (Campbell *et al.*, 2012). Nitrogen fixing bacteria added to very small immature plants could have a deleterious effect on plant growth through a high metabolic load that would be correlated with bacterial numbers impairing the progression of plant growth.

It is also possible that the reaction observed was due to the existence of normal plant immune responses. Plants lack an immune system that could be likened to that in animals which produces T cells that can attack and disable pathogens before eliminating them, but instead have more general defences (Lam *et al.*, 2001). One of the most common responses to a perceived pathogen threat is the initiation of programmed cell death (PCD) which forms part of the hypersensitive response (HR) pathway of immune response (Keller *et al.*, 1999). The HR response occurs at the site of pathogen entry and initiates PCD in and around the infection site (Lam *et al.*, 2001). This response can often be seen as lesions on leaves, but as the point of entry for the plants in this experiment was root based, it would be more difficult to see necrotic lesions as a result if a resistance response. Systemic disease resistance responses are

usually initiated when pathogens are not recognized by the host, however have also been observed even when they are known to the host. In a study on plant growth promoting bacteria and grape vine by Bordiec *et al.* (2010) perception of infection by non-host bacteria *Pseudomonas syringae*, was met with an extreme release of defence genes and mechanisms, however, when the same plants were artificially infected with a known beneficial pathogen (*Burkholderia phytofirmans*), a reaction was still noted, although much less intensely. This proved that plants could produce a generalized HR response when treated with bacteria, before recognition of beneficial or negative species. Quantity of bacteria likely plays a large role in the scale of the plant response. Within humans, there exists a vast microbiome of microbes, which generally cause no ill effects, and the majority of interactions are symbiotic, with bacteria playing a vital role in the function of the tissues they inhabit, and thus playing an important role in the balance between health and disease (Lee and Mazmanian, 2010). These bacteria only become an issue when they begin to dominate, for example in immunocompromised patients, which suggests that numbers of bacteria are key to the difference between beneficial interactions and systemic infection (Taur and Pamer, 2013). It is likely a similar reaction is being seen within infected seedlings in these experiments. In the GNT14 seed in particular, a highly negative response was noted to initial infection (Figure 4.30). It is highly possible that the plants were compromised as a result of broken seed and mould infestation as a result, and as such were less able to deal with initial infection responses as a result of added endophytes.

The nature of the interactions between host and endophyte is complex and not fully understood. The results of inoculation with endophytes could be altered by a variety of factors such as host species, bacterial species, bacterial concentration, soil type, and by the presence of existing endophytes within the host. Chamberlain *et al.* (2014) describe these interactions as context dependent species interactions. These can vary from negative, to mutual, to positive along a spectrum, varying in magnitude, and is highly dependent on the biotic and abiotic conditions in which they occur. Therefore, the most likely context-based reason for the effects seen is that the endophyte added to the plugs was too concentrated from either high endophyte concentration within the polymer, or too much polymer added to the compost, and/or the plants were too weakened by seed coat damage and biotic stress from mould infiltration. A study analysing root endophyte additions in Norway Spruce

seedlings reported variable results ranging from beneficial to negative, and discovered a strong positive link between the extent of colonization and virulence (Tellenbach *et al.*, 2011). It would also be highly informative to extend experiment 1 and grow plugs with a range of different concentrations of endophyte to discover how close are the commercial concentrations of endophyte to levels that are inhibitory. A further consideration is the possibility of interspecies interactions between added endophytes and those already existing in the seed as a result of vertical transmission from parent plants. Tellenbach *et al.* (2011) discovered dark septate endophytes effects on Norway spruce growth and survival, had a high virulence effect when they originated in the same region as the Spruce seedlings, as opposed to those gathered elsewhere. A meta-analysis of the effects of fungal root endophytes on plant growth suggested that plant responses can be effected by several factors (Mayerhofer *et al.* (2013). Firstly, host specificity; inoculation with an endophyte isolated from the same plant species could increase root biomass by up to 88%, compared with plants inoculated with endophytes isolated from different plant species. Secondly, different hosts respond differently to endophytes; tree species in particular appear to have mainly negative responses, but that monocots such as grasses and sedges mainly responded positively. Thirdly, experimental conditions; such as variation in nutrient status of growth medium, soil pH and organic content, the impact of such environmental factors can be greater than that of host specific impacts.

4.8b Field trials

Seedling behaviour under glasshouse conditions for the HCK11 (experiment 1) field trial saw a significant height increase between seedlings of GNT3 grown under the higher endophyte concentration (treatment A) than the control treatments with and without N and the lower concentration (Figure 4.8). Phenotyping assessments showed consistently good performance in standard control treatment, with controls without N often having the lowest growth averages. Biomass yield was not assessed for the first growth year. Data from the second and third harvest year indicate a much lower dry matter accumulation from the control without N plots, although this improved slightly in the latter (2019) harvest (Figure 4.12). The addition of endophytes within nutrient free soil undoubtedly aided plant growth and was comparable with the standard treatment in the nutrient rich compost, clearly demonstrating the nutrient fixing abilities of the endophytes, under limited nutrient conditions. It is unlikely that

commercially grown plug plants would be grown in nutrient free compost and therefore it would be of interest to assess endophyte additions to standard germination compost.

The larger multi-hybrid (Experiment 4) trial provided less conclusive results. Seedling development was initially similarly stunted as the previous seedling trial described in this chapter. Germination was also comparably poor within the GNT 14 plugs, especially in treated populations (Figure 4.28). Three-week-old hybrid seedlings all had reduced stem growth and leaf number when treated, in comparison to control populations, with the smallest plants in endophyte treated GNT14, and the largest in control GNT5 (Figure 4.29). As with the previous experiments the controls are seen to initially grow larger than the endophyte treated plants, and growth is slower and more homogenous within the population which may reflect lower levels of available nutrients.

The levels of establishment in the field of the seeded hybrids was comparable to that achieved by rhizome propagated *Mxg*, with the exception of GNT14. Biomass yield was not significantly different between endophyte treated and control plots in any growth year or for any hybrid (Figure 4.34).

Harvested biomass was higher in *Mxg* established without pots, but with SRS. There was no significant difference observed between any *Mxg* treatments until the third harvest year when plots that had been propagated without pots but with SRS, had significantly higher actual yield than those with SRS that were propagated in pots. This difference became non-significant when gap corrected however, suggesting that higher survival within the quadrat area may have been the largest difference, as opposed to actual individual plant biomass, however both SRS treatments were yielding more than the controls by this point. The data from this trial is extremely variable within and between treatments, and genotypes, making it difficult to identify significant effects. This data also proves that the majority of difference and growth promotion observed when beneficial endophytes are added, is seen at the young seedling and pot phase, as opposed to later on in the open field trial phases. This result is not uncommon, however, positive effects of endophyte inoculation have been reported to a higher degree than neutral or negative, for example in Perennial ryegrass (Lowe *et al.*, 2008). It is likely that growth promotion of *Miscanthus* by *H. frisingense* and *P. fluourescens* is genotype specific. Kim *et al.* (2012) tested the ability of one endophyte (*Burkholderia phytofirmans* strain PsJN) on switchgrass cultivar Alamo and other switchgrass cultivars, and

results suggested specific genotype effects exist, as some genotypes were highly responsive to the growth promotion effects of PsJN, and others not.

It is likely that higher levels of responsiveness to inoculation are observed under pot conditions because those conditions are nutrient and space limited, unlike conditions found in the field. *Miscanthus* species are a highly sustainable crop, in part due to their low input requirements, ability to grow under marginal conditions, and their high nutrient use efficiency. Studies have shown, that *Miscanthus* requires relatively little nitrogen fertilization once established, and that yields differed only slightly in *Miscanthus* trials grown on poor, eroded soil, when compared with yields on productive soil (Yost *et al.*, 2017). Several field experiments have been conducted on plantations of *Miscanthus*, showing that the use of nitrogen fertilization had little to no effect on biomass (Schwarz *et al.*, 1994). One such assessment labelled added nitrogen with ^{15}N and examined the distribution and balance after application, discovering just 19% of total nitrogen was derived from the introduced fertilizer (Christian *et al.*, 2008).

Important consideration should be given also, to the potential of extensive bacterial population already existing within the seed. Cope-Selby *et al.* (2017) demonstrated a diverse range of bacterial populations within *Miscanthus* seed that had been thoroughly surface sterilized. This suggests a high probability that beneficial bacteria populations are passed down from one generation of *Miscanthus* to the next, via vertical transmission (Cope-Selby *et al.*, 2017). Throughout their research into the identification of endophytes within *Miscanthus*, Cope-Selby *et al.* (2017) found that *Herbaspirillium frisingense* was not among those found, although *Pseudomonas fluorescens* was sequenced along with 17 other phyla within the sterilized seed. This suggests a high level of bacterial diversity within even sterilized plant material. Within natural systems a large variety of plant growth promoting bacteria exist, all with high diversity in their genomic composition suggesting numerous common and strain specific effects for potential interactions (Straub *et al.*, 2013). Not only are numerous bacterial species found within the seed, but also within the soil microbiome and any effects of inoculated bacteria will competing against the effects of this large and diverse population.

4.8c Conclusion

There is a case to be argued for the use of beneficial endophyte species within *Miscanthus* growth plantations. This assessment was limited in terms of experimental design, by company partner requirements for generation of the SRS product to be undertaken at the company labs due to industrial patents. As such some methodology was confidential, and thus in-depth experimental designs and project control over endophyte concentration and amount supplied to plants were not possible due to these restrictions, placing a great deal of experimental design into the hands of the commercial partner.

Based on results obtained in these experiments and field trials, it is unclear as to whether bacterial endophytes can enhance establishment or biomass yield under reasonable growth conditions. Glasshouse results prove that the SRS formulated by the company Nutriss ltd, is successful in delivering micro-organisms to the plants. The endophytes chosen are known to exist in *Miscanthus* material and have been specifically noted for their potential roles in stress tolerance and enhanced biomass in the book 'Endophytes for a Growing World' (Beekwilder *et al.*, 2019). In many cases, the endophyte additions had positive effects on seedling growth, after initial negative effects after inoculation. Further assessment is necessary to determine optimum concentrations and combinations of bacteria presented to young seedlings. It is also important that quantities of SRS given to pots or plugs be effectively standardized. Given the common belief that endophytes reveal their greatest advantages under conditions of plant stress, it is probable that this would be the optimum and most economical use of them as biofertilization. Results of field trials are inconclusive at best for the most part; however, the positive result for *Mxg* plots seen at the end of the multi-hybrid trial is interesting as this result followed a severe drought year, potentially confirming the stress resistance hypothesis. It would be interesting to observe whether this positive trend continues over subsequent harvests.

The financial costs of endophyte biofertilization must be considered when results are as variable as have been observed here. There is little point in adding expensive micro-organisms into plug plants if the benefits are too variable or small to be considered an economic gain further down the line. It is entirely possible that with more research and fine-tuning of best practice, that *Miscanthus* plug plant growth would be greatly enhanced under glasshouse conditions by additions of micro-organisms. Potentially this could mean shorter amounts of

paying time spent under commercial conditions, which would be a gain both financially, and in terms of energy used to provide lighting and heat. An additional aim would be the occurrence of an economical yield in the second harvest year, which could see a faster return on grower investment. If additions of endophytes have little to no yield benefit, then their additions would be an unnecessary cost.

4.8d Further work

For the best possible benefits of these plant – micro- organism relationships to occur in *Miscanthus* seedlings, a multifaceted approach must be considered. Context of plant genotype, geographical location and likely biotic and abiotic conditions in the field, should be considered collectively when selecting for the most useful combination. In addition to the requirements of best plant husbandry, it is likely that it would be of great benefit to identify the regions of plant genome responsible for governing these relationships, so that it may be exploited for future plant breeding. A visual assessment of bacterial colonisation using confocal microscopy could provide interesting information regarding speed of bacterial colonisation and population density within each part of the plant. As mentioned previously, an in-depth study into bacterial species selection and concentration of bacteria required would be greatly beneficial to the growth promotion of *Miscanthus* plug plants. Many positive results using endophytes have come from using them to enhance plant abilities under less than ideal conditions. Therefore, further assessments should also consider testing of endophytes under abiotic or biotic stress conditions including drought or flood, salt levels within soil, severely nutrient deficient soils, contaminated soils, and upon exposure to herbivores. In order to test for the existence of the inoculated endophytes, primers should be designed that would be specific enough to amplify only the endophyte of interest, as there is likely to be a rich variety of species within plant material, and surrounding soil. It may not be sufficient to be able to prove that the endophyte in question simply exists in the plant material, due to the potential for vertical or horizontal transmission of the species from other sources. It may be more informative to assess concentration levels of the inoculated species, in comparison to other species of bacteria present. This would allow a quantitative analysis of bacterial species present, as opposed to simply proving their existence in the plant material, which could have come from a number of sources. The presence of a high quantity of artificially inoculated species would allow a greater certainty of successful transmission to

plant material. Assessments of concentrations of essential nutrients including nitrogen and phosphorous in surrounding soil and plant material of treated and control plants, could also be informative when testing bacterial efficacy.

5 Growth and development of *Miscanthus* seedlings when grown in one of four different module sizes, and transplanted to field at three separate dates.

5.1 Introduction

5.1a *Miscanthus* and propagation methods

Within *Miscanthus* species, the typical propagation method of choice until recently has been to plant rhizome fragments. However, in recent years there has been research into developing a seeded *Miscanthus* variety. This would allow, among other benefits, the ability to upscale the crop faster and to produce more young plants at a cheaper rate (Clifton-Brown *et al.*, 2017). As this is an innovation in its relative infancy, there is much room for improvement, refinement and analysis to identify methods of best practice for commercial seeded *Miscanthus* varieties. *Miscanthus* seeds are small and have high thermal requirement for growth and establishment (Clifton-Brown *et al.*, 2011) (Figure 5.1). Smaller size seed has been correlated with lower competitive advantage under vegetation cover conditions in monocarpic perennials (Gross, 1984). Studies have also shown that larger seeds are more likely to have a 'reserve effect' containing a potentially greater amount of reserves to aid a germinating plantlet (Westoby *et al.*, 1996). There is also a higher risk of seeds being lost in soil crevice's and reduced seed to soil contact. Direct seeding of *Miscanthus* has so far remained an unreliable method of commercial planting, due to low seed germination rates, climate heterogeneity and seed to soil contact issues. This often results in the appearance of gaps within a field plot (Ashman *et al.*, 2018). These gaps due to plant death leads to an increase in the need for labour, as replacement plants must be acquired and placed in the spaces to achieve homogeneity across the field site. These new plants often have reduced growth by the end of the season due to being planted later. Without the nutrient reserves found within the rhizome, and the temperatures needed for seed germination, a seed is very unlikely to germinate and subsequently survive under field conditions. As such the current standard method has centred around *Miscanthus* plug plant technology, using plug techniques originally developed for the vegetable industry (Lewandowski *et al.*, 2016). Plugs are grown in glasshouses for the first 8-10 weeks of their lifecycle before being planted in the field (Figure 5.2). This allows the plants to have some development of root and stem biomass

Figure 5.1 Seeds of GNT14 hybrid. Authors own image

and a better chance of field survival. This system has much scope for optimisation, with variables including genotype, module size, soil type, nutrient additions, age at field planting and the physical characteristics most suited to survival in the field, such as root systems, which are of huge interest.

Figure 5.2 *Miscanthus* plug plants growing in the 126A standard plug size tested in this experiment. Plants are nearing planting readiness in the photograph. Image courtesy of Bell brothers Nurseries, Boston, Lincs.

Direct seeding into field trials, currently requires considerable optimisation due to low establishment, especially under the temperate conditions of the UK (Ashman *et al.*, 2018). Whilst these improvements are pending, the focus is on the optimisation of yield produced from the plug planting method. Growing plug plants in more controlled environments prior to transfer to the field provides the opportunity to manipulate factors that are hypothesised to alter plant performance. These factors include sowing and planting timing, maturity of plugs, module size, and environmental conditions after planting. A balance will need to be struck between the impact of controlled treatments and their cost. One considerable benefit would be if controlled treatments of developing seedlings could shift, to earlier in the perennial cycle, the attainment of economically harvestable yield by the end of the second year. Such improvements would likely see a rise in uptake of the crop from potential growers.

Conditions of establishment are a significant factor in the vigour and productivity of a field trial (Tejera *et al.*, 2019). One group of variables of high importance regarding the plug planting technique, is the size of the module the plant will be grown in during the glasshouse phase, and the timing of sowing and planting based on above and below ground maturity within the module. The potential impact of module size is illustrated in a study of the effects of pot size on *Pinus pinea* by Dominguez-Lerena *et al.* (2006), where it was shown that container volume had the greatest influence on plant morphology. Containers with a larger volume allowed greater height and diameter, higher nutrient content and better field performance.

5.1b Sink-source relationships and the importance of pot size

In order to maximize growth and survival throughout a plant's life, an individual plant must strike the optimum balance of sink-source nutrient partitioning, and growth, depending on internal and external factors (White *et al.*, 2015). This balance must be achieved both below ground in the plant's root system, and above ground in the plant's aerial shoots (Iwasa and Roughgarden, 1984). Both the root/shoot ratio and growth rate are highly dependent on several, often co-occurring variables; including plant species, time of year, weather, age of plant, water availability and photosynthetically active solar hours. The ability of a plant to effectively manage its own source-sink relationship is a key element to survival in the transient environmental conditions it may be subjected to (White *et al.*, 2015). An effective balance will allow the plant greater chance of surviving during times of resource limitation, and flourishing during times of plenty (Rogers *et al.*, 1995). This system is seen globally in wild and cultivated plants in all environments, with species flourishing and attuned to specific environmental niches. However, within the controlled environments of plant biology study, these conditions will be largely dominated by what growers provide for individual plants.

The majority of studies into plant biology will start off by using individually grown plants, which are grown in a specific container best suited to the desired end result or analysis (Poorter *et al.*, 2012). This system, while necessary within experiments, cannot adequately replicate the growth and development a given species would exhibit in the unrestricted rooting conditions prevalent in the field. Container grown plants in general tend to have differing root morphology than field crops (NeSmith and Duval, 1998). Often an experimental or commercial venture based on individual plants will ideally adopt the smallest container or

pot possible for each individual plant, with the aim of reducing costs associated with materials such as compost, and making more efficient use of space for growing and transportation (Poorter *et al.*, 2012). Glasshouse space can be a particularly costly commodity for commercial plant production, and so making the most out of a plant in the smallest module/pot possible is of great interest to growers.

When attempting to grow the most viable plants per unit area the importance of the root system must be adequately considered to effectively acquire below ground resources (Ho *et al.*, 2005). There are many possible variables associated with pot design, including depth of the pot, volume of soil it can hold, and lateral surface area for more surface level root systems. Root systems are highly complex, and the source of nutrients for the plant to produce aerial growth. A smaller pot will naturally allow for a greater number of replicates and easier handling but may also impede plant growth due to a lower quantity of soil, water and nutrients and cause root binding in many species if left in the container too long.

The length of time a plant remains in the container is an important factor that needs to be optimised (NeSmith and Duval, 1998). A comprehensive meta-analysis study into the effects of pot size and rooting volume in plants conducted by Poorter *et al.* (2012), highlighted that in the majority of studies growth of plants was restricted, and in many cases highly stunted in smaller pot sizes. This phenomenon was seen increasingly as experiments progressed, with later analysis showing the largest differences were seen in plant growth rate and overall biomass, which is not unexpected as pot size should have minimal effect on plants in their infancy.

5.1c Root morphology and the *Miscanthus* seedling

It is not certain what the ideal plug characteristics would be for *Miscanthus*. Impacted and root bound plants signal that the plant has outgrown its current container. Due to the sink-source feedback loop, if roots ordinarily act as a sink then limiting root growth could limit above-ground growth by potentially down-regulating photosynthesis (Campany *et al.*, 2017). Alternatively, root impaction could potentially encourage more and competitive above ground growth if other sinks were available such as stem, which is a sink in grasses (Harris, 1992). Plant roots and shoots work in close co-ordination, controlled by complex signalling pathways of various hormones, further complicated by interactions with various other

external factors (Di Benedetto, 2011). These mechanisms are not clearly understood, especially in *Miscanthus* research due to the infancy of the crop as a commercial product (Clifton-Brown *et al.*, 2017).

In commercial *Miscanthus* trials, a more impacted root ball allows for easier high throughput planting using conventional planting machines, because the plug is easily removed from its container and planted intact. Some trials have reported that less compacted plugs with a looser and more "feathered" root architecture can establish and grow faster. This phenomenon is likely due to the presence of free growing lateral roots which could quickly establish themselves in the soil for improved resource capture, as opposed to bound roots which need time to grow into the newly available space (Schultz and Thompson, 1997). Often, when root restricted seedlings are planted in the field, they are unable to compensate for evapotranspiration due to reduced root-to-soil adhesion, even when well-watered directly after transplanting (NeSmith and Duval, 1998).

Of highest concern to the end user, grower, or farmer, is the post-planting performance of the seedlings. Survival and yield across the whole botany sector is of utmost importance, with variations in container size showing some mixed results (NeSmith and Duval, 1998). Many morphological and physiological responses of plants to varying container sizes and root restrictive conditions have been reported across a wide range of species, and the improvement and testing of available options in this experiment is of great importance to further improve establishing complete and high yielding *Miscanthus* plantations in the field. The aim is to optimise glasshouse space, module size, length of time in modules and planting window.

This chapter focusses on assessing the impact of four different commercially available plug module sizes ranging from small and compact with high glasshouse space efficiency, to large rooting depth with increased space requirements. In addition to testing the impact of different plug designs the interaction with growth time prior to field planting was tested. Three separate field trials with different plug sizes were planted at varying times 'early', 'mid', and 'late' season planting over Spring and Summer in 2017. It was hypothesized that best plant performance at the end of the first growth year, as well as highest yield at harvest would occur in the plants given the largest module size and that were planted at the beginning of the growth season. This, in theory, would allow the plant to achieve maximal biomass

accumulation as a result of the time spent in the ground. However, it is uncertain whether more time in controlled environments with optimal temperatures would result in better overall seasonal growth and establishment due to the limitations to root development and subsequent associated effects.

The hypotheses for this experiment are:

1) Large plug modules will increase plant biomass, faster growth under glasshouse conditions, and produce higher establishment when planted into field conditions
2) Earlier planting will extend the growth season available to plants, encouraging higher accumulated biomass, in comparison to later planting

5.2 Methods

5.2a Plant material and growth conditions

Seeds of *Miscanthus* hybrid genotype 'GNT 14' from the late 2016/early 2017-threshing batch were acquired by Bells Nurseries, Boston Lincs. Multiple trays of four different module sizes and volumes were purchased and filled with John Innes germination compost. The first tray size was the current commercial standard; the '126 blue', containing 126 separate wells, each with a volume of $25cm^3$ and a soil capacity of approximately 8.5g each, at a rooting depth of 4cm. The second tray size also contained 126 individual modules, with a volume of $35cm^{3,}$ soil capacity of approximately 11.9g and rooting depth of 5cm. The third tray contained a higher number of 144 individual modules, with a volume of $45cm^{3,}$ soil capacity of approximately 15.3g and rooting depth of 5cm. The fourth tray contained a lower number of 104 individual modules, with a much larger available volume of $70cm^{3,}$ a soil capacity of approximately 22g, and rooting depth of 5.5cm.

Seeds of GNT14 were multisown (3 – 5 seeds) into each of the individual seedling wells for all trays on February 2nd 2017 and trays placed into a temperature controlled commercial glasshouse. Temperature was kept within 18 - 28°C where possible on a 12hr night/day cycle, with occasional temperature peaks under sunny conditions. Table 3 shows module details, and Figure 5.3 shows images of each module type.

Table 3 Plug module information and parameters for each of the four plug sizes used in this experiment

Plug module type	126A	126B	144	104
Volume of soil (cubic centimetres)	25	35	45	70
Depth (cm)	4	5	5	5.5
Soil capacity (g)	8.5	11.9	15.3	22
Cost per tray (£)	2.6	2.55	2.5	2.42
Trays per 100m2 of glasshouse	63	63	61	61
Plants per 100m2 of glasshouse	7925	7925	8764	6330
Approximate price per plug plant	£0.021	£0.020	£0.017	£0.023

5.2b Glasshouse measurements of selected tray of each plug type

One tray of each type was assessed throughout the glasshouse period from February to mid-June 2017. Germination assessment was undertaken at approximately 14 days post-sowing and recorded as the number of cells in a single tray containing a seed with a green shoot. Ten plants were chosen at random per tray on a near weekly basis and extension of tallest stem measured as the height from the base of the stem, to the newest fully expanded leaf. In May, ten seedlings were again randomly selected from each tray, but were then destructively harvested. Each plug was cut just above the root mass of the seedling and above and below ground biomass were separated, roots washed and weighed individually. Samples were then dried at approximately 70°C and re-weighed.

5.2c Planting and trial design

Plants from each tray type were sown into three replicate blocks in three different trials, separated by planting date, all at a density of approximately 15,000 plants per hectare. Trial sites were situated within the same experimental field in Hackthorn, Lincolnshire UK (Latitude 53°19'50.82"N, Longitude 0°28'13.46"W). The first field trial (HCK 25) was planted on the 26th of April 2017, when plants were approximately 11 weeks old. The trial was planted with the four different module sizes divided into four plots, with three replicates of each plot. Each plot contained 100 plants in 10 rows of 10. Intra-row spacing was approximately 1 metre, and inter-row spacing approximately 0.75 metres. Including paths between plots, the entire area is approximately 0.16 ha.

The second trial (HCK 30) was also planted with three replicates of four plots containing plants from one of the plug sizes, but was planted 3 weeks later on 17th May 2017. Due to field space requirements and remaining plug numbers, this trial had a halved number of plants in each plot compared to the first planting. Individuals were planted in 50 plots with 5 columns of 10 plants. Intra- and inter-row spacing remained the same as with the previous trial. The third trial (HCK 33) was planted on the 14th June 2017, when plants had been in plugs for approximately 18 weeks. The trial was set up to the same design as the second trial, with three replicates of the four plug sizes, orientated in 5 rows of 10. All plots were hand planted, before being sprayed with a pre emergence herbicide, watered, and covered in a layer of biodegradable mulch film. Plants were left to grow through the mulch film over the summer and autumn, being weeded where necessary. Exact placing of each trial can be seen in Figure

5.4 Trial sites as environmentally similar as possible were chosen, and all within the same field. The soil over the field is described as sandy clay loam. The soil becomes slightly heavier however, to the left of the field where the early and late planting are situated.

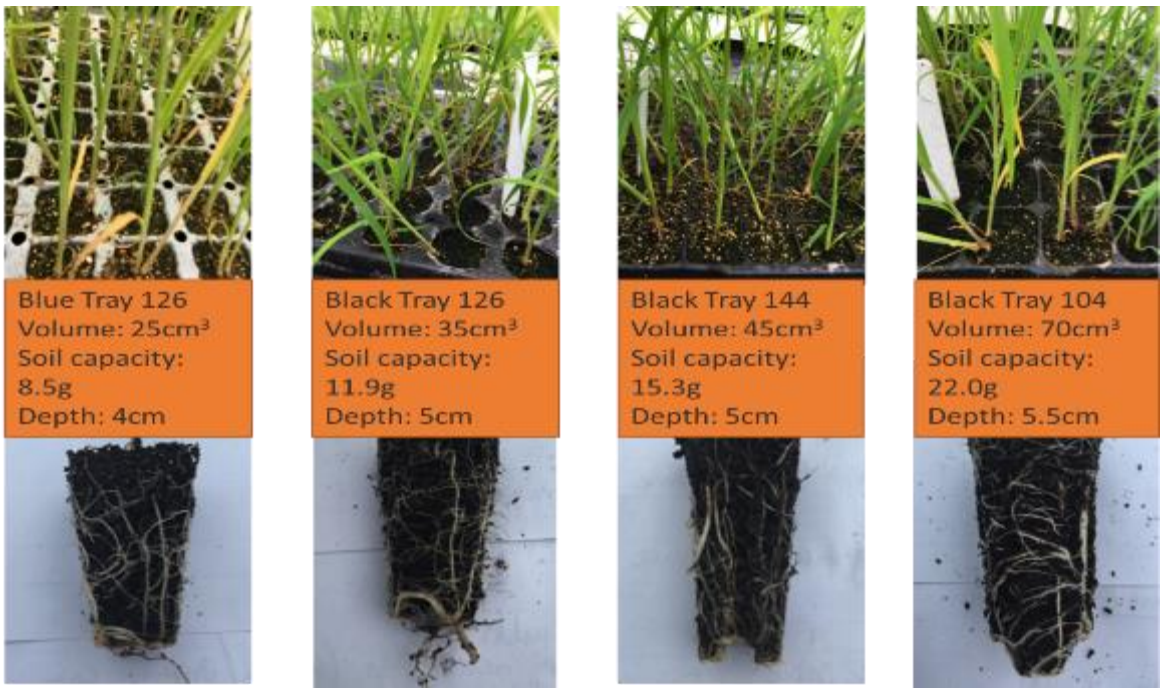


Figure 5.3. Sample photographs of each of the four different module size trays as they look from above, with module information and representative root ball for each module. Images taken in May 2017 after approximately 6 – 8 weeks in modules. Image courtesy of Bells nurseries, Boston Lincs.

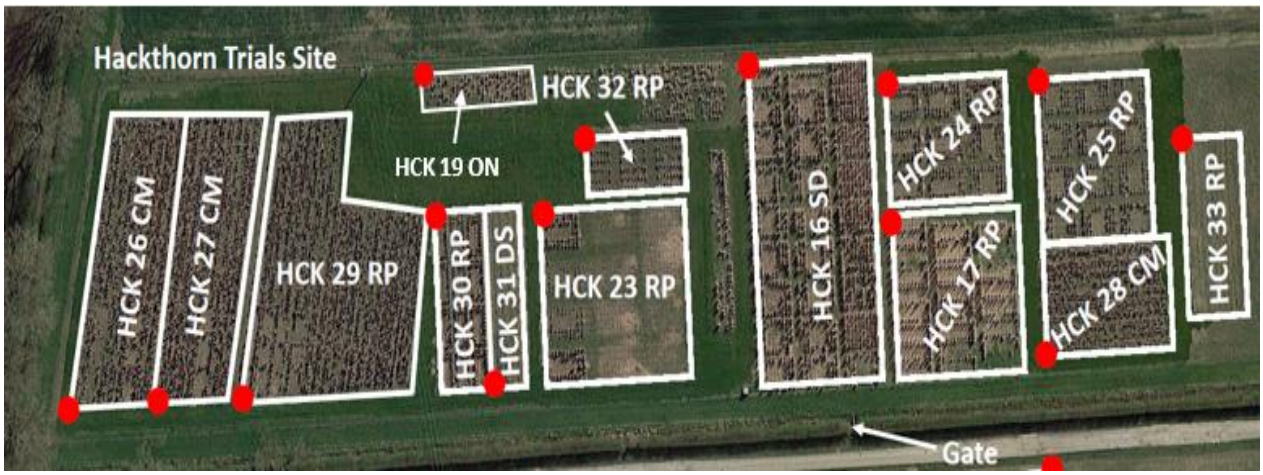


Figure 5.4 Layout of Hackthorn field trials including sites for HCK 25, HCK 30, and HCK33 plug sizes trials. HCK 25 – earliest planted (26th April) to the right of the field. HCK 30 – mid planting (16th May) to the mid – left of the field, and HCK 33 the latest planting (14th June) at the far right).

5.2d Autumn phenotyping, rhizome harvest and overall harvest for all plug size trials

All plots were measured for growth parameters in November 2017. Within each plot, eight plants were selected from the innermost column of the plot to avoid potentially confounding edge effects. This method was chosen to increase the accuracy of the plot means but to avoid pseudoreplication treatment means were calculated from averaged plot values only. This method was repeated for all three trials. Selected plants were assessed for canopy height; a trait described as the height of the canopy where maximal light interception begins, functionally described as where leaves begin to bend. Shoot height was measured as the height of the tallest stem from the base of the plant, to the highest ligule on the newest fully expanded leaf. Number of stems was also counted, as any stem over the height of approximately 5cm. This assessment was repeated at the end of 2018.

Assessment of below-ground biomass was done during March 2018 on one plant per replicate plot, totalling three replicates per plug size for each trial. Plants selected were not within the quadrat area of plants being harvested and phenotyped for other analyses. For the first planting, the HCK 25 plot (which has 100 plants per plot), rhizomes were able to be taken from plants which were not on the edge and therefore subject to edge effects. For the mid-season and late season plots HCK 30 and HCK 33 trials, which had 50 plants per plot, plants had to be selected from the edge of a plot, but care was taken that they not be from the exposed (outer) edges of the trial. Plants chosen were from the same row and column from each plot where possible. Once selected, the above-ground portion of the plant was cut with a hedge strimmer as close to the base as possible. The fresh biomass was weighed in field using scales and a tripod. A representative subsample was taken from the cut biomass, containing three whole stems chosen at random, with whatever leaf material remained on them, and the subsample weighed for fresh biomass. Subsamples were later dried at 80°C to reach a constant dry weight. The base of the plant was dug from the soil at approximately 30cm diameter from the base in all directions, and as far down as was necessary to ensure all rhizomatous material was collected. The remaining above-ground attachments were removed. All rhizome samples were placed in nets and labelled. Samples were pressure washed to remove as much soil as possible. When as much soil was removed as was practically

achievable, a fresh weight of the sample was taken, and the sample placed in a drying oven at 105°C until constant dry weight was recorded.

Harvesting of plot biomass was during March 2018 to allow ample drying down of senesced plant matter, and assimilation of nutrients to the rhizome. In the larger plots of HCK 25 an inner 24 plant quadrat was harvested. The number of live plants within the quadrat were recorded for an accurate assessment of yield within the quadrat. Live plants were then cut from the base, from approximately 5cm height, using a hedge strimmer, and the entire biomass weighed using a tripod and scales. Three representative leaf and stem subsamples per replicate plot were collected in separate paper bags, and then weighed, and labelled. The subsamples were dried in ovens at 105°C until constant weight, and moisture content calculated by difference. An average could then be taken of all three subsample moisture contents. Using this moisture content, it was possible to estimate the total dry biomass of the harvested quadrat. Knowing the quadrat area, maximum plant count, and surviving plant count, these parameters could then be scaled to estimate the total biomass per hectare.

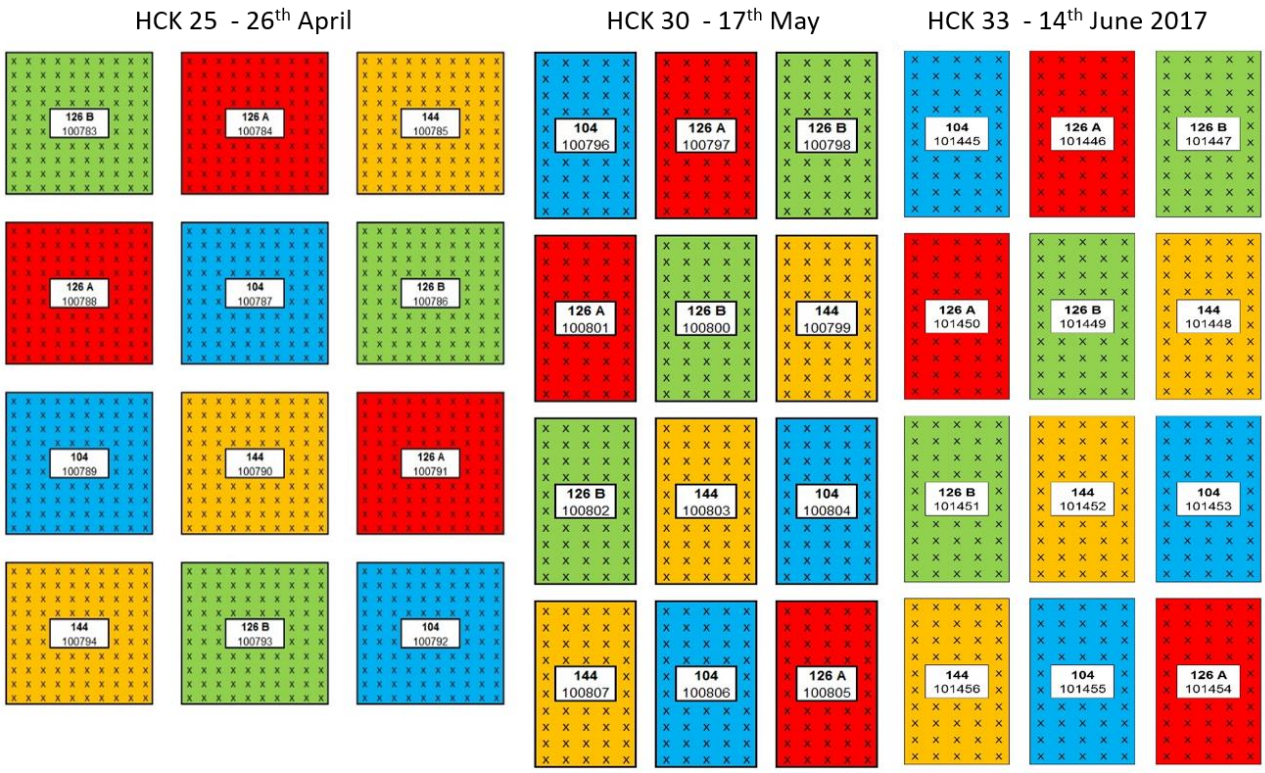


Figure 5.5 Field design for *Miscanthus* trial of 4 plug designs and 3 sowing times. Each sowing time comprises three replicate blocks for each plug type: Red – 126A, Green - 126B, Yellow – 144, Blue – 104, X represents one plant. HCK 25 the early planting consists of 100 plants per plot, HCK 30 and 33 trials consist of 50 plants per plot.

5.3 Results

5.3a Glasshouse phase germination and growth prior to planting in spring 2017

Germination rates did not differ significantly between module size, reaching between 80 – 90% for each tray measured (Figure 5.6).

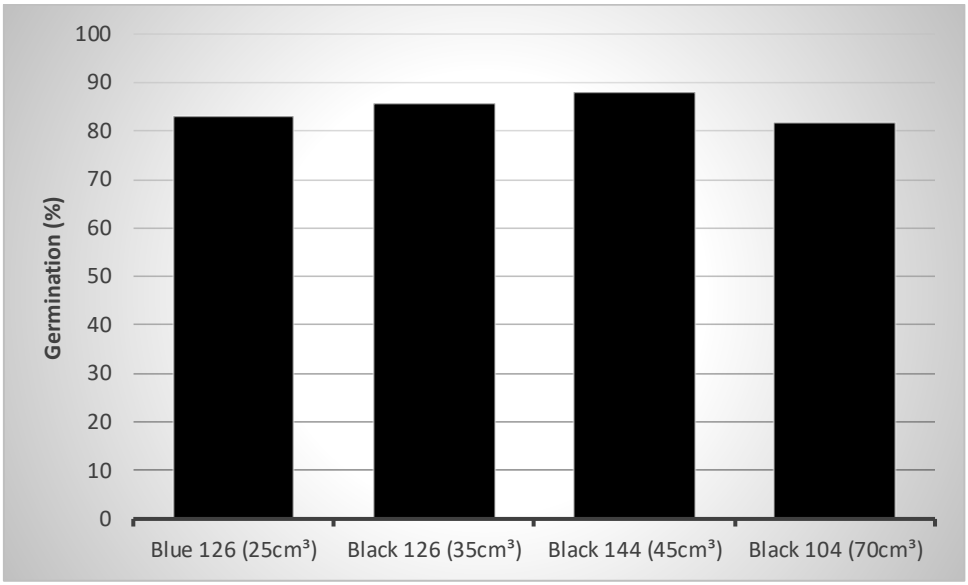


Figure 5.6 Germination rate of one complete tray of each of the four different plug tray designs of different sizes and volumes. Seeds (3-5) were sown in February 2017 and the presence of green shoots recorded from each plug after approximately 10-14 days

5.3b Assessments of height over four months in glasshouse conditions

There was no significant difference in heights between module sizes for the first three months (Figure 5.7). In early May, however, there was a significant difference of $p = 0.009$ between groups, with post-hoc tests confirming that the black 104 module produced plants that were significantly taller than all others ($p<0.05$ in all cases), reaching an average of 23.4cm per plant, compared with between 18 – 20cm in all other module sizes. The result was the same in mid-May. By the start of June, all average heights had increased dramatically under glasshouse conditions. Between groups there was a significant difference of $p = 0.030$, and further analysis confirmed that the 104 module with an average of 30.54cm stem length, was significantly taller than the 126A (blue) module with 23.9cm average per plant ($p = 0.022$).

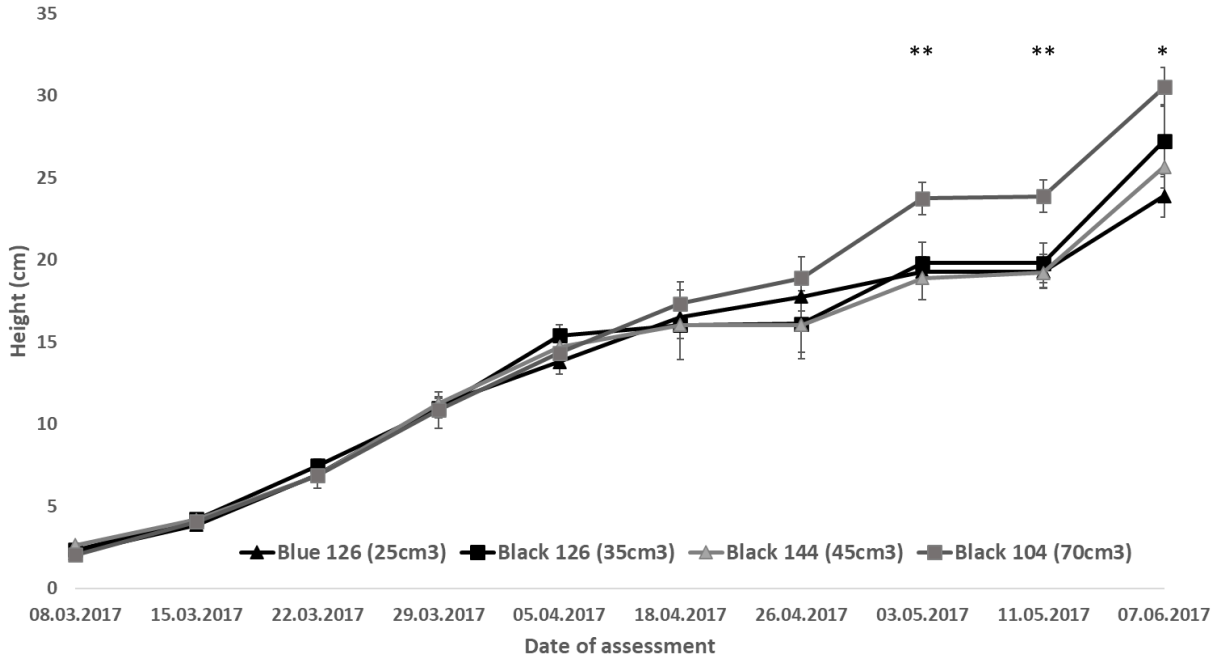


Figure 5.7 Height of *Miscanthus* from four different plug tray designs of different sizes and volumes growing in glasshouse conditions. Black lines indicate the two smallest plug sizes (triangles = blue 126A tray, squares = black 126B tray). Grey lines indicate the larger two trays (triangles = black 144 tray, and squares = 104 tray). In each case n = 10 based on individual plants in one tray of each size. Error bars show ± 1 se. Means were compared by one-way ANOVA at each time point (indicates where p<0.01, * indicates where p<0.05)**

5.3c Destructive harvesting of plants in May and again in June

Destructive harvesting of plants from the glasshouse in May 2017 revealed a significant effect of plug design on above-ground dry biomass ($p < 0.01$) (Figure 5.8A). Further analysis found that the 104 modules produced significantly higher above-ground biomass than all other module sizes ($p<0.01$). Below ground analysis produced more variable results, with the 104-module producing the highest average below-ground dry biomass but no significant differences were found (Figure 5.8B).

Biomass harvests of glasshouse plants in June 2017 produced a significant effect of plug size on above-ground dry biomass ($p < 0.01$), and Tukeys HSD post-hoc analysis confirmed that the 104 module produced significantly higher above-ground biomass than all other module sizes ($p < 0.01$ for all) (Figure 5.8A). Below-ground biomass was also significantly different between groups ($p < 0.01$). Post-hoc analysis showed that the 104-plug produced significantly

higher below-ground biomass than all other modules ($p < 0.01$ for all), but also that the 144-well tray produced significantly more biomass than the 126A blue module ($p = 0.014$). Photographs of a representative root mass from each module size show the large variation between larger and smaller modules.

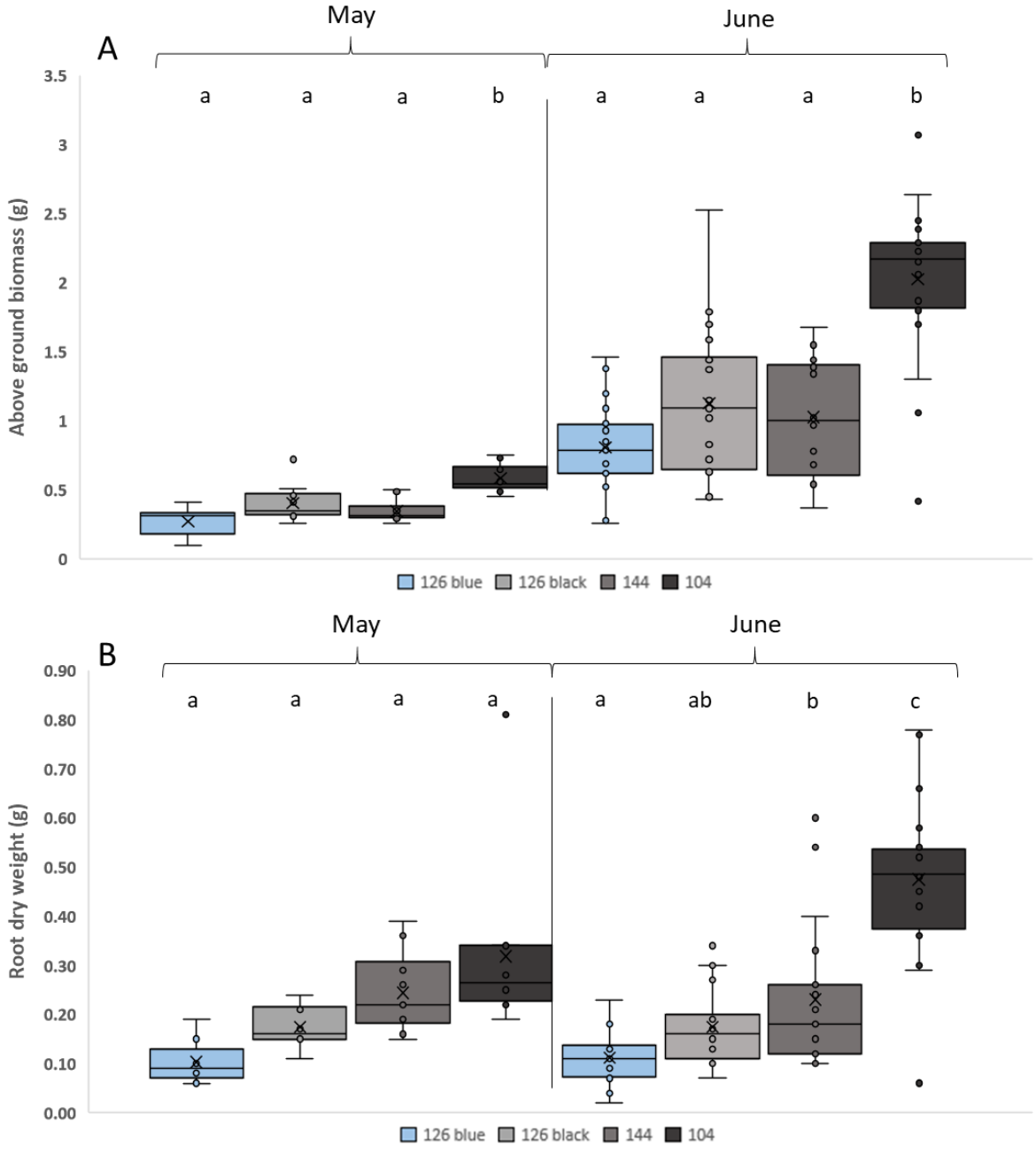


Figure 5.8 Results of destructive harvest for aboveground (A) and belowground (B) biomass assessment of plants grown in one of four different module sizes. Pale blue boxes show result for the 126A blue module (25cm^3). Light grey show result for the Black 126B (35cm^3) module. Medium grey shows result for the 104 module, and darkest grey result for the 144 module. Charts are divided into the results for May and results in June. Letters denote significant groupings by Tukey's HSD. In May n=10, and in June n=20.

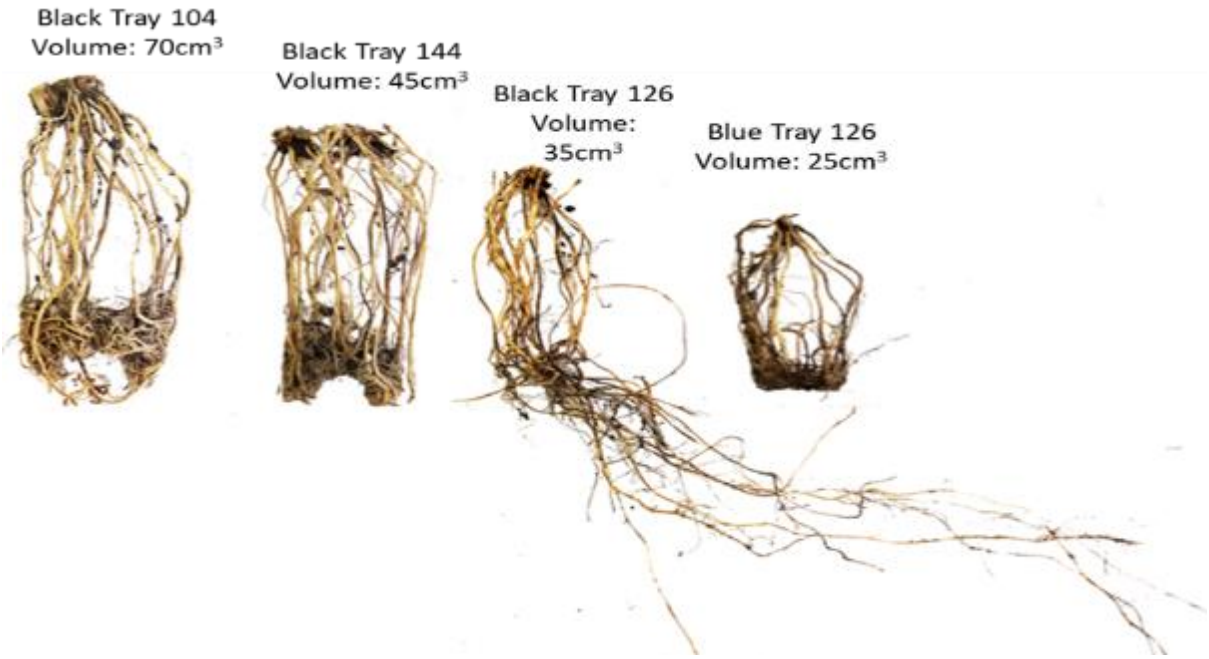


Figure 5.9. Representative image of the typical intact root system taken from one *Miscanthus* plant growing in each of 4 different plug module designs in June 2017. Plants were approximately 5 months old. Image courtesy of the *Miscanthus* Upscaling project and commercial partner Bells Nurseries, Boston Lincs.

5.3d Establishment and growth characteristics from autumn 2017 and 2018, and harvest results from early spring 2018 and 2019

Establishment after the first and second growth season

Two-way ANOVA analysis of the number of surviving plants in Autumn 2017 revealed a significant effect of planting date ($p < 0.01$), and plug size ($p = 0.006$) on the establishment rates over the first season (Figure 5.10Figure 5.11). The interaction of factors was also significant ($p = 0.002$). The highest establishment rates were achieved from the mid-season planting, every plug size produced 100% establishment after the first growth season. The earliest planting also produced high establishment with over 90% establishment from each of the 4 module types. Both early and mid-season planting times displayed no significant difference in establishment between module sizes. Late season planting formed a separate group and was significantly different to the two earlier plantings. There was a significant difference between module designs in the late season planting group ($p = 0.024$). Lowest

establishment was observed in the two smaller module sizes with averages of 76% in 126A and 67% in 126B. The largest module size (104) produced the best establishment rate in the late season planting at 99%.

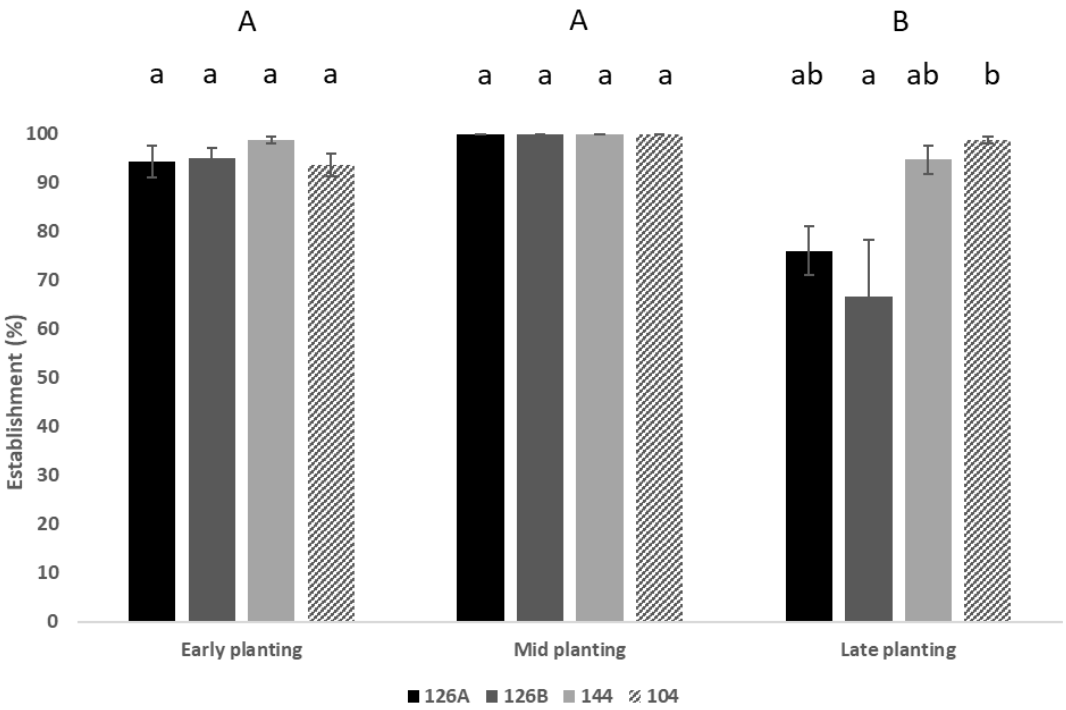


Figure 5.10 Establishment rate of *Miscanthus* plants grown in 4 different plug designs before transfer to the field. Percentages were calculated from a count of live plants in Autumn 2018 following the first growth season from three replicate plots for each module size. Plots were planted at 3 dates; early, mid and late season. Black bars = blue 126A module. Dark grey = black 126B module. Light grey = 144 module, and hashed = 104 module. Capital letters above chart show Tukey's HSD analysis of survival between 3 planting times. Lower case letters denote Tukey's HSD analysis of module size within individual planting dates. Error bars show ± 1 se

Results of establishment after the first winter and through the next growth season did not differ from the first assessment (Figure 5.11). Two-way ANOVA showed a significant effect of planting date ($p < 0.01$) remained after the second growth season, but no significant effect of plug size over all sowing times ($p = 0.066$). The interaction between plug design and planting time was no longer significant ($p = 0.148$). Establishment remained high at 98-99% over all module sizes after the mid-season planting. Establishment rates from the early planting were lower after the second growth year than the first at between 81 – 88%, but no significant effect of module size within the early planting data. Survival remained variable in the late

planting data, but had not declined further from the previous year. The largest module size produced the best establishment rate over all planting dates.

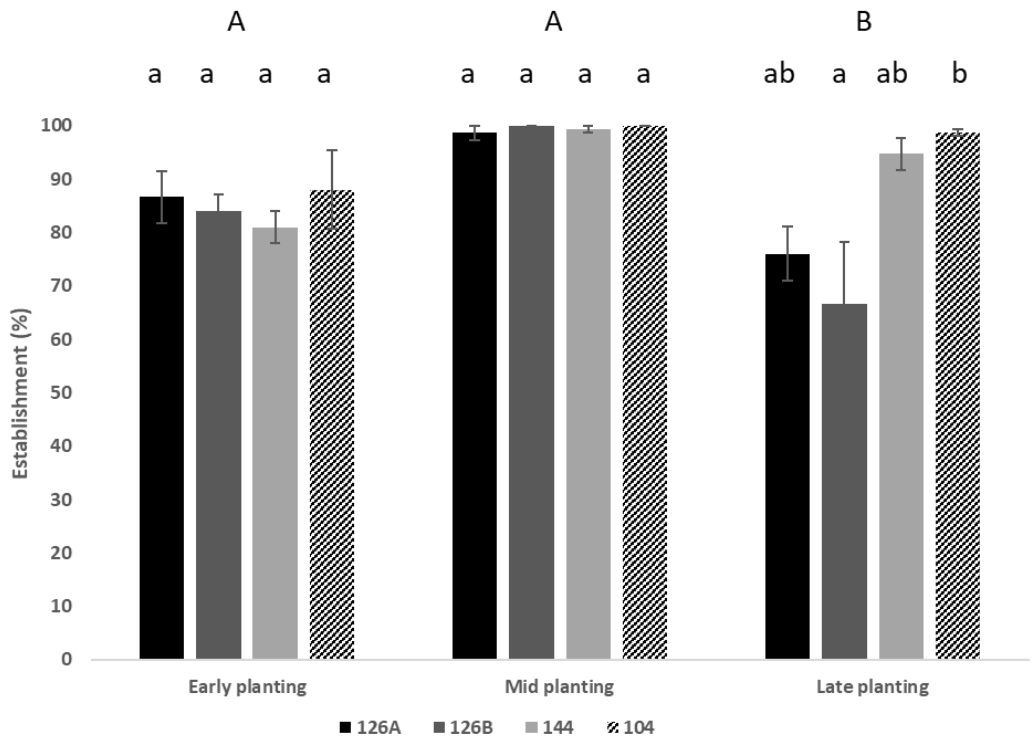


Figure 5.11 Establishment rate of *Miscanthus* plants grown in 4 different plug designs before transfer to the field. Percentages were calculated from a count of live plants in Autumn 2019 following two growth seasons from three replicate plots for each module size. Plots were planted at 3 dates; early, mid and late season. Black bars = blue 126A module. Dark grey = black 126B module. Light grey = 144 module, and hashed = 104 module. Capital letters above chart show Tukey's HSD analysis of survival between 3 planting times. Lower case letters denote Tukey's HSD analysis of module size within individual planting dates. Error bars show ± 1 se

Weather data at planting

The first planting was conducted in the coolest temperatures, with the average temperature during the planting week between 4 - 5°C, with moderate rainfall (Figure 5.12). Temperature averages increased greatly to 10 – 12 °C for the few days following the planting. Planting 2 was in slightly warmer temperatures of between 11 – 16°C and consistent rainfall. The third planting was in the highest planting temperatures of between 15 – 19°C, climbing to a temperature of 25°C a few days later. There was very little rainfall until around 2 weeks post planting at this time (Figure 5.12).

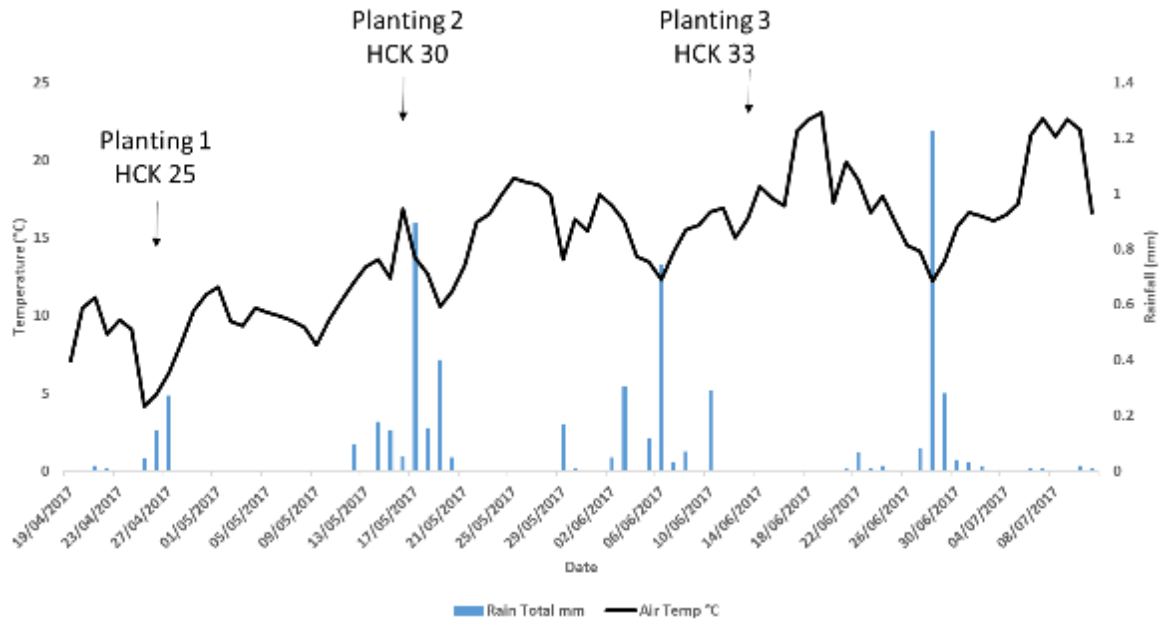


Figure 5.12 Meteorological data at Hackthorn, UK in 2017 across the planting times of *Miscanthus* growing in 4 different designs of plug tray. Three planting dates are indicated by arrows along with associated trial names along with air temperature (black line) and rain fall (blue bars).

Table 5.4. Stem counts, canopy height, and shoot height of *Miscanthus* in November 2017 after one growth year following planting at 3 different dates and growth in 4 different plug tray designs

Stem count						
Planting	**Early planting**		**Mid planting**		**Late planting**	
Tukeys subset	B		A		C	
Module size	**Mean (± StdE)**	**Tukey's subset**	**Mean (± StdE)**	**Tukey's subset**	**Mean (± StdE)**	**Tukey's subset**
126A	23.41 (±1.62)	a	29.9 (±2.7)	a	14.8 (±1.3)	a
126B	29.04 (±2.93)	a	34.3 (±3.1)	a	18.3 (±1.79)	ab
144	23.43 (±2.81)	a	40.3 (±2.8)	a	19.7 (±2.2)	ab
104	29 (±2.93)	a	37.6 (±2.7)	a	22.3 (±1.7)	b
P-value	0.220		0.06		0.026	
Canopy height						
Planting	**Early planting**		**Mid planting**		**Late planting**	
Tukeys subset	B		A		C	
Module size	**Mean (± StdE)**	**Tukey's subset**	**Mean (± StdE)**	**Tukey's subset**	**Mean (± StdE)**	**Tukey's subset**
126A	111.4 (±4.5)	a	131.9 (±7.3)	a	71.3 (±5.8)	a
126B	123.9 (±7.7)	a	140.4 (±5.7)	a	65.8 (±4.4)	a
144	107.8 (±7.4)	a	155.5 (±6.7)	a	69.7 (±4.3)	a
104	127.1 (±7.2)	a	151.5 (±6.5)	a	81.5 (±3.4)	a
P-value	0.179		0.052		0.092	
Shoot height						
Planting	**Early planting**		**Mid planting**		**Late planting**	
Tukeys subset	B		A		C	
Module size	**Mean (± StdE)**	**Tukey's subset**	**Mean (± StdE)**	**Tukey's subset**	**Mean (± StdE)**	**Tukey's subset**
126A	95.5 (±4)	a	133.7 (±6.9)	a	53.6 (±5)	a
126B	103.8 (±6.61)	a	120.04 (±5.7)	a	49.8 (±3.9)	a
144	89.3 (±7.1)	a	135.1 (±6.3)	a	52.7 (±3.5)	a
104	107.6 (±6.9)	a	128.7 (±6.1)	a	63.8 (±3)	a
P-value	0.17		0.085		0.066	

Means are shown ± s.e. for each module size along with Tukey's HSD analysis between module at each sowing date (lower-case letter). Upper case letters denote the results of Tukey's HSD analysis of differences between planting dates. Early season planting – 26th April 2017, mid-season planting – 17th May 2017 and late season planting – 14th June 2017. For each result n = 8.

Phenotyping assessments of stem counts, canopy height and shoot height in November 2017

Two way ANOVA identified a significant effect of planting date ($p < 0.01$) and module size ($p = 0.005$) on stem counts assessed in autumn after the first growth season (Table 5.4). There was no significant interaction ($p = 0.311$). Stem counts were significantly different between all planting dates regardless of module size ($p < 0.01$) for all multiple comparisons placing them all in individual subsets. Within the early planting, module size did not have a significant effect on stem counts ($p = 0.220$). The effect of plug module design on stem number was also not significant in the mid-season planting ($p = 0.06$). There was a significant effect of plug module design on stem number after the late-season planting ($p = 0.026$); this planting time consistently produced the lowest stem number across all modules. Post-hoc testing of stem counts in the later season planting identified a significant difference of the small module (126A module) and the 104 module ($p <0.05$), the other two module sizes were in both groups.

Two way ANOVA identified a significant effect of planting date ($p < 0.01$) and module size ($p = 0.035$) on canopy height assessed in autumn after the first growth season (Table 5.5). Canopy height was significantly different between all planting dates regardless of module size ($p < 0.01$ for all multiple comparisons). Lowest canopy heights were in the late season planting (between 65.8 ±4.3cm and 81.5 ±3.4cm) and the highest were in the mid-season planting (between 131.9 ±7.3cm and 155.5 ±6.7cm). Within planting dates, there was no significant effect of module size ($p>0.05$).

The results of shoot height followed the same trends as canopy height. There was a significant effect of planting date ($p<0.01$) and module size ($p = 0.051$) and no significant interaction. Largest differences were seen between planting times ($p < 0.01$ for all comparisons), placing them all in individual subsets. Within planting dates, there was no significant effect of module size ($p>0.05$).

Table 5.5. Stem counts, canopy height, and shoot height of *Miscanthus* in November 2018 after one growth year following planting at 3 different dates and growth in 4 different plug tray designs

Stem count						
Planting	**Early planting**		**Mid planting**		**Late planting**	
Tukeys subset	B		A		A	
Plug size	**Mean (± StdE)**	**Tukey's subset**	**Mean (± StdE)**	**Tukey's subset**	**Mean (± StdE)**	**Tukey's subset**
126A	24.2 (±1.6)	a	19.2 (±3)	a	15.4 (±2.9)	a
126B	28.3 (±3)	a	19.9 (±2.9)	a	13.5 (±2.9)	a
144	25 (±2.6)	a	25.4 (±3.2)	a	19.2 (±2.2)	a
104	30 (±2.9)	a	21.3 (±2.6)	a	21.7 (±2.2)	a
P-value	0.343		0.452		0.103	
Canopy height						
Planting	**Early planting**		**Mid planting**		**Late planting**	
Tukeys subset	A		B		A	
Plug size	**Mean (± StdE)**	**Tukey's subset**	**Mean (± StdE)**	**Tukey's subset**	**Mean (± StdE)**	**Tukey's subset**
126A	119.9 (±5)	a	138 (±26)	a	91.3 (±15)	ab
126B	127.4 (±8.2)	a	147 (±13.8)	a	76.4 (±14.3)	a
144	124.9 (±5.2)	a	163 (±15)	a	129.1 (±11.3)	bc
104	136 (±7.6)	a	159 (±13.7)	a	145.7 (±12)	c
P-value	0.386		0.614		0.001	
Shoot height						
Planting	**Early planting**		**Mid planting**		**Late planting**	
Tukeys subset	AB		B		A	
Plug size	**Mean (± StdE)**	**Tukey's subset**	**Mean (± StdE)**	**Tukey's subset**	**Mean (± StdE)**	**Tukey's subset**
126A	100.7 (±4.6)	a	109.4 (±13)	a	71 (±12.3)	ab
126B	104.3 (±6.9)	a	114.7 (±11.9)	a	58.7 (±11.4)	a
144	98.8 (±5.8)	a	131 (±12.5)	a	106 (±9.5)	bc
104	113.3 (±7.1)	a	125.3 (±11.5)	a	118.6 (±9.8)	c
P-value	0.357		0.586		0.00	

Means are shown ± s.e. for each module size along with Tukey's HSD analysis between module at each sowing date (lower-case letter). Upper case letters denote the results of Tukey's HSD analysis of differences between planting dates. Early season planting – 26th April 2017, mid-season planting – 17th May 2017 and late season planting – 14th June 2017. For each result n = 8.

Phenotyping assessments of stem counts, canopy height and shoot height in November 2018

After two growth seasons two way ANOVA identified a significant effect of planting date on stem counts ($p < 0.01$) (Table 5.5). The effect of module size on stem counts was no longer significant ($p = 0.117$). There were no significant effects of module size on stem counts within the early, mid or late season plantings ($p = 0.343$, $p = 0.452$ and $p = 0.103$ respectively). The early season planting produced significantly higher stem counts ($p<0.05$) than the mid- and late-season planting, placing it into a different group by Tukeys HSD.

After two growth seasons there was a significant effect on canopy height of planting date ($p< 0.01$) and module size ($p < 0.01$) and there was also a significant interaction ($p = 0.002$). Canopy height was significantly different between early and mid-season planting ($p = 0.011$) and late and mid-season ($p < 0.01$). There was no significant difference between module sizes in the early and mid-season planting; however, there was a significant effect of module size in the late season planting ($p = 0.001$). Tukeys HSD placed stem heights in 3 groups depending on module size, the lowest containing the 126A and 126B modules (a), the highest containing the 104 and 144 modules (c). The 144 and 126A results were additionally placed in the intermediate group (b).

The results of shoot height followed the same trends as canopy height. There remained a significant effect on shoot height of planting date ($p < 0.01$) and module size ($p = 0.002$) and no significant interaction ($p = 0.062$). Large differences in shoot height were seen between planting times. Mid-season plantings were significantly taller than late season ($p < 0.01$), but not the early planting ($p = 0.070$). Within planting dates, there was no significant difference in the early and mid-season plantings based on module size ($p>0.05$), although the late-season planting followed the same differences seen in the canopy heights ($p = 0.00$).

Rhizome harvest from one plant per block in March of 2018

Above and belowground biomass was removed for one plant per plot (3 replicates of each plug size and planting date combination) and the harvested below-ground biomass for each is illustrated in Figure 5.13. Two way ANOVA of plants dug up in March 2018 (after one growth year) identified a significant effect of planting date on both above- (Figure 5.14A) and below-ground biomass (Figure 5.14B). There was a significant effect of planting date ($p < 0.01$), but not plug size ($p = 0.235$). There was more below ground biomass after the mid-season planting than both early season ($p = 0.008$) and late season ($p < 0.01$). No significant difference in belowground biomass was found between module sizes at each planting date. Above ground biomass followed a similar pattern. There was a significant effect of planting date ($p < 0.01$), but not module size ($p = 0.085$). Multiple comparisons between planting dates showed all were significantly different from each other, and Tukey's HSD post-hoc tests placed them in separate groups. There was no significant effect of module size on aboveground or belowground biomass at each planting date when analysed individually. The above and belowground biomass for each of the plants was moderately correlated ($R^2 = 0.604$) (Figure 5.15).

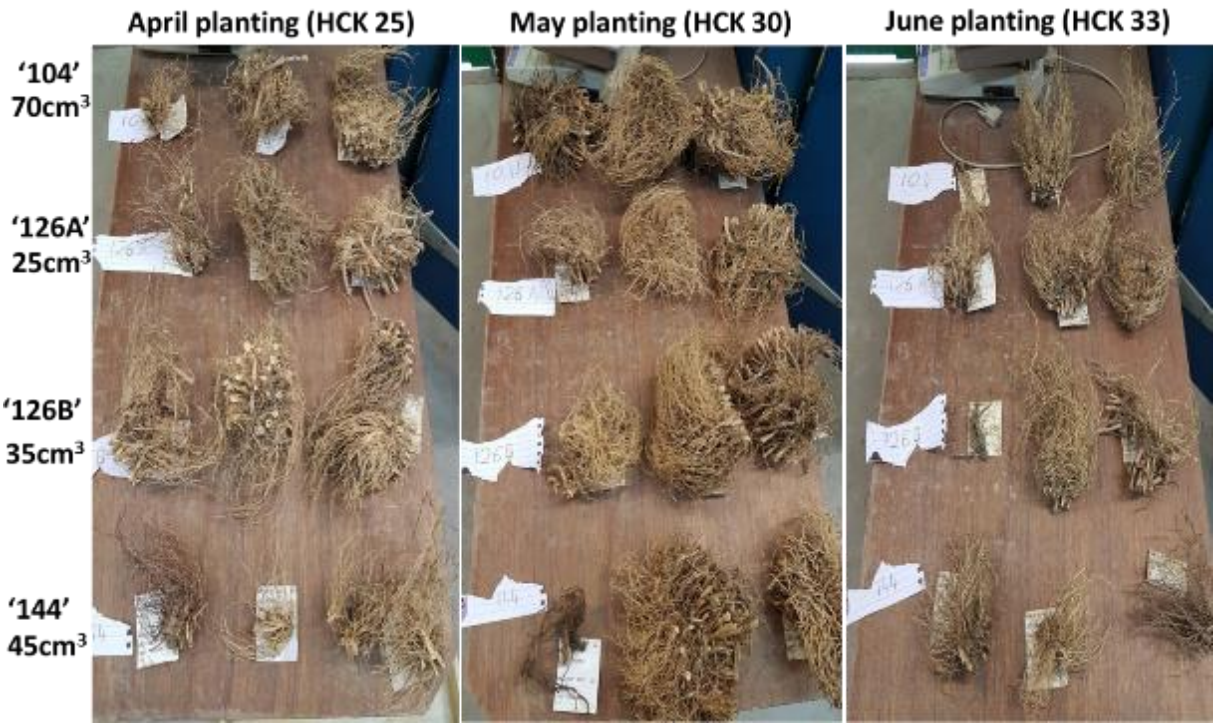


Figure 5.13 Photographs of the dried below-ground harvested material of one *Miscanthus* plant dug from each replicate plot, of four module sizes for each of the three planting dates. Plants were harvested in March 2018, almost 12 months after growth in 4 different module sizes (shown at left).

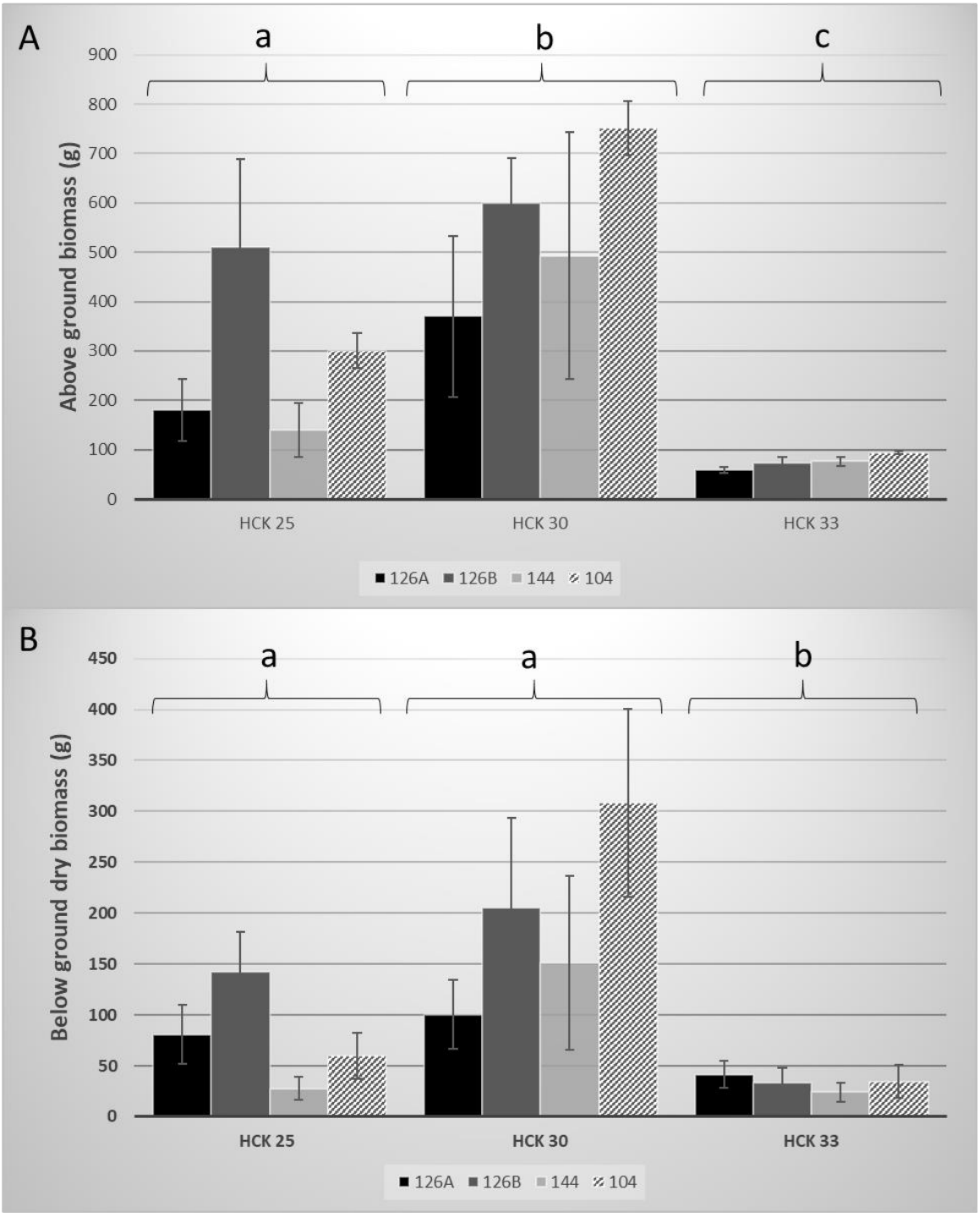


Figure 5.14 Aboveground (A) and belowground (B) dry biomass harvested in March 2018 from plots planted with variable module sizes and at three planting dates. HCK 25 – early planting (26th April 2017), HCK 30 – mid-season planting (17th May 2017) and HCK 33 – late season planting (14th June 2017). Black bars denote smallest plug size 126A. Dark grey denotes module size 126B, pale grey denotes module size 144, and hashed bars show largest module size – 104. Means are of one plant per replicate plot ± s.e., n = 3. Letters above indicate significant groups by Tukeys HSD comparison for planting date.

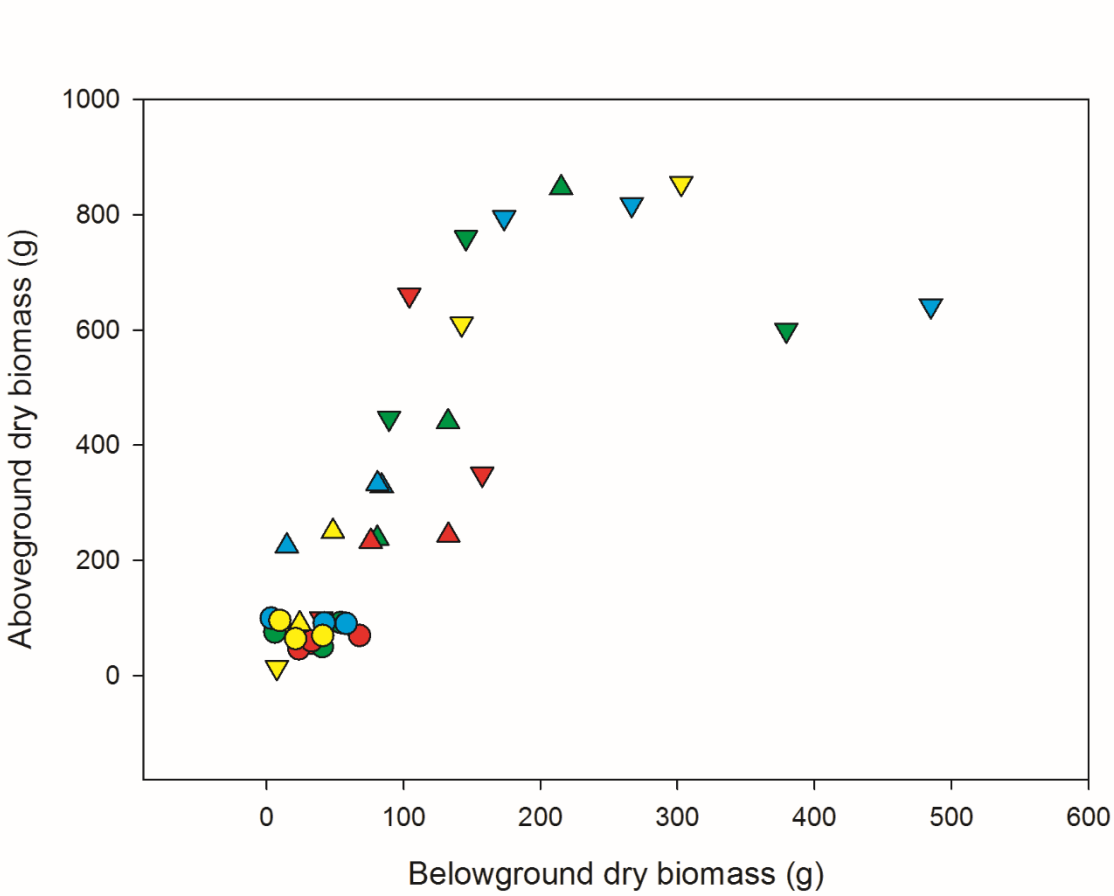


Figure 5.15 Correlation of above and below ground biomass for all plants dug from experimental plots in March 2018, for all module sizes and planting dates combined. Upwards pointing triangles signify planting 1. Downwards facing triangles signify planting 2. Circles signify planting 3. Red – 126A module. Green – 126B. Yellow – 144. Blue – 104.

Harvest of plot biomass in spring 2018 and 2019

Plot harvests to assess biomass production after winter (Spring 2018) revealed a significant effect of planting time ($p<0.05$) and module size ($p = 0.028$) and no significant interaction ($p = 0.330$). Post hoc analysis of the effect of planting dates identified three significant groups, with the mid-season planting produced the most biomass across all module sizes (3.5 - 5 estimated tonnes ha^{-1}), and the latest planting the lowest (0.8 - 1.2 estimated tonnes ha^{-1}). Module size had no significant effect on biomass produced from either the early or mid-planting dates; however, module size had a significant effect on biomass produced from the late season planting ($p = 0.006$). Post-hoc analysis indicated a significantly lower yield for the smaller module sizes in comparison to larger modules.

Plot harvests after the second growth year (Spring 2019) showed a retained significant effect of planting date on biomass ($p = 0.002$), but the effect of module size was no longer significant ($p = 0.312$). Mid-season planting produced significantly more biomass (7.1 – 7.9 estimated Tonnes ha^{-1}) than the late season planting (4 – 7 estimated Tonnes ha^{-1}) ($p = 0.001$). There was no significant effect of module size within planting dates ($p>0.05$).

There was a significant effect of planting date ($p < 0.01$), but not of module size ($p = 0.643$) on moisture content (Figure 5.17.1) of biomass harvested in spring 2018. The earliest planting produced biomass across all modules with significantly lower ($p < 0.01$) moisture contents of between 34 – 37% (Stdev 2.4 & 2.5 respectively), than average moisture content of biomass from the mid-season and late season plantings (between 40 – 48% across all module sizes, Stdev 7.9 & 6.4 respectively).

After the second growth season the biomass harvested in 2019 (Figure 5.17.2) showed a significant effect of planting date on moisture content ($p < 0.01$), but not module size ($p = 0.517$). Each planting date formed a significant group after Tukeys HSD analysis, the lowest moisture content in the mid-season planting (between 37 – 40%), and the highest in the later planting (42 – 44%).

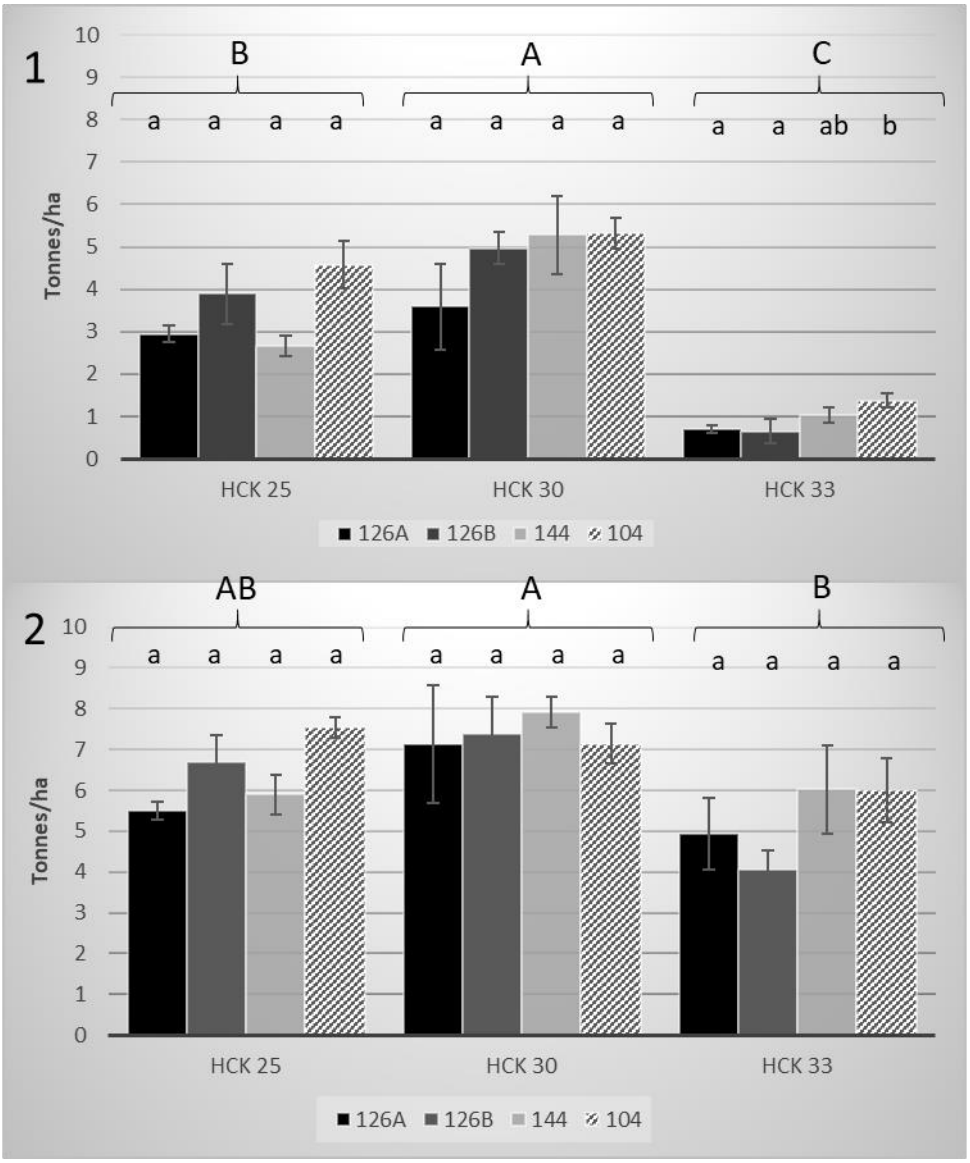


Figure 5.16 Estimated biomass yield of *Miscanthus* from plots planted with different module sizes, and at three planting dates. HCK 25 – early planting (26th April 2017). HCK 30 – mid-season planting (17th May 2017), and HCK 33 – late season planting (14th June 2017). Black bars denote smallest plug size 126A. Dark grey denotes module size 126B, pale grey denotes module size 144, and hashed bars show largest module size – 104. Harvests were in spring 2018 after the 1st (1) growth year and spring 2019 (2). Values are means ± s.e. n = 3. Tukeys HSD identified significant groups denoted by Upper case letters for groups comparing between planting dates and lower case letters for groups comparing the effect of different module sizes within planting dates.

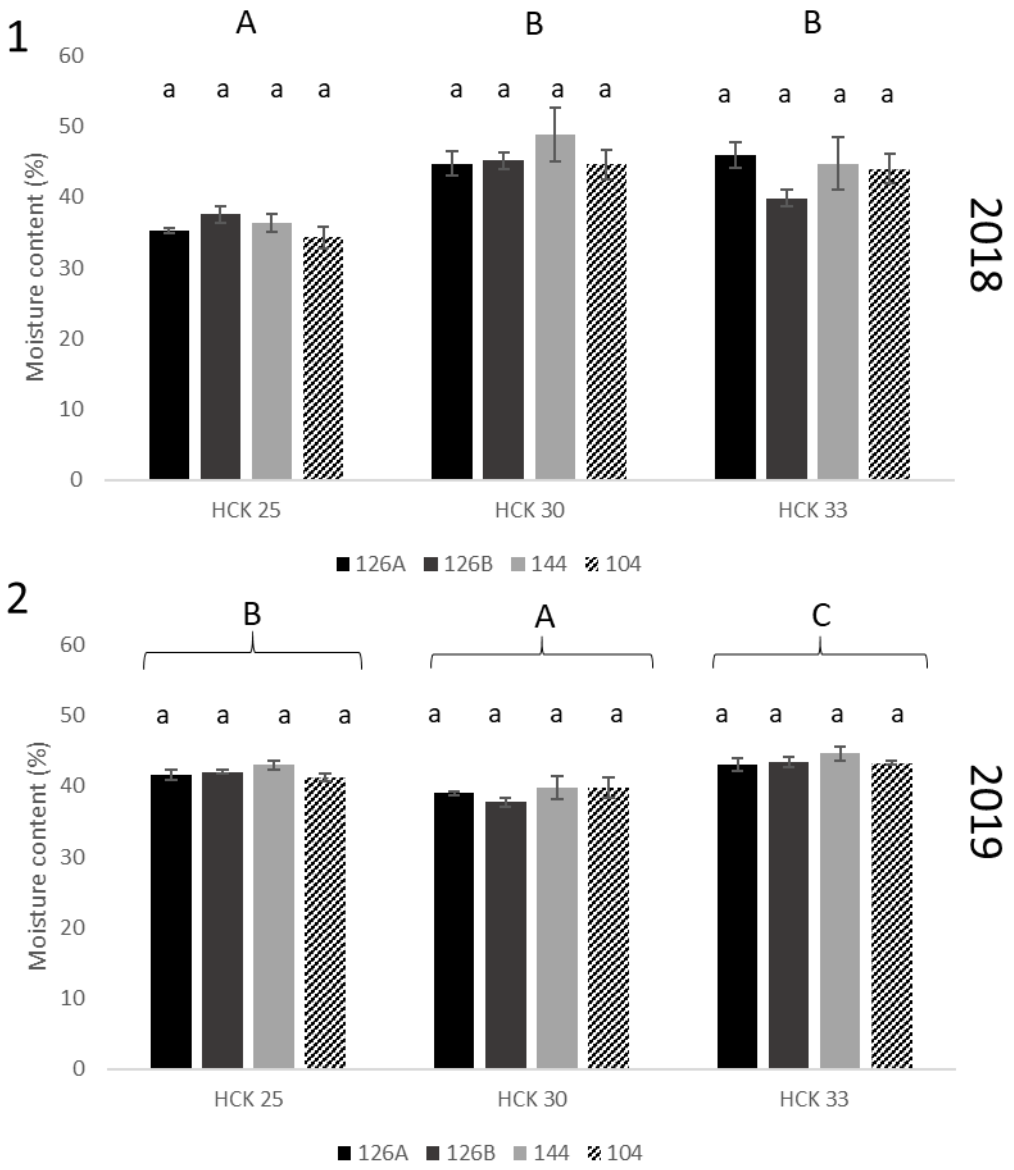


Figure 5.17 Estimated moisture content of biomass from *Miscanthus* plots planted with different module sizes, and at three planting dates. HCK 25 – early planting (26th April 2017). HCK 30 – mid-season planting (17th May 2017), and HCK 33 – late season planting (14th June 2017). Black bars denote smallest plug size 126A. Dark grey denotes module size 126B, pale grey denotes module size 144, and hashed bars show largest module size – 104. Harvests were in spring 2018 after the 1st (1) growth year and spring 2019 (2). Values are means ± s.e. n = 3. Tukeys HSD identified significant groups denoted by Upper case letters for groups comparing between planting dates and lower case letters for groups comparing the effect of different module sizes within planting dates.

5.4 Discussion

A key area of research into innovations in *Miscanthus* propagation for mass-scale roll-out of the crop is the optimisation of plug planting methods for hybrids developed from seed (Clifton-Brown *et al.*, 2017). There are a large number of factors that could potentially affect the establishment and growth of a new field of *Miscanthus* plants, including population, seed size, growth temperature, growing medium and seedling age at planting. The focus of this experiment was to assess the effects of four different commercial module designs used to establish plants in the glasshouse in combination with the effects of three different field planting times during the Spring and Summer of 2017.

It is common practice in agronomical studies to grow experimental plants in pots or containers (Poorter *et al.*, 2012). Commercially grown plants are usually grown under restricted conditions due to the cost of greenhouse space. There will, therefore, be a commercial balance between the cost of controlled growth and the returns that growth produces once the crop is in the field. Economics of the plug costs were analysed (Table 3) but important traits such as early and complete establishment of the crop, the resulting yield and moisture content at harvest were the main focus of the analysis.

The size of the pot required depends on a number of factors, including the size and the age of the plant, and the desired end product from a grower. There is a balance to be struck between the size of a pot or container and the costs and space taken up to accommodate more moderate sizes. Restrictions imposed on the root system in smaller containers decrease the above ground dry weight accumulation (Di Benedetto and Klasman, 2004). Commercially, it is more profitable to produce higher numbers of plants in smaller modules to save on space and growth substrate requirements; however, on average doubling the pot size can increase biomass production by 43% (Poorter *et al.*, 2012). Pot size has attracted relatively little consideration in the fields of agronomy as most agricultural species are either direct seeded or propagated by other methods. Transplanting methods are, however, the preferred method for ensuring good crop establishment in ornamental bedding crops and as such more research exists in these areas. In species such as *Euphorbia* increasing pot size resulted in higher photosynthetic activity resulting in greater leaf number, leaf area, root length and shoot dry biomass (Fascella and Rouphael, 2017). Quality and marketability of bedding plants, is also reduced when grown in small containers commercially, with smaller cells increasing the

vulnerability of the plant to fluctuations in moisture, oxygen and nutrients available in the soil (Di Benedetto, 2011, De Lojo *et al.*, 2017).

5.4a The effects of module size and shape on the growth and biomass accumulation of *Miscanthus* seedlings

Miscanthus plug plants would ideally have as large root mass as possible after growth in the glasshouse as they must face cold spring temperatures and unpredictable field conditions after field planting, conditions which smaller root morphologies may be more vulnerable to. This phenomenon was described in a paper by Westoby *et al.* (1996) as the 'reserve effect' , whereby during the initial growth period, larger seeds (or in this case plug plants) contain a larger percentage of reserves, which are available to support plant growth and repair any damage sustained under less optimal conditions. Over the last few decades *Miscanthus* has typically been propagated clonally by rhizome splitting, providing new plantlets with some rhizome reserves which presumably bolster resilience against environmental stresses (Xue *et al.*, 2015). *Miscanthus* plug planting is a relatively new innovation and the module of choice until the current study has typically been a 25cm^3, 4cm deep module in trays of 126 individuals. These are small volumes but provide the ability to produce many more plants in a small amount of glasshouse space. This was one of the four module sizes tested in this experiment. Other module designs increased the volume and depth of substrate and the largest (104) had just under three times the volume of the original design and a rooting depth of 5.5cm (Figure 5.3). This plug size produces fewer plants in a given space however, with 104 individuals in similar size tray, compared to 126 or 144 plants for others modules. Assessments of the maximum potential plug quantity produced within 100m^2 of glasshouse space were calculated for each module size. The small 126 modules could produce 7925 plugs each, based on 100% survival. The larger 144 module would produce 8764 plugs in the same area, and the largest 104 module would produce 6330 plugs. Combining this with the approximate costings per plug (Table 3), and the resulting seedling resilience and size, it was decided that the 144 size was likely the most profitable of the modules assessed here.

Within the glasshouse each module size produced variable root morphology as well as overall biomass. Photographs of bare roots from each module (Figure 5.9) show the current commercial 25cm^3 126A tray produced a compact, small plug with a strong mass of white root at the base of the module, whereas the 126B with slightly increased volume of 35cm^3 had

visible escaping of roots through the bottom of the module due to a larger drainage hole. As such, roots were not so impacted. The larger modules produced comparable morphology with more root mass overall, but also more impaction at the base of the module. In ornamental systems it is commonplace to find a mat of white roots at the base of a pot, even in larger modules, due to vertical root restriction effects even when plants are under optimal conditions (Di Benedetto, 2011). The destructive assessments in May and June revealed the lowest above- and below-ground biomass in the 25cm^3 126A current plug size. The 126B 35cm^3 volume had slightly higher biomass, and surprisingly displayed higher average above-ground biomass than the larger 144 45cm^3 module in May, and was comparable in June (Figure 5.8A). Across all assessments the 104 70cm^3 volume plug produced consistently and significantly larger plants under glasshouse conditions. Reduced growth in smaller pots is caused predominantly by a reduction in photosynthesis per unit leaf area, as a result of biological constraints including small soil quantities, and therefore reduced water holding capacity and available nutrients, as well as impedance of root growth (Poorter *et al.*, 2012).

Plug plants for *Miscanthus* planting are typically sown in late January or early February, and so plant age at field planting is also an important factor to consider. If a larger module size, potentially in addition to other optimum external conditions, can accelerate a plant's growth to the point it reaches a field ready stage earlier, then this could negate the extra cost of larger modules by requiring less time spent in energy consuming glasshouse conditions. However, the optimum morphology for plug grown *Miscanthus* plants to survive and flourish in early season field conditions is unknown. The variable morphology as a result of module size provides part of the assessment of this trial.

5.4b Establishment in field

The effects of module designs on field performance of *Miscanthus* were less significant in the longer term than the effects of the date of planting which produced large effects on establishment and growth. It was accepted that there was potential for confounding factors in terms of field position effects, despite all plots being within the same field, however, the by the nature of field trials some degree of microclimate variation across a field site is to be expected.

The age at planting and the time of year in which plugs are planted can have significant effects on the growth and yield of various species such as Hemp (Cosentino *et al.*, 2012) and Zoysiagrass (Sladek *et al.*, 2011). Across all measured parameters over the first year, the mid-season planting produced superior survival rates and biomass across all module sizes, whereas the later season had much poorer results. Date of planting is a concluded to be a highly important determinant of the success of a field of *Miscanthus* plants. Ideally, the date should be early to maximise the potential for longer time spent in field accumulating biomass, and not be so early that late winter or early spring frost events kill the plants before the crop is established. Sladek *et al.* (2011) describe this phenomenon in studies on Zoysiagrass as the accumulation of 'growing degree days' or 'GDD'. They found that planting grass plugs slightly earlier in Spring/Summer produced a more enclosed grass canopy, than those planted in later summer, which required a higher density of planting to achieve the same turf cover. This 'sweet spot' in planting time is transient and dependent on weather conditions over the season. In the trials reported here, the early season planting was at the end of April. Weather assessments of the weeks leading up to, and the days following the planting revealed that the planting took place in a moderate dip in temperature at the time to around 4°C on average but rising to approximately 12°C over the next week. The rainfall was low beforehand, but moderate at the time of planting and over the next couple of days. During the mid-season planting in mid-May, average temperatures were higher at around 15°C and there was moderate rainfall before, during and after the planting. The late season planting in mid-June was planted during a dry spell, with no rain the week leading up to planting, or after planting for approximately 8 days. Temperatures were higher on average, with a spike of temperature reaching 24°C on average a few days later, before reducing again in later June. These variable weather conditions appear to have a significant effect on plant performance, especially in the later planting, which had lower survival than the earlier season, particularly in the smaller plug sizes. The warm and dry conditions of the later planting could potentially have had a negative effect on the plants under film, as temperatures can rise rapidly under direct sunshine under fresh film layers (Ashman *et al.*, 2018). This, in combination with dry conditions, and a shallower rooting depth, and thus reduced ability to search for deeper ground water, is likely the reason for the 25 – 30% establishment failure seen in the smallest plug volumes. In the mid-season planting, the survival rate was 100% over the first few months for all plug modules, suggesting the weather and environment conditions at the time

were optimal. Survival in the early planting was excellent overall but still reduced by 2 – 6% compared to the mid-season, although it should be noted that the number of plants per plot in this planting were double compared to the mid- and late-season planting, increasing the statistical likelihood of some mortality.

The length of time spent in plugs could potentially be a contributing factor to establishment differences seen, as well as environment. By June, all plants had been in root-restricted conditions for a considerable period, although results of analysis of all module types prior to planting suggested that the plants grown in larger modules continued gaining above- and below-ground biomass from May to June (Figure 5.8). The smaller plugs had increased aboveground biomass over time, but the belowground biomass accumulation appeared to halt between May and June. It is likely that small plugs would have been totally root bound by the final planting. Root binding could cause issues when planting out into free soil. In species of pine, tree survival and growth after planting is directly related to the ability of the root system to rapidly colonise and grow into the surrounding soil (Schultz and Thompson, 1997). It is possible some degree of free growing feathered roots for quick anchoring and establishment into the new medium would be optimal for rapid establishment in *Miscanthus* plugs. Schultz and Thompson (1997) trialled the innovation of planting pine seedlings into modules with open slots in the container walls for free root growth and 'air pruning'. This method appeared to have little positive effect however, with roots and growth medium becoming more susceptible to drying out, resulting in reduced overall biomass. With the results of the late planting here, it is probable that the lack of free growing roots reduced the root growth potential when planted, reducing the seedling's ability to deal with water stress after planting (McTague and Tinus, 1996). When comparing the success of smaller modules with the larger two modules under the late planting condition, an observation of great commercial importance is the successful establishment of the larger modules, even though they had been under glasshouse-restricted conditions for a longer period than is ideal. This result is of substantial importance because it allows for a greater potential planting window, without negatively affecting the plant vigour as a result. A number of things including machinery and labour availability, particularly where travel is necessary, can affect planting timing but it is principally weather dependent. Waiting for more ideal planting conditions with plants grown in small modules could negatively affect plant vigour as roots become

increasingly restricted, and nutrients within the plug more depleted. Under larger module sizes, glasshouse time could be extended if necessary, allowing a level of flexibility that could be of huge benefit to planters and growers. In a study by Dohleman and Long (2009) *Zea maize* and *Miscanthus* were grown side by side in the corn belt of the US. They found *Miscanthus* to be 59% more productive than *Zea maize.* They describe that productivity is a product of the total solar radiation per land unit area, and the efficiency of light interception and its resulting conversion into above ground biomass. The results show that *Miscanthus* produces more yield by intercepting more light with a larger leaf canopy. This suggests a strong potential to increase biomass accumulation by extending solar hours over the growth season. This is difficult to achieve in temperate regions due to temperature limitations, but the glasshouse allows an artificial extension of growth period, which is likely particularly impactful in seedlings. Growing from seed means the plants are small and will need to compete with weeds to intercept light with an initially poorly established leaf canopy. These considerations increase the requirement of a module size that will allow longer growth under restricted conditions where needed.

5.4c Growth and biomass yield under field conditions

The rate of growth of plants can vary widely, not just between species but also within. This is, in part, due to adaptation methods to match growth rate to the resources available to the plant at the time, with the goal of keeping the plant alive and/or producing the next generation. It is widely accepted that roots and shoots complement each other in plant physiology, with the roots relying on the aerial parts of a plant to photosynthesize and produce various hormones, and the aerial parts relying on the roots for stability, water, nutrient uptake, and hormonal control. This delicate balance between the sink and the source relationship of a plant is the driving force behind good, maximal growth and can be easily upset when root systems are restricted (NeSmith and Duval, 1998). This imbalance can have short- and long-term effects on a plant's growth, over the course of its life cycle. Growth is a hugely complex balance of physiology and feedback loops, depending mainly upon ecological adaptation and evolutionary history (White *et al.*, 2015). The majority of models for plant growth focus on growth as the outcome of the balance of carbon entering and exiting the plant. The mass balance is between the net photosynthetic gain of carbon, balanced by the allocation of this carbon to areas such as growth and storage, and that which is lost to

respiration and tissue turnover (Campany *et al.*, 2017). The theory behind a sink-source paradigm for growth is one method of explanation for growth rate in plant species. This theory describes that growth can be limited in one of two ways; by the source activity - the amount of carbon a plant can access via photosynthetic parts of the plant, and by sink strength - the amount of carbon a plant can utilize during its growth (Campany *et al.*, 2017).

When assessing the growth and vigour of the plants at phenotyping and harvest at the end of the first growing season, the effects of differing planting dates were the most obvious initial observation. Planting dates were all significantly different from each other for each growth parameter. The mid-season planting had higher stem counts, and taller plants consistently, and for each module type. The late season had by far the smallest plants regardless of module size. As such, it is difficult to pick out an overall front-runner for plug size; In early season the 126B and 104 perform best, in mid-season the 144 appears to produce average largest plants, and at the later planting the 104 was best overall.

Assessments of the rhizome harvest are surprising. The 126B tray produced the largest average biomass both above- and below-ground for the early-planted plots. The largest module produced largest averages for the mid-season, which had significantly larger biomass overall, although the 126B was still producing comparable results. The later planting produced significantly lower biomass over all plug sizes, with no significant differences between them (Figure 5.14). The success of the smaller 126B module here is interesting, and potentially a result of the 'escaping' roots seen in the glasshouse harvests for that module size seen in Figure 5.9, producing the reduced restriction and subsequently more rapid soil colonisation mentioned by Schultz and Thompson (1997).

Assessment of harvest biomass again revealed that planting time produced the greatest difference. Mid-season planting produced the highest biomass overall and late planting produced consistently lower biomass. This is not unsurprising on the basis that a later season planting allows fewer growing degree days, and thus less time for plants to grow and accumulate biomass before winter. This theory is conflicted when results indicate early season biomass was significantly less than mid- season however, suggesting that length of growing season not the only factor. The two more successful planting times revealed no significant difference between module size, but in the late planting, larger plugs performed significantly better, but still poorly in comparison to all other groups. This slight increase in

comparison to small plugs is likely due to starting out with larger photosynthetic biomass and larger below ground biomass, with less root binding, which allowed them to begin biomass accumulation at a more efficient rate and more rapidly post-planting.

Survival after winter was slightly reduced in the early-season planting across all module sizes. This is surprising as the hypothesis would be that plants within that field trial would have had the longest time to developmentally mature in field conditions and should ideally have senesced more sufficiently. Mid-season survival was still the highest, with a small percentage of losses in the 126A and 144 modules. Late season planting seemed to not have reduced further from the first season but remained lowest. The second year harvest followed similar trends to the first. Biomass increased across all groups; the largest yield coming from the mid-season planting 144 modules and the lowest in the smaller modules of the late-season planting, which was significantly lower overall than mid-season. There were no significant differences between plug sizes, however.

Moisture content assessments after the first and second winter provide crucial results relating to developmental maturity of the stand, senescence ability, and nutrient remobilization for the next growth season (Robson *et al.*, 2011). Results of moisture content at harvest one revealed significantly lower moisture content across the entire first planting biomass, by a margin of up to 10% reduction on average. Lower moisture content is a desirable trait by harvest time, for multiple reasons. Firstly, it improves the quality of the offtake for the desired end uses, reducing the costs involved in transportation by a reduction in weight and reducing spoiling, and secondly by improving the combustion quality of the biomass as high water levels lower the heating value of the fuel (Lewandowski and Kicherer, 1997). Reduced moisture contents also suggest that the crop had sufficient time to ripen and remobilize nutrients before winter dieback, allowing a greater chance of improved new growth for the next season (Robson *et al.*, 2012). This result suggests that plants that had been in the ground for longer during the planting year had been able to optimize their growth via increase of thermal time and degree days, and produce a developmentally advanced crop in comparison to the latter two plantings, between which there was no significant difference (Figure 5.17). During the second harvest this difference had altered, with the lowest moisture contents seen consistently in the mid-season planting stands. It is likely that the improved growth and

establishment seen during the first year in the mid-season stands produced competitive new season growth as a result of likely larger rhizome systems, and overall improved vigour.

5.4d Conclusions and further work

Overall, it is possible to draw multiple conclusions from the results gained over the trial period. A move away from the small module sizes is suggested for multiple reasons. The larger modules encourage a higher rate of glasshouse growth, with reduced root restriction allowing source-sink balances that encourage maximal above and below ground growth within the restricted time frame of glasshouse time. The larger depth of the modules also allows newly planted seedlings to access deeper soil moisture reserves, a vital requirement under dryer planting windows. Overall, the module size that provides the best balance of increased rooting depth and soil volume, with good survival and growth under most field conditions, and crucially, allowing more plugs to be grown per unit area in the glasshouse, is the 144 45cm^3 module. The 104 70cm^3 largest module provided comparable survival and growth rates in field conditions, despite a significantly larger above and below ground biomass, but with an approximately 28% reduction in the number of plugs produced per unit area.

The length of time spent growing in plugs should also be taken into consideration. Large plug volumes allow plants to mature more rapidly, reducing necessary glasshouse time, but also provide a longer window for planting them, as pot binding takes longer to achieve. The planting window needs to be considered carefully, but weather is unpredictable, and managing to plant under optimal conditions will not always be possible, particularly at longer distances as much preparation and planning is required. Here it is suggested that optimal planting conditions involve a period of rain beforehand, and warm temperatures after planting. Mulch film covering provides plugs with greatly improved chances of survival after planting, but under high temperatures and direct sunlight can increase the risk of weaker plug desiccation. Therefore, it is also important that the plants be able to establish under a variety of conditions. The results strongly suggest that late planting times should be avoided where possible, as the growth period in the field is not sufficient time enough to develop and mature to a high yielding and developmentally mature sward, regardless of initial biomass. This reduction in biomass continues into subsequent growth seasons. The May planting appeared, in this study, to be the optimal planting time, with the best environmental conditions.

In future work, more emphasis should be placed on assessing the morphology of plug rooting that could produce the fastest establishment when planted into free soil from a restricted module. Planting under more variable planting windows between April and May should also be trialled, with seedlings at varying maturities.

6 First year field establishment, growth and yield of ten morphologically differing, greenhouse propagated populations of the *Miscanthus* seeded hybrid 'GNT27'

6.1 Introduction

One of the main barriers to the successful implementation of seed propagation in *Miscanthus* is achieving good first year yield and a high rate of establishment. Gaps within the field trial can be costly to fill both monetarily and in labour, and can reduce the vigour of neighbouring plants (Zimmermann *et al.*, 2014). Also production per Ha will decrease if the crop contains gaps (Hastings *et al.*, 2017b). Much experimentation has been targeted toward perfecting methods of planting and plant aftercare to ensure the best chances of a full field (complete canopy). Additionally, a strong plant at the end of its first year is more likely to have a strong root and rhizome system and be able to survive the low winter temperatures in some temperate regions. Such a plant would therefore have sufficient stored resources in the rhizome to produce strong growth in the second year, potentially allowing for an economically harvestable yield after 2 years.

The first year is the most critical time for *Miscanthus* plants as they are at their most vulnerable to weeds, water availability and low temperatures. The objective therefore is to ensure adequate growth and development of plants when planted in their first year.

Previous experiments using *Miscanthus* plug plants showed low correlation between large plug plants at the planting stage, and large plants at the end of the growing season (chapter 3). These surprising and unexpected results prompted a deeper exploration into the morphology of plug plant seedlings. The aim was to create a large range of different seedlings morphologies and from the variation identify the optimal plug plant.

Different glasshouse treatments were used, with the primary aim of altering the physiology of the *Miscanthus* seedlings. The glasshouse conditions themselves were not the primary focus of this experiment, but were a means to induce different plant morphologies. The experiment was focussed on generating variation in stem height, aerial biomass, stem number, below-ground biomass, leaf number and leaf chlorophyll in the *Miscanthus* seedling prior to field planting. In addition, because the original studies suggested plant size *per se* did not correlate well with field performance. One aim of the experiment was to generate variation that may not necessarily be intuitively associated with yield or easily quantified in terms of gross morphology.

One individual plant genotype can produce a variety of different phenotypes in different environments, as a result of a fundamental property of plants known as phenotypic plasticity (Sultan, 2000). Environment plays a crucial role in plant development and morphology, particularly variation in irradiance, temperature, water and nutrients, although the influence of these variables differs between physiological development and phenotypic growth (Atkinson and Porter, 1996). For much of the past century, phenotypic changes in response to environment were regarded as 'environmental noise' that hid the actual characteristics of a particular organism, but more recently has been recognized as an important survival strategy in varying environments (Sultan, 2000). Plant growth and development are affected by several environmental factors. Light and temperature are particularly impactful and are amenable to manipulation in controlled environments (Chory *et al.*, 1996, Atkinson and Porter, 1996). Plant responses to light are complex and difficult to predict, as light quality and quantity initiates signalling cascades of specific photoreceptors, which can alter gene expression over a large number of genes, which can trigger a multitude of different responses (Olle and Viršile, 2013). This complexity may be exploited to generate large variations in phenotype from simple manipulations of light. Red light is the most common requirement for lighting plants, as it is the primary wavelength absorbed by chlorophyll and therefore has a large effect on biomass yield (Olle and Viršile, 2013). Blue light is also absorbed by chlorophyll at a lower level and is vital for other physiological characteristics such as leaf colouration and nutrient content (Lin *et al.*, 2013). Effects of rooting depth and growth medium also have significant effects on the growth and development of plants, and are easily manipulated as illustrated by studies of the impacts of pot size on plants (Poorter *et al.*, 2012). Using knowledge of how plant development could be altered by environmental factors, ten different glasshouse treatments were formulated as described in Materials and Methods (Section 6.2b) to generate a highly variable *Miscanthus* population at planting. We previously hypothesized that seedlings with larger biomass at planting will perform best under field conditions. However, this was not adequately proven and our previous observations suggested the anticipated correlation was low. Therefore, we hypothesised that generating high levels of variation from different environmental sources would maximise the likelihood of identifying a seedling phenotype with improved success in the field if such a phenotype existed. We define success in the field as a combination of high yield and high rates of establishment.

6.1a Aims of the experiment

- To identify a number of different growth conditions or treatments that could produce highly variable *Miscanthus* seedling phenotypes
- Produce multiple populations of variable traits within the same hybrid. Variation should be significantly different between treatments, but consistent within treatments.
- Assess and characterize the morphological differences in the resulting populations
- Produce two randomized field experiments to compare the first-year growth and survival of the individual populations in two contrasting field sites. The field sites chosen were, on average, a cold and wet site versus a warm and dry site to test if different morphologies are associated with superior performance at different sites.
- Test for associations of seedling traits and superior best first year field plant performance as measured by either biomass accumulation or percentage establishment.

6.2 Materials and Methods

6.2a Sowing and plant material

The hybrid used in this experiment had previously exhibited strong, homogenous growth and high rates of germination and sufficient seed was available to ensure the required quantities of plug plants could be produced. The genotype used was a seeded hybrid (GNT 27), a cross between a *Miscanthus sinensis* and *Miscanthus lutarioriparius*.

Tray and plug sizes were selected based on the findings of previous experimentation on plug plant growth and survival. Commercially grown *Miscanthus* plug plants have, since 2017 been grown in modular plant trays containing 144 wells, each one holding 45 cm^3 volume of soil. Two full commercial standard plug trays of seedlings were placed into each of 10 treatment conditions. 100 plants from each treatment were randomly selected to go into a completely randomized field nursery of 1000 plants, in Aberystwyth, and in Hackthorn, Lincolnshire.

Due to the unreliable germination rates of grass seed in general, and the requirement for this experiment to have almost full trays, seeds were sown and germinated on large trays of wet, course sand beneath blue roll, for one week in a greenhouse compartment. Trays were placed on capillary matting on the floor of the growth compartment, with Son T heat lamps approximately 1.5 metres above. The temperature was maintained between 18-26 °C on a 12 h night/day cycle. The majority of the seedlings were sown on the 6th of February 2018 and were left for a week with irrigation from capillary matting underneath, with supplementary top misting where necessary. Many seeds were lost to mice during the first few days, so more were sown to make up numbers within 7 days of the original sowing. These slightly younger seedlings made up the populations of treatments I and J, the two monochromatic light treatments, and were planted out at the same time as the other treatments.

Twenty plug trays filled with standard John Innes No. 2 compost. Once germinated with approximately 2-4mm of emerged green shoot, seedlings were gently removed from the blue roll with tweezers and placed in small ready prepared holes in each plug of each tray at a density of one seedling per plug. Two full trays of seedlings were then placed into each treatment where they remained until hardening off and planting in May.

6.2b Growth environments

In order to produce the highly variable plug plant morphology required, a range of environmental conditions or treatments were used within four separate environments. Plants remained in the treatments for 12 weeks and were watered daily with a manual sprinkler. Treatments were colour coded and labelled from A – J (see appendix 7).

Most treatments used the standard glasshouse environment commonly used to produce plugs for commercial planting. This was the same glasshouse compartment used to germinate the seed (18-26°C with a 12 h night/day cycle). Lighting was natural daylight supplemented by SonT lamps 1.5 metres above the trays, and moved up when seedlings grew to reduce leaf burning. Within this standard environment there were six treatments. Other environments used included a cooler glasshouse and two growth cabinets. Specific treatments are detailed below.

A - The standard treatment – colour coded white

Standard or 'normal' treatment plants were sown at the usual time (6th February), kept in a high temperature environment (18-26°C with a 12-hour night/day cycle) with standard lighting SonT provided at 250 µmol m^{-2} s^{-1}, standard soil compaction of approximately 18-20g of soil per plug and standard nutrient additions of one dose of Miracle-Gro (All purpose). Most other treatments were compared to this control or 'standard' treatment.

B - Reduced photosynthetically active radiation (PAR) – colour coded orange

Plants for this treatment were grown with everything standard, as in treatment A except PAR available to the plants was reduced to 100 µmol m^{-2} s^{-1} by raising the lighting.

C – No added fertilization – colour coded black

Plants were grown in the standard manner as in treatment A. The treatment received no supplemental nutrient additions.

D – Higher soil compaction – colour coded brown

This treatment differed by having 0.5x extra soil within the tray cells filling them with approximately 23-25g of compost per plug. Trays were filled to the standard level with compost, compressed and half again more compost added and pressed down until

compacted. Seedlings received the same temperatures, PAR and nutrient additions as treatment A.

E – Cutting treatment – colour coded green

Plants were grown under standard treatment conditions A, but the main stem was cut to below the lowest ligule two weeks before planting.

F – Later sowing – colour coded purple

Seed were sown two weeks later than previous treatments; all other growth conditions remained the same as treatment A.

Two treatments used cooler temperatures in a different glasshouse compartment. This glasshouse was 12-20 °C, with a 12 hour night/day cycle. Treatments G & H were in the cooler environment.

G – Cooler temperature – colour coded yellow

Everything standard as in treatment A, however the growth temperature was cooler (12 – 20 °C, with a 12 hour night/day cycle).

H – Cooler temperature and lower PAR – colour coded pink

Everything standard as in treatment A except growth temperature was lower (12 – 20 °C, with a 12 hour night/day cycle), and available PAR was reduced to 100 µmol m^{-2} s^{-1}.

The final two treatments used growth cabinets with coloured LED lighting.

I – Red light alone – colour coded red

Plants were grown in a controlled Fitotron growth cabinet, Weiss Technik UK, Loughbourough UK, and the temperature set to 19-25°C 12 h night/day cycle. Growth conditions included standard compost and compaction. Light was provided by red LEDs at approximately 650 nm wavelength (Figure 2A); PAR was approximately 100 µmol m^{-2} s^{-1}. Seedlings *in situ* are shown in Figure 6.1.

J – Blue light only – colour coded blue

Plants were therefore placed in a Conviron Europe Ltd Iselham UK controlled environment cabinet. Temperature was set to 19 – 25°C. Growth conditions included standard compost and compaction and the only light available was from blue LEDs, at a wavelength of approximately 415 nm (Figure 2); PAR was approximately 100 µmol m^{-2} s^{-1}. Seedlings *in situ* are shown in Figure 6.1

Table 6.1 Matrix summary for all 10 treatments explaining the presence or not of each factor with a Y – yes or N – no. Treatments were designed to encourage variable growth characteristics in populations of the same *Miscanthus* hybrid and are labelled A – J.

Treatment:	Low lighting	Low temperature	Normal nutrients	Normal lighting	Normal soil compaction	Normal temperature	Seedlings cut back	Red light grown	Blue light grown	Late sowing
A	N	N	Y	Y	Y	Y	N	N	N	N
B	Y	N	Y	N	Y	Y	N	N	N	N
C	N	N	N	Y	Y	Y	N	N	N	N
D	N	N	Y	Y	N	Y	N	N	N	N
E	N	N	Y	Y	Y	Y	Y	N	N	N
F	N	N	Y	Y	Y	Y	N	N	N	Y
G	N	Y	Y	Y	Y	N	N	N	N	N
H	Y	Y	Y	N	Y	N	N	N	N	N
I	N	N	Y	N	Y	Y	N	Y	N	N
J	N	N	Y	N	Y	Y	N	N	Y	N

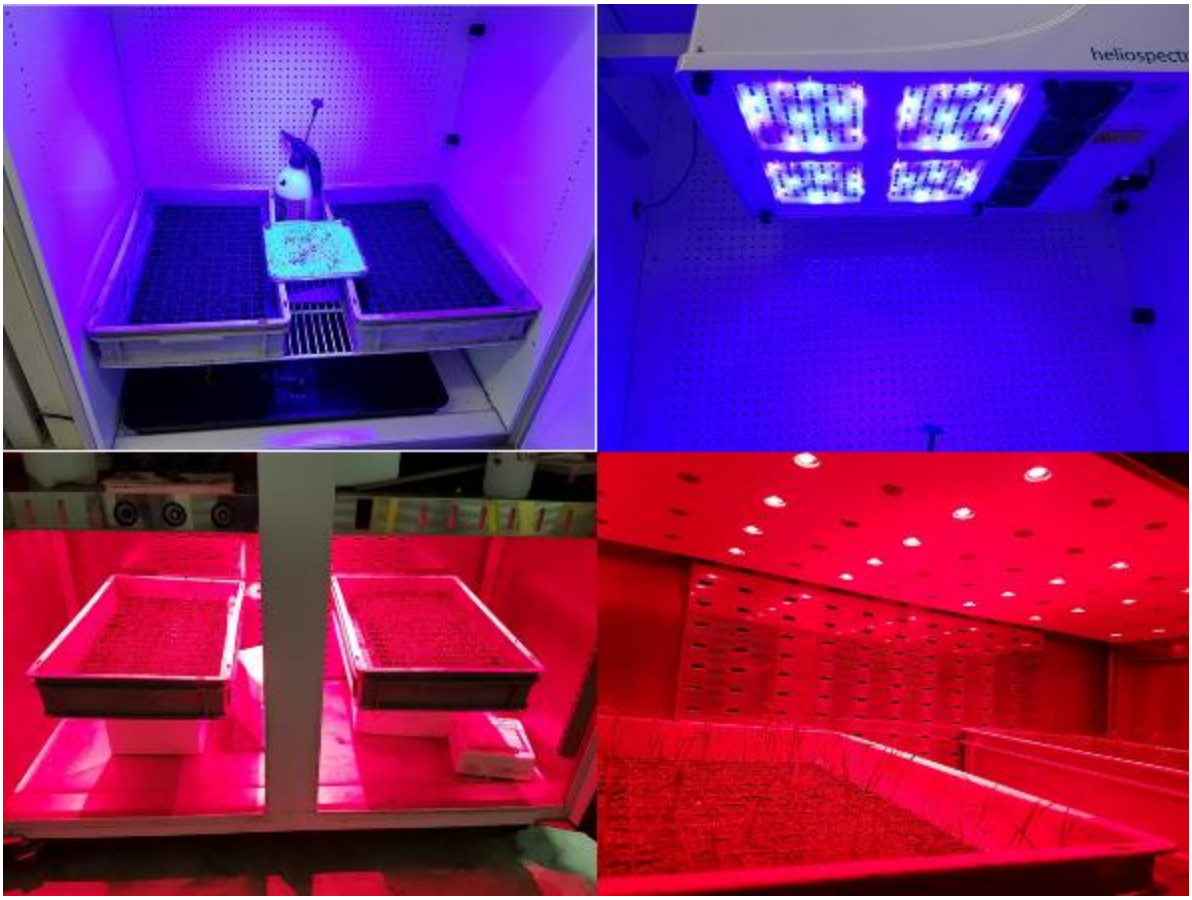


Figure 6.1 Plants in trays in blue light Conviron cabinet, and red light Fitotron cabinet. Plants received no other light source except when cabinet was opened for watering or measuring.

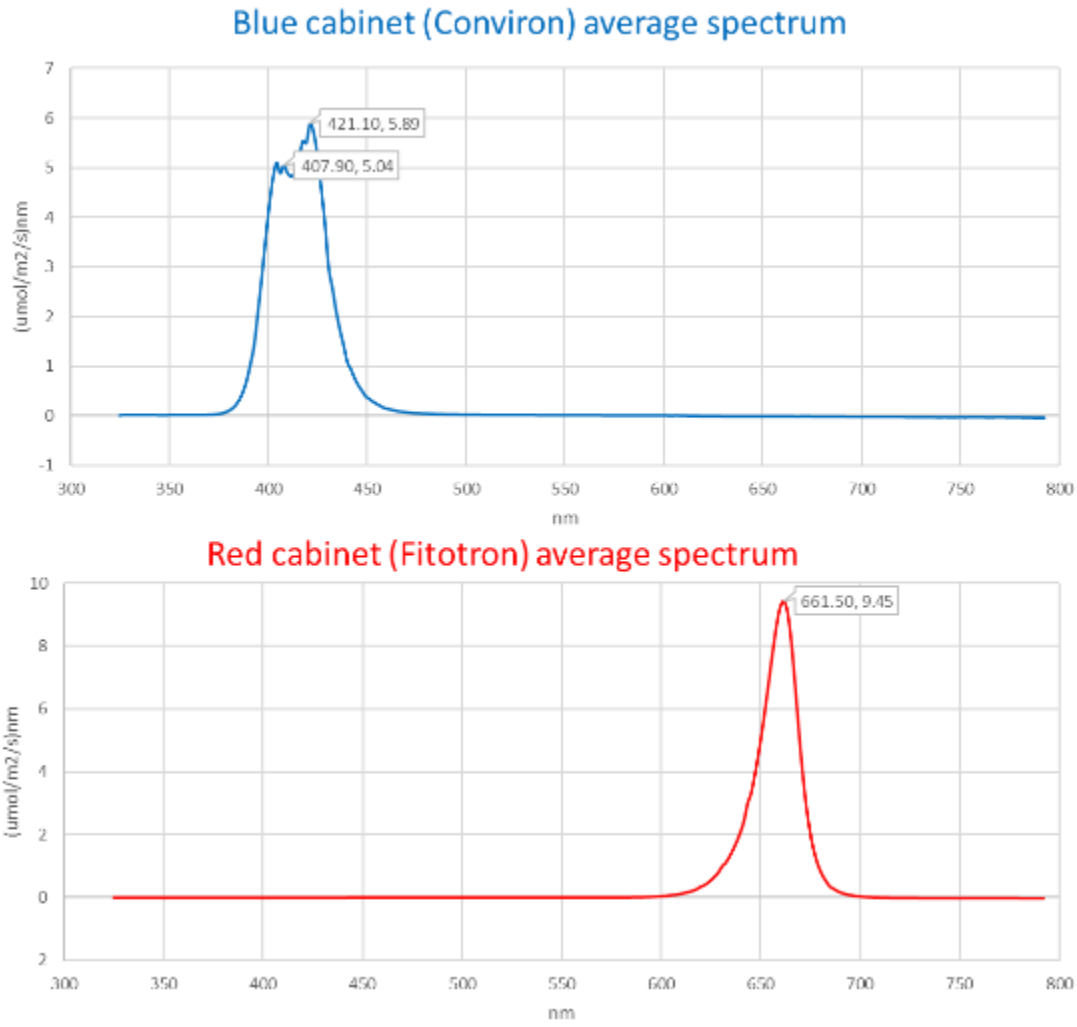


Figure 6.2 Average spectra of red and blue LEDs used to treat *Miscanthus* seedlings before transfer to field trials. Red light was provided by inbuilt lighting in Fitotron growth cabinet by Weiss Technik UK. Blue light was provided by heliospectra lamps built into Conviron growth cabinet (Conviron Europe Ltd).

In addition to the trays grown with individual treatments, four additional trays of 144 plugs, containing the same genotype, were planted and grown as standard in the warm compartment, to fill a double row of plants as an edge barrier around the experimental plots.

6.2c Field trial design and preparation

Two 1000 plant field trial observation nurseries were created comprising 25 columns by 40 rows and 4 edge rows each (21.75m x 29.92m). The trials comprised ten treatments of 100 plants each, in a completely randomized design. The distance between columns (0.75m) was to allow tractor access for film laying. The width between rows was 0.68m. Mulch film could then be placed over two columns of plants at a time, with one empty column at the end. The design was identical at both sites.

Two sites were chosen based on differences in climate and soil type. It was important that the trial sites be accessible by car for planting, phenotyping and harvesting so sites within the UK were chosen. The Aberystwyth site (Latitude52°26'0.40"N Longitude 4°1'25.72"W) was stony soil, classed as sandy loam, with large clumps and agricultural grassland with little previous use. The site was flat but at the base of a small hill, with a large copse of Willow trees approximately 10m away from the end column of the trial (Figure 6.4A).

The Hackthorn site in Lincolnshire UK (Latitude 53°19'50.82"N Longitude 0°28'13.46"W) (Figure 6.4B) was flat agricultural land; soil was clay loam with a fine tilth that was regularly rotated and treated. Day length did not vary between sites due to being on a similar latitude (Figure 6.3) but meteorological conditions were different due to a West-to-East gradient of rainfall and temperature as described in Ashman *et al.* (2018).

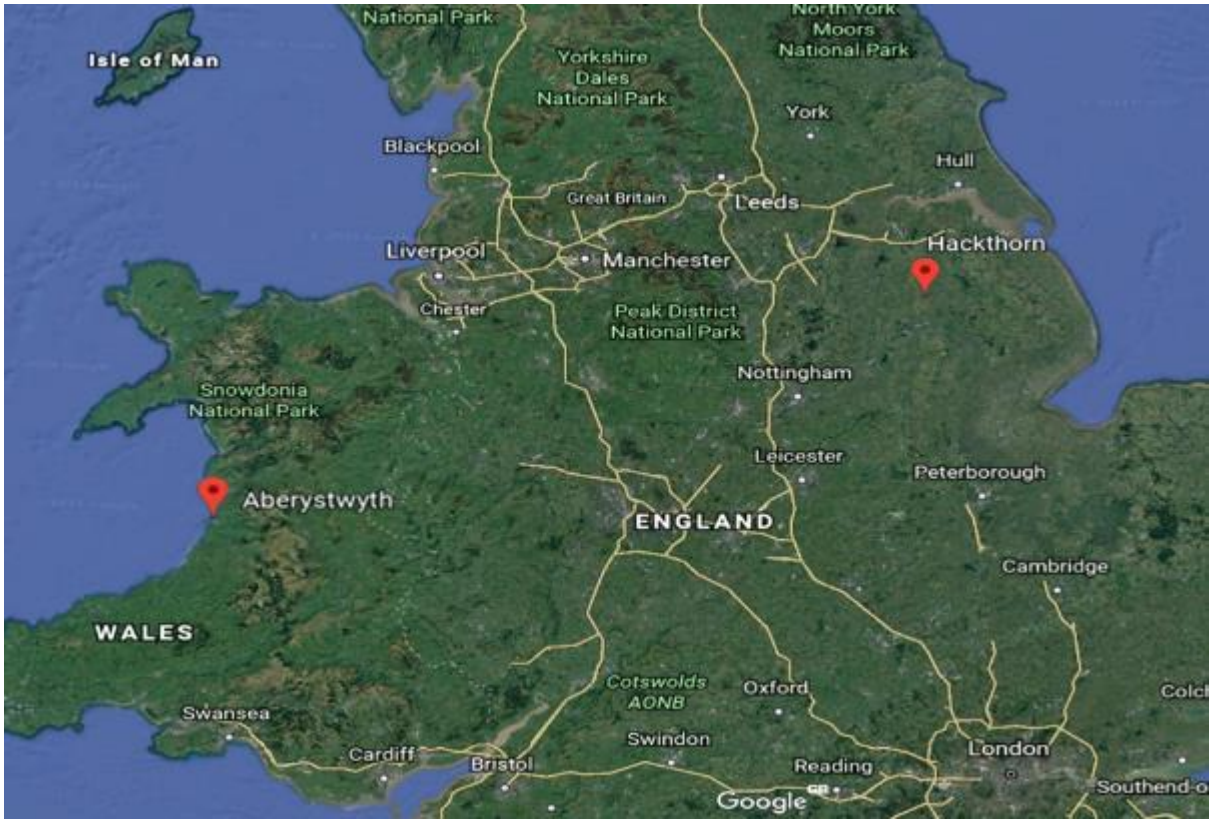


Figure 6.3 Position of field trials within the UK. Aberystwyth site contains a 1000 plant nursery of 100 plants grown under 10 glasshouse treatments designed to encourage morphological differences in seedlings (trial name - ABR 71). Hackthorn site contains identical nursery at a different location (trial name - HCK 35) to test plant vigour under different temperature and rainfall conditions. Image taken from Google Earth

Figure 6.4. Aerial view of exact locations used to test two identical 1000 plant trials, comprised of 100 plants grown in 10 different glasshouse treatments designed to encourage morphological variation within the same genotype, at two sites in the UK for additional assessment of plant performance under variable climatic conditions. White squares show the location and approximate size of the plots in relation to other trials A – Aberystwyth IBERS field site. B – Hackthorn Terravesta trial fields in Lincoln

Randomization plans were completed and coloured using the randomisation and conditional formatting functions in Excel (Figure 6.5). Each of the two sites had a different randomisation design (see appendix 8 & 9). .

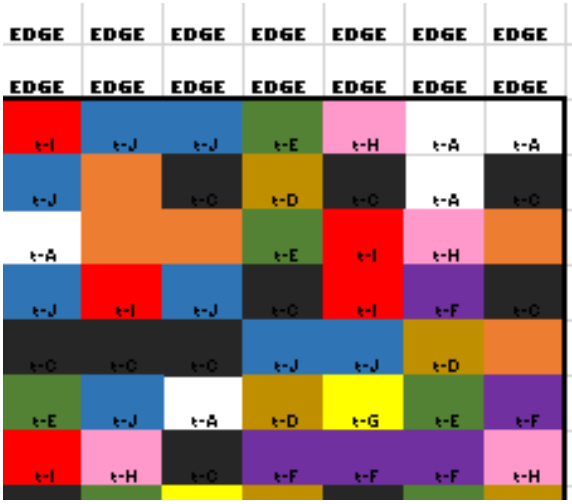


Figure 6.5 A section of the completely randomised design showing the corner of one of the field plans, surrounded by a double row of edge (barrier) plants. Firstly, plans are randomized into treatments (A-J) and each treatment colour co-ordinated to aid in differentiating seedlings for measurements and planting. Complete field plans are in the appendix.

Each individual plant was given a Unique Identifier (UID) number which would identify it from tray simulation to the field design, so each plant could always be identified temporally and spatially. UID order began in the top right corner of the field, spanning across the top row in sequence, and following a 'zig-zag' pattern through all rows of the trial.

Once plug plants had been grown in their respective environments for approximately 10-12 weeks, a randomly selected aliquot of 200 per treatment were selected to be placed in the trays according to the field plan. The selected plants were carefully removed from their nursery trays, root ball intact and placed in the same size plugs in the field plan trays. The complex design of the field plan meant that planting could not be done in the typical way using a mechanised planter. The randomization was completed in the glasshouse and plantlets hand planted within the trays to match the desired placement on the field. In order to accurately replicate the colour coded field plans in the trays, trays were marked with coloured stickers in each well, for ease of quick assessment of which plug from which treatment was assigned which location (Figure 6.6). This was done for each usable plug well, for each of the 9 trays to be used in each trial.

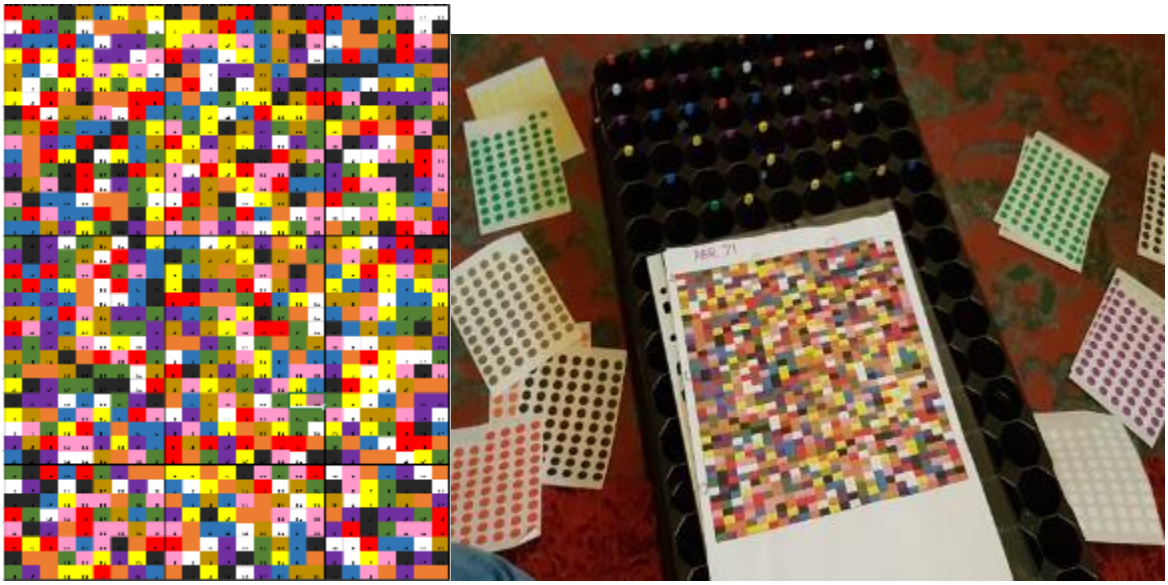


Figure 6.6. Colour coded field plan for each trial randomisation divided into trays to simulate the design in the field. Left figure shows the Aberystwyth field plan with each treatment colour coded, with an outline of trays. Right picture is the plug trays being given a sticker per well to match the plan, to make placing the plugs into the randomisation easier

6.2d Pre-field phenotyping

Once plants were in the correct field plan orientation for each site and had UIDs assigned, baseline data for each plant was collected. Photographs were taken using a Canon DSLR camera with two hooded anti–glare photography lights (Figure 6.7 A). The base was a non-shine white background with measurement ruler drawn on. The camera was attached to a remote shutter release in order to reduce the risk of affecting the focus while taking the large numbers of standardized photographs. Each photo contained a QR code label (Figure 6.7 B) created using R studio version 1.1.383 – 2009 – 2017 containing the plant UID, treatment ID and column and row number. The QR code was included to be read using an R studio extension and code reader to sort photographs into groups. Plants were measured before photographing for longest stem length and stem number.

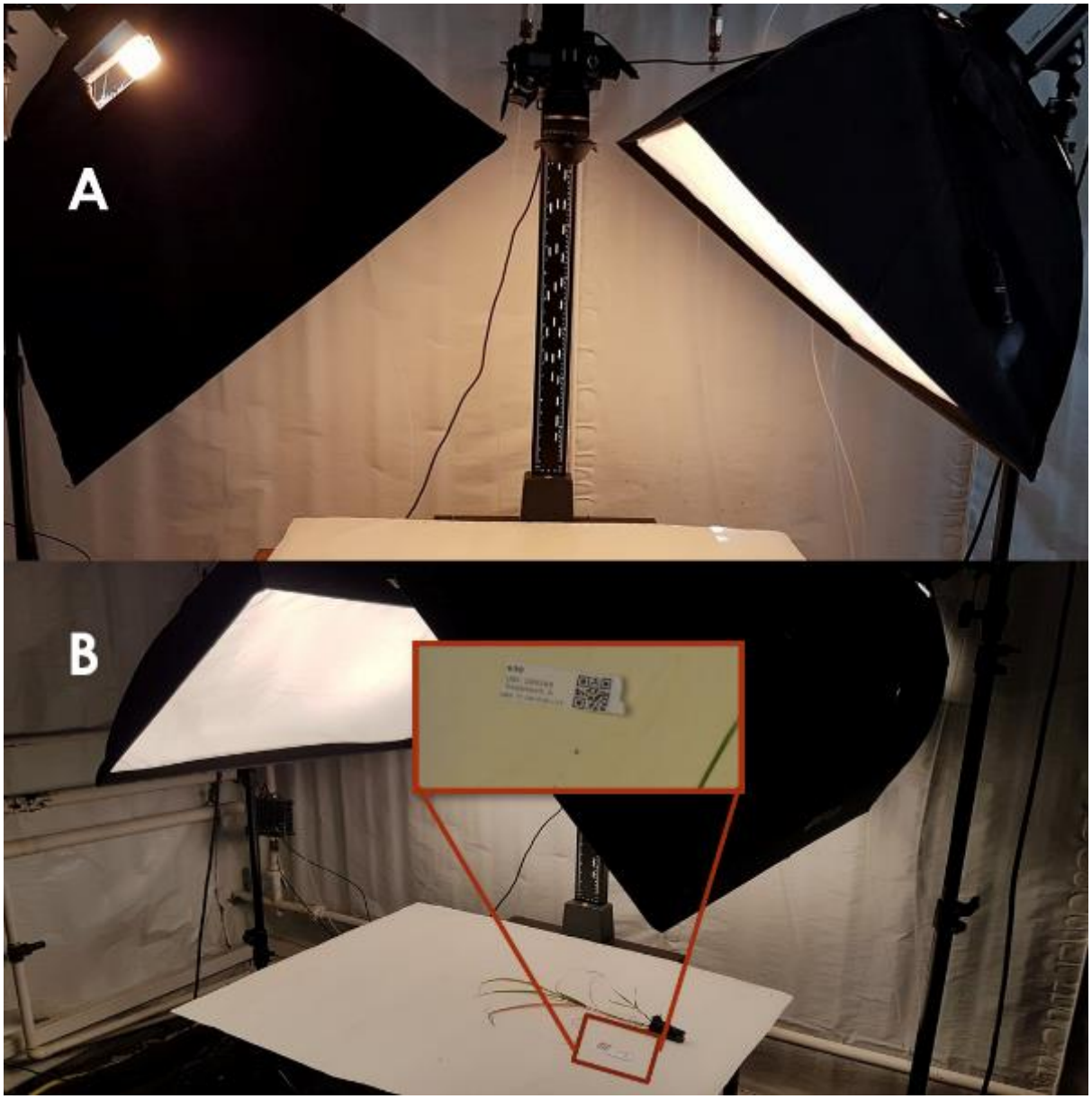


Figure 6.7 Lighting and photography set up within a light restricted growth chamber. Image A – camera mounted on stand with two hooded lights to reduce shadowing. B – Plant with QR code label in the set up.

6.2e Sacrificial pre – field assessments

After the 200 plants per treatment has been randomly selected for field planting, a further representative sample of remaining plants from each of the trays were taken for detailed analysis. Due to larger than anticipated rates of plant death in some of the treatments the number of replicates was lower for some treatments. Each of the representative plants was photographed in the same way as the field populations plus the longest stem measured and the number of stems counted which were measurements in common with the field

population. Relative leaf chlorophyll was measured using a SPAD 502 meter (Konica Minolta, Osaka Japan) at three places along the youngest leaf with a ligule, and the results averaged, and the number of leaves counted. Roots were carefully washed to retain as much fine root matter as possible, while removing the soil. Roots, leaves and stems were weighed separately before being dried at 80°C until constant dry weight, to assess total dry biomass and moisture content.

6.2f Regression analysis

Using stem data (stem length and stem number) from seedlings destructively harvested in May a regression model was estimated to predict above ground total biomass using R Studio. The stem data was in common with the field population, so the model was used to estimate biomass of these plants.

6.2g Field planting and maintenance

The two trials were planted over the course of one day each. The Aberystwyth trial was planted on 18th May 2018, and Hackthorn on the 30th May 2018. The trial area was measured out using posts and string, with cable ties at each 0.68 cm section down the columns to standardize plant spacing. Holes were pre-made for each plug which was hand planted from the tray in order of the field plan, and the soil tamped to ensure good soil-root contact. Guard plants were added in double rows around the perimeter of the experimental plants. All plants were well watered before being covered in a layer of biodegradable mulch film by tractor, which was dug into the soil at the edges to ensure it stayed in position. Once film had begun to degrade hand-weeding between plants as often as was possible was used to control weeds. The Aberystwyth site had especially high weed levels. Some supplemental watering was required at both sites due to the 2018 summer being unusually dry.

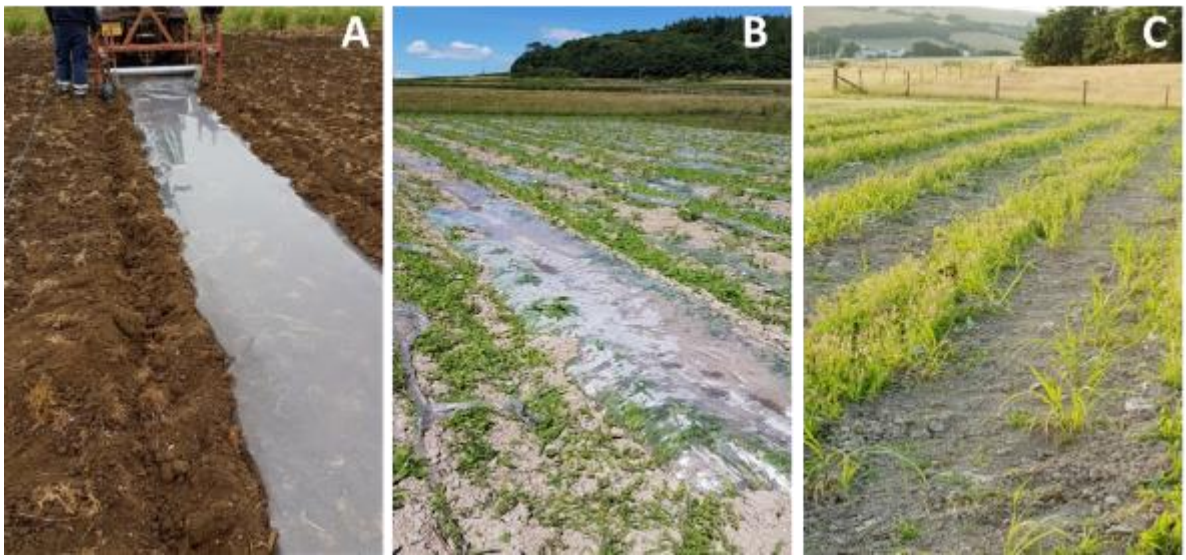


Figure 6.8. Progression of field plan over first 2 months. A - Film layer being applied on day of planting, B - Film layer beginning to degrade and weeds becoming prevalent, C - Film layer mainly gone, and weeding being done between rows

6.2h Autumn phenotyping

Field plans were downloaded onto a Samsung tablet using the *Miscanthus* database app. which was used to record stem counts and shoot height on each plant in both trial plots in October 2018. Dead or missing plants were marked on the plan so that establishment per population could be assessed.

6.2i Spring harvesting

Both field trials were harvested for aboveground biomass in March 2019 using the same method. 15 large builders' buckets were weighed individually and given a number. Following the zig zag field plan, rolls of plant labels were printed out containing plant UID and location, and a QR code of the information. Each plant was cut approximately 5cm from the soil surface and placed into one of the numbered buckets with the corresponding UID label. When the buckets were filled, they were brought to a weighing station in the field (Figure 6.9). Each plant label was scanned into an excel document, the whole plant weight recorded and a subsample taken and placed in a paper bag with the label. The plant weight minus subsample was recorded and the bulk plant material discarded. The bucket number was also recorded, to be taken off the total weight later. This process was repeated for all experimental plants in both trials. Subsamples were placed in drying ovens at 105°C until a constant weight was

reached. Subsample dry weight was then recorded to estimate whole plant dry biomass and moisture content.

6.2j Data analysis

Statistical analysis used IBM SPSS statistics Version 21. Data was analysed for normality (Shapiro Wilks test), and means compared by One Way ANOVA, with Tukeys HSD post hoc tests to identify significantly different means. Seedling charts were created using SPSS chart building GGplot software. Other charts were made using Microsoft Excel. Regression analyses used R (R Core Team, 2015)

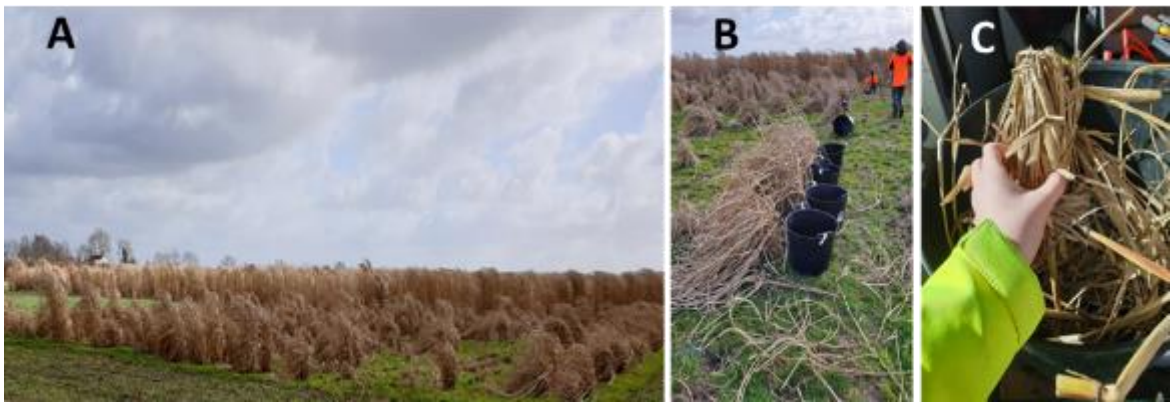


Figure 6.9 Harvesting of the Hackthorn field trial October 2018. A - Senesced entire trial prior to harvest. B - Buckets being used for the whole plant weight. C - Representative subsample taken from each plant

6.3 Results

6.3a Survival of all populations following the glasshouse phase, and growth characteristics of destructively harvested seedling subsamples from each population

Plant survival was assessed at three time points, survival rates at the end of the treatment period, survival after field planting to Autumn and survival through winter. Treatment resulted in large differences in plant survival. All populations grown in the warm glasshouse-controlled environment (treatments A-F), and the red light cabinet (treatment I) had high survival rates (95-100%). Populations grown under cooler conditions survived treatments less well (percentage survival from treatments G+H were 79% & 75% respectively) and the lowest survival rate was under blue light (J) with 61% survival.

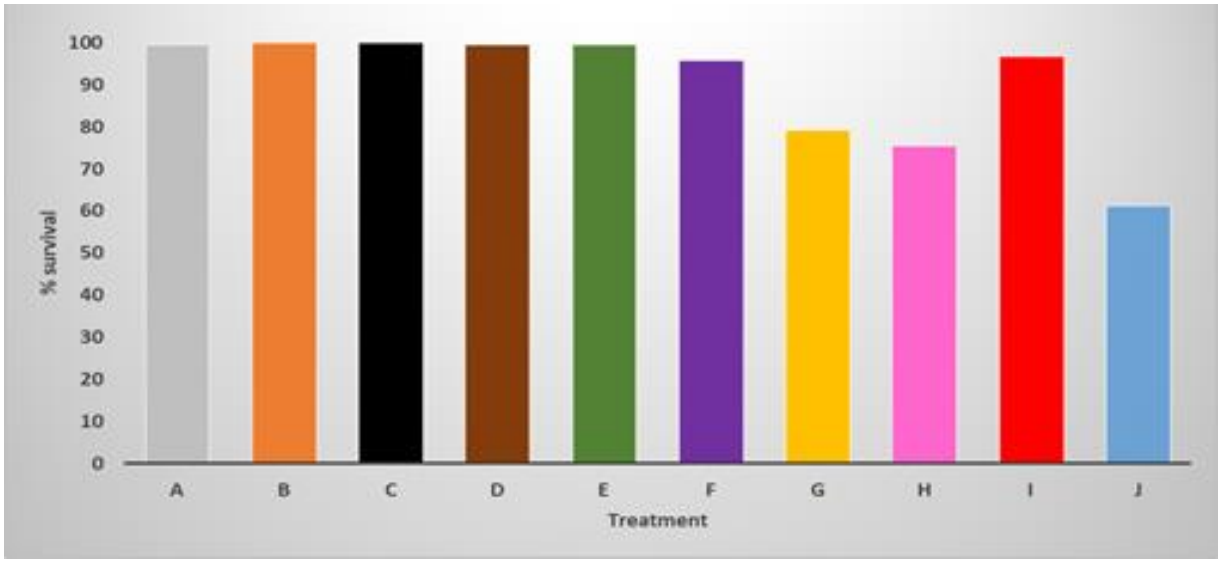


Figure 6.10 Survival of each population as a percentage of the plant number for each population required to fill two 100 plant field trials, and have 16 plants left over for representative harvesting and biomass analysis. Colours are used to represent each treatment in the field plans so have been added here.

A typical representative plant of each treatment environment is shown in Figure 6.11. Little difference can be seen visually between treatments A – D. Treatment E was cut back a week prior to assessment so, along with later grown treatment F, appears smaller. Colder treatments (G & H) are much smaller than seedlings from other treatments, as are the blue

light grown population (J). Red light treated plants had much longer single stems than other treatments. Detailed morphological assessments were completed as detailed below.

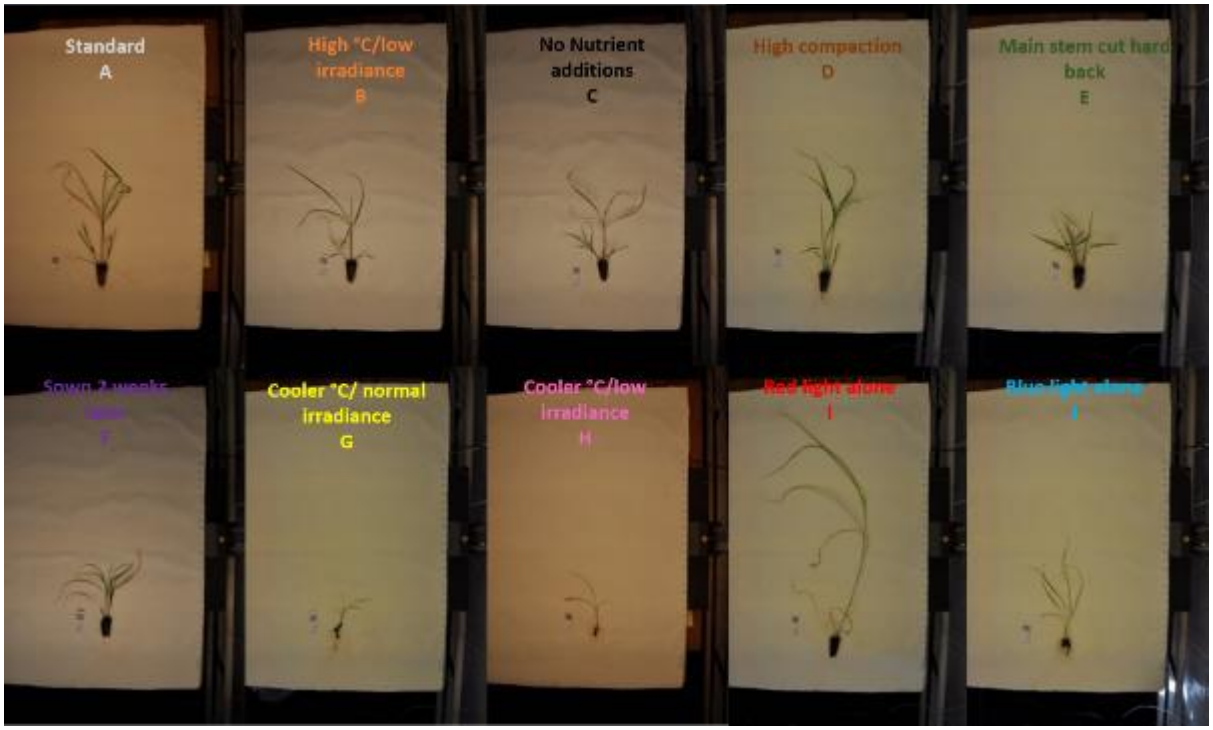


Figure 6.11 Representative plant morphology produced from each of the ten environmental variations, prior to field planting. Images are annotated with the treatment and the corresponding field plan colour

Results of the representative sacrificial plant growth parameters revealed 5 homogenous subsets for overall total biomass, and within this, 4 subsets for above and below ground biomass separately. There was a significant effect of treatment on whole plant biomass ($p < 0.01$). A Tukeys *post hoc* test identified several subsets (Figure 6.12). The range of values for total biomass per treatment was from the lowest of between 0.02g – 0.12g (a) to the highest of between 0.5-0.7g (e) and many populations were assigned to more than one subset. Seedlings grown under normal light and temperature conditions, but in compacted soil plugs (treatment D), produced significantly higher total biomass ($p < 0.05$). This treatment group had significantly higher biomass than most other treatments with the exception of the population grown under lower PAR in the high temperature cabinet (treatment B), and the population that were not given extra nutrients (treatment C), which were also classed as homogenous group 'e'. Lowest overall biomass (homogenous subset 'a') contained both treatments grown under cooler conditions (treatment G & H). Those grown two weeks later

(treatment F), and those grown under the blue light conditions (treatment J) were also placed in subset ‘a’, although were additionally part of the slightly higher lower biomass subset ‘b’ which also contained populations grown under red light.

When above and below ground biomass were assessed separately, a similar result was seen for both (Figure 6.13 A and B). There was a significant effect of treatment on below ground biomass ($p < 0.01$). Below ground biomass had a significant difference of $p < 0.01$ between groups. Tukeys post hoc test divided the treatments into four homogenous subsets from ‘a’ (lowest root biomass) to ‘d’ (highest root biomass) which ranged from 0.023g (seen in treatment G) to 0.5g (treatment D). Highest root biomass was again observed in the plants grown under compacted soil (D) which had significantly higher root mass than all other subsets ($p<0.05$), with the exception of treatment C. Lowest below ground biomass subset ‘a’ contained treatments F, G, H & J, all of which had dry root mass averages of consistently less than 0.05 grams. There was a significant effect of treatment on above ground biomass ($p < 0.01$) (Figure 6.13 A). The high compacted treatment (D) was the highest biomass subset, with a significantly higher ($p < 0.05$) above ground weight than all other treatments with the exception of B & C. Lowest above ground biomass (subset ‘a’) comprised of the cool cabinet populations (G & H), and the later sown population (F).

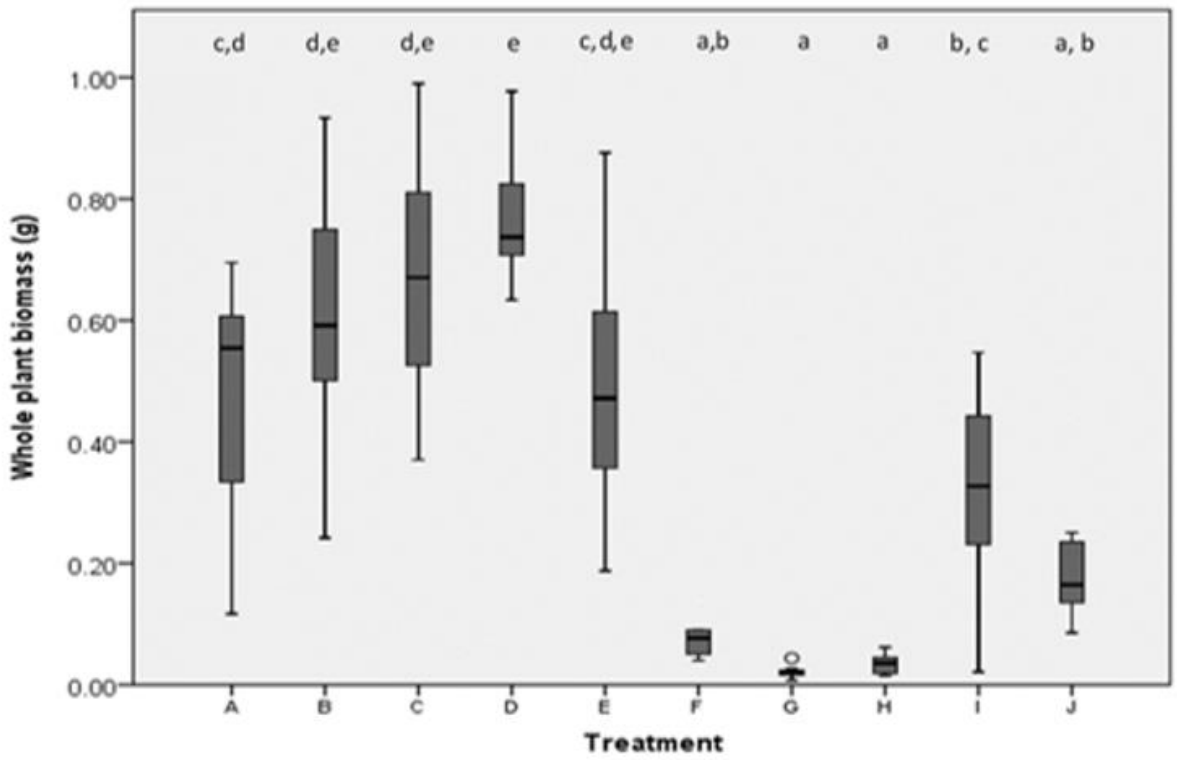


Figure 6.12 Total biomass from destructively harvested *Miscanthus* plantlets sampled from 10 treatments that were used to create a highly variable population for field planting. N = 5 – 16 plants depending on survival rate. Letter groupings denote the homogenous subsets for each growth parameter. Data was analysed using ANOVA in SPSS, and homogenous groupings made using Tukey - B post hoc testing.

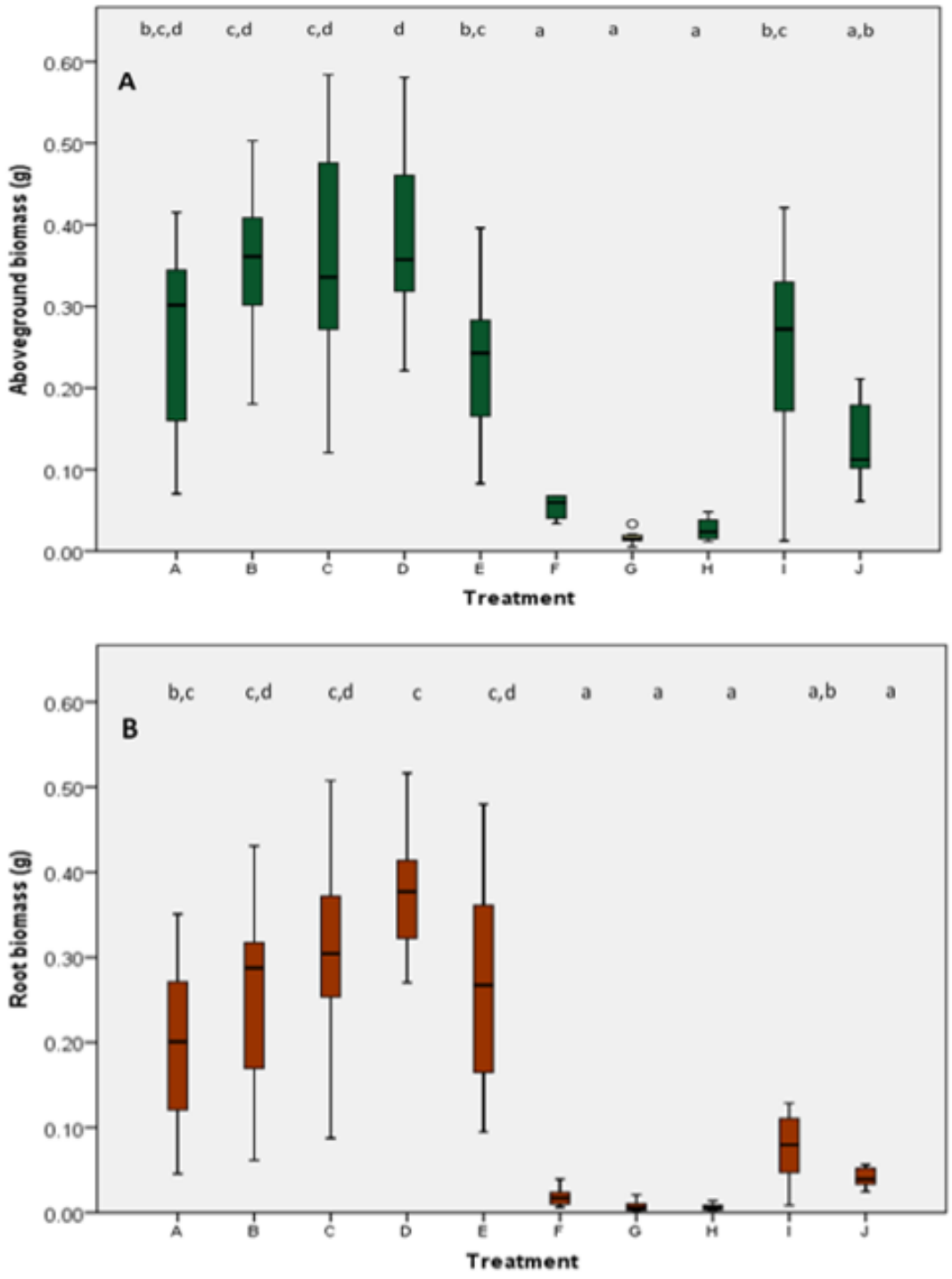


Figure 6.13 Total above (A) and below (B)ground biomass from destructively harvested *Miscanthus* plantlets sampled from 10 treatments that were used to create a highly variable population for field planting. N = 5 – 16 plants depending on survival rate. Letter groupings denote the homogenous subsets for each growth parameter. Data was analysed using ANOVA in SPSS, and homogenous groupings made using Tukey - B post hoc testing.

Number of leaves was significantly affected by treatment (ANOVA $p < 0.01$) between groups (Figure 6.14A). Treatments were in four significant groups by Tukeys HSD. As with previous measurement parameters the lowest leaf numbers (subsets 'a' and 'b' with between 4-7 leaves per seedling on average) were seen in the colder temperature and the red and blue light environments (treatments G,H,I,J) but also in the later sown plants (F). Higher leaved subsets ('c' and 'd') consisted of all those grown under warmer temperatures. Highest average leaf number overall was observed in the warm but lower light PAR treatment (B), which had significantly more leaves ($p=0.05$) at an average of 16 leaves per seedling, than all other treatments, with the exception of the compaction treatment (D).

There was a significant difference of relative leaf chlorophyll content between treatments ($p = 0.036$); however, Tukeys HSD test did not reveal any individual differences, classing all treatments as the same homogenous subset 'a' (Figure 6.14B).

There was a significant effect of treatment on stem height ($p < 0.01$), (Figure 6.15A). Tukeys HSD post hoc tests revealed five subsets. Tallest stems (subset 'e') were observed in plants grown under red light alone with an average height of 16cm. This was significantly higher ($P<0.01$) than all other treatments with the exception of B,C & D, all of which fell into subsets 'e' or 'd' or both. The smallest plants in terms of height (subset 'a' alone) were seen in treatment G, the cooler temperature with normal lighting conditions, with an average of 4cm high main stem, significantly lower ($P<0.01$) than B, C, D & I.

There was also a significant difference in the number of stems between groups ($p < 0.01$) (Figure 6.15B). Tukeys HSD produced four subsets, ranging from the lowest average of one stem (subset 'a'), to the highest average of 3 stems with some variation (subset 'd'). Lowest stem numbers (subset 'a' only) were seen in the same plants that had significantly lower biomass (treatments G & H). Treatments I, J and F also produced lower averages (group 'ab'). Consistent higher stem number seen in treatments A (commercial standard) & B (warm temperature and low irradiance), and the highest overall in treatments D (higher compaction) and E (main stem cut back 1 week previously).

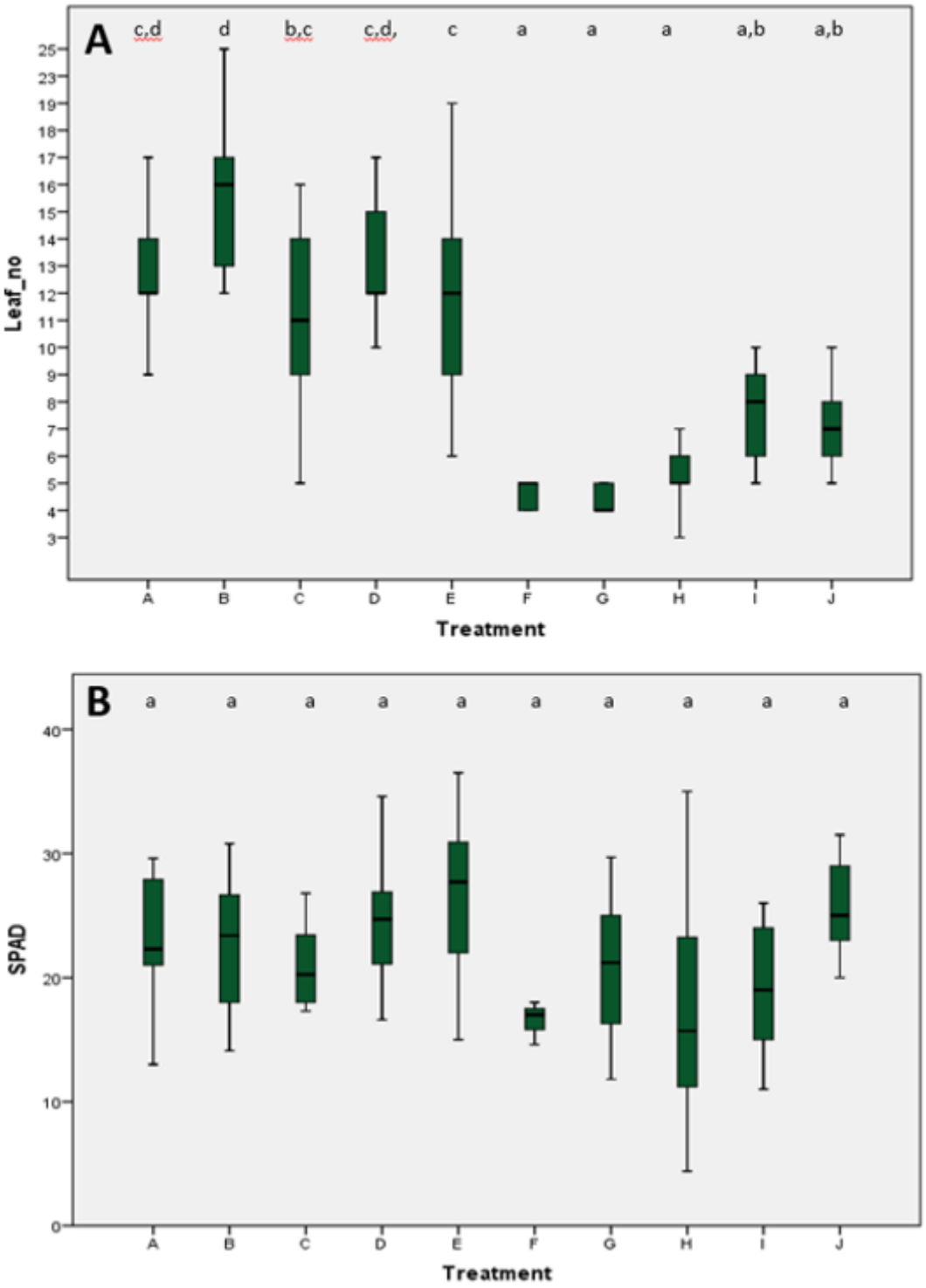


Figure 6.14 Leaf number (A), and greenness (SPAD) (B) of the destructively harvested representative sample of each population. N = 5 – 16 plants depending on survival rate. Letter groupings denote the homogenous subsets for each growth parameter. Box plots were made using SPSS GGplot. Data was analysed using ANOVA in SPSS, and homogenous groupings made using Tukey –B post hoc testing.

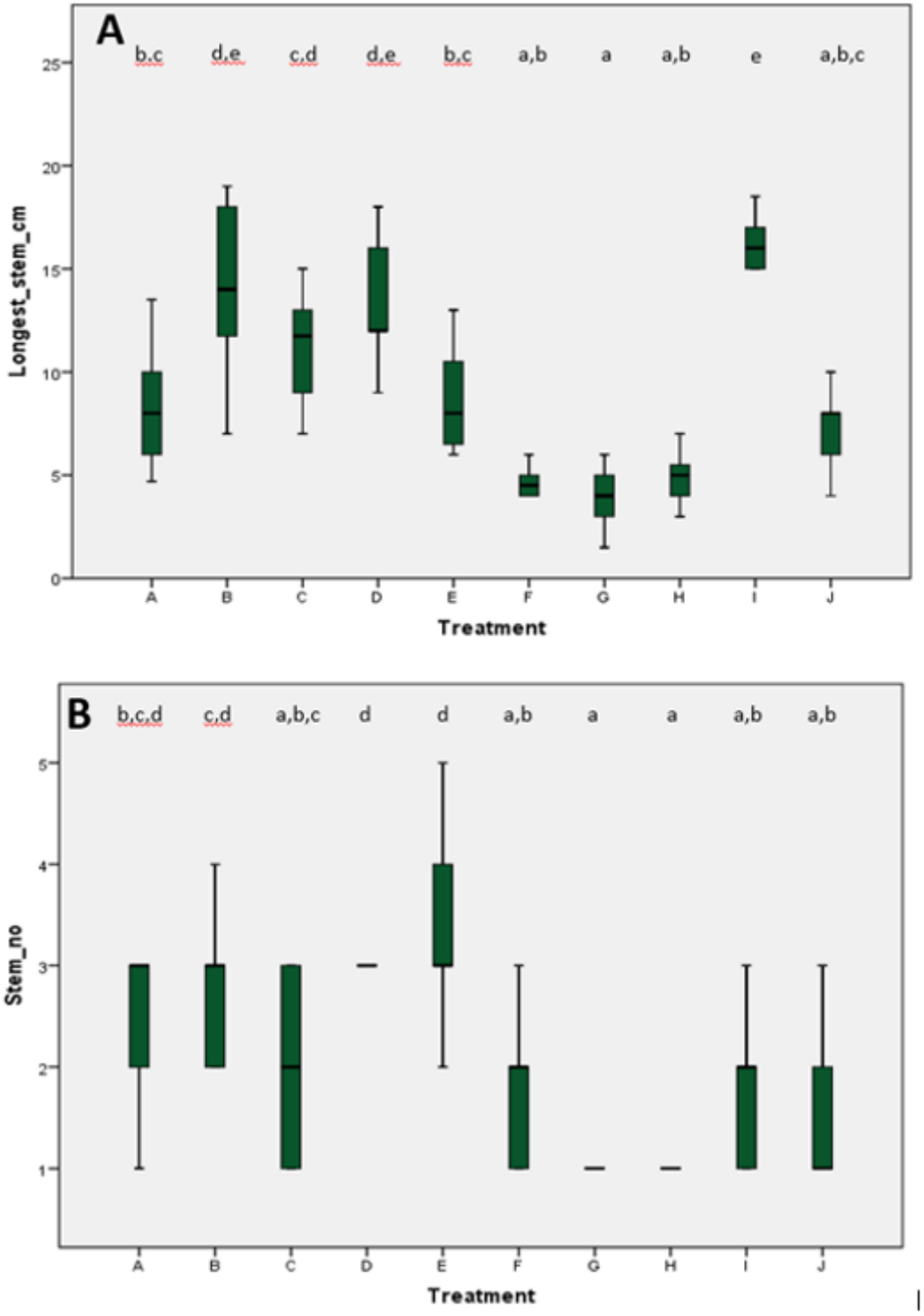


Figure 6.15 Length of longest stem (A), and stem number (B) of the destructively harvested representative sample of each population. N = 5 – 16 plants depending on survival rate. Letter groupings denote the homogenous subsets for each growth parameter. Box plots were made using SPSS GGplot. Circles identify outliers. Asterisks identify extreme outliers. Data was analysed using ANOVA in SPSS, and homogenous groupings made using Tukey –B post hoc testing.

Shoot: root ratio was significantly different between treatments ($p < 0.01$) (Figure 6.16). Plants grown under warm conditions produced root: shoot ratio's closest to 1; in particular treatment D (high compaction), produced the most biomass overall and significantly ($p < 0.01$) higher root: shoot ratio than treatments F, G, H, I & J. Plants grown under cooler conditions were significantly smaller and had a much lower root: shoot ratio. Plants grown under red or blue light also had a low root: shoot ratio and lower overall biomass than warmer grown plants, but higher than those cold treated.

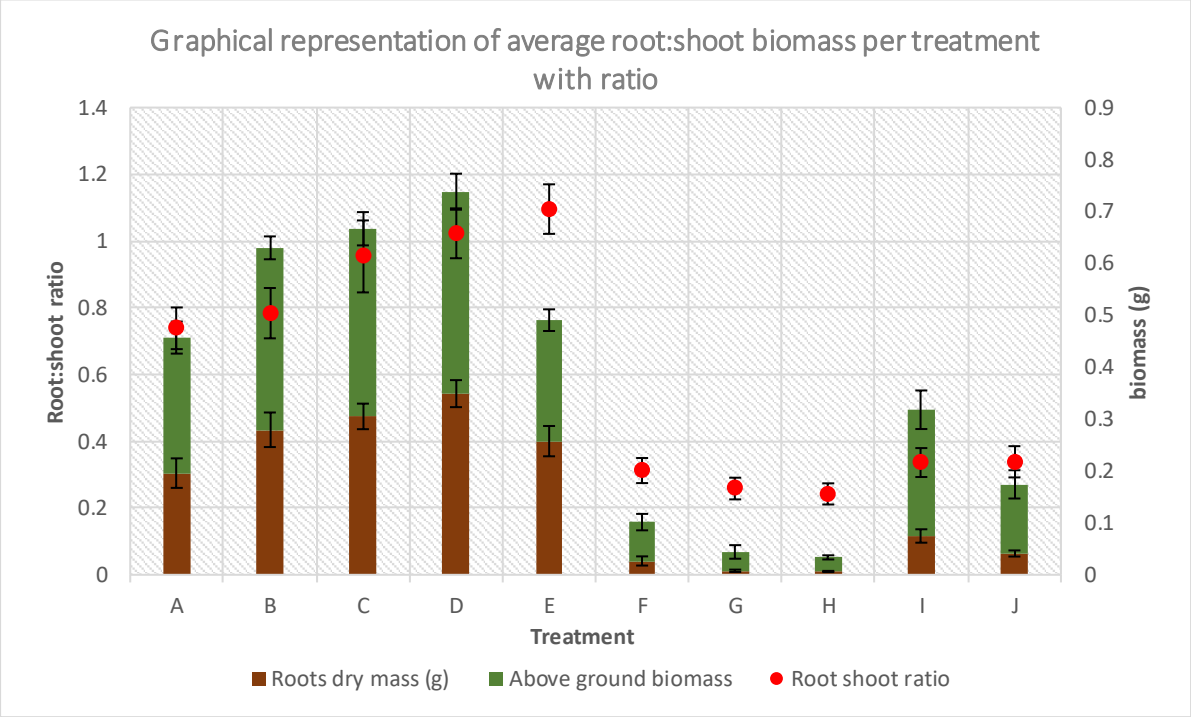


Figure 6.16. Above to below ground biomass and the root: shoot ratio for each population. Data is based on the destructively harvested populations for each treatment.. N = 5-16 depending on survival rates within populations. Error bars use StdE mean for each parameter. Brown bars show average below ground dry biomass, and green bars show average above ground dry biomass for each treatment. Red points shows the average root: shoot ratio seen for that treatment.

Assessments of the relationships between average seedling growth variables per treatment and the average total plant biomass from the seedlings selected for destructive harvest showed the highest positive relationship between leaf number and total biomass (R^2 = 0.8468). Most populations followed the line of best fit for leaf number (Figure 6.17A). Length of tallest stem was positively correlated with seedling biomass (R^2 = 0.6102), the red-light treatment (red) was most divergent with one of the highest average stem lengths but lower

overall biomass (Figure 6.17B). Stem number was positively correlated with biomass (R^2 = 0.6134) (Figure 6.17C). Stem number showed more variation around the line of best fit, particularly for the treatment group grown with no added nutrients (black), which had slightly lower average stem number to correlate with higher seedling biomass, in comparison to other higher biomass populations.

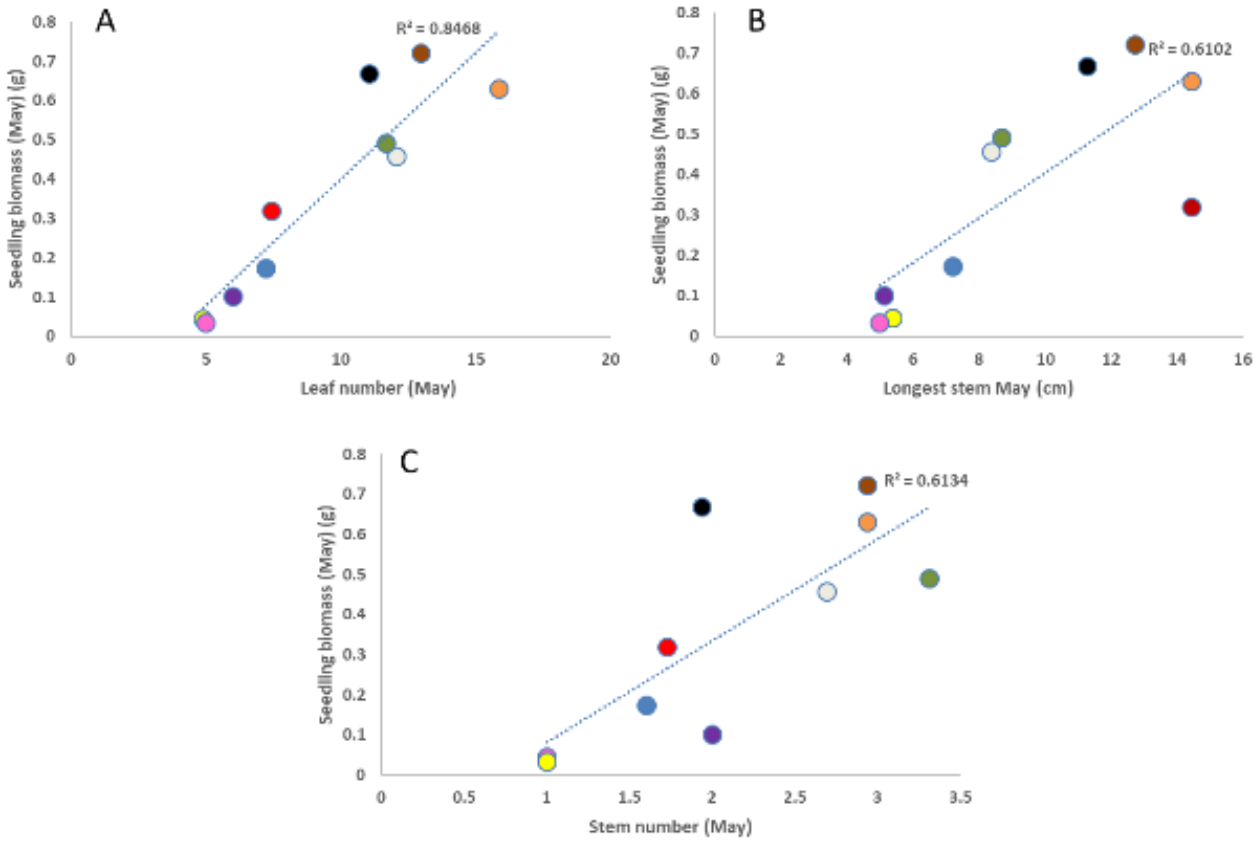


Figure 6.17 Correlations between *Miscanthus* seedling characteristics from destructively harvested subsamples in May, to assess the relationship between plant biomass and 3 growth characteristics leaf number (A), longest stem length (B) and stem number (C). Correlations use population means from 10 seedling treatments, the colours refer to treatment (See appendix for colour code for treatments).

6.3b Analysis of stem length and stem number from all plants from all populations prior to field planting in May 2017

Figure 6.18 displays pre planting phenotyping results for the approximately 2000 plants in both field trials. There were significant differences in height of tallest stem ($p< 0.01$). Five significant groups were identified by Tukeys HSD, the tallest of which conatined treatment I, which had significantly higher stem length than all others treatments ($p < 0.01$). Lower average heights were seen in treatments E, G & H, which were placed into the 'a' lowest height homogenous subset. Correlation of the destructive samples results, against the whole population produced a positive correlation ($R^2 = 0.5511$) (Figure 6.19 A).

There was a significant effect of treatment on stem number for the whole plant population ($p < 0.01$). Four subgroups appear overall, congruent to the destructively harvested plants. Highest average stem number is seen in treatment E in both assessments, with the lowest stem numbers seen in cold cabinet and red and blue cabinet treatments, in agreement with the destructively harvested data (Figure 6.14D). Correlation of the destructive samples results, against the whole population produced a strong positive correlation ($R^2 = 0.8201$) (Figure 6.19B)

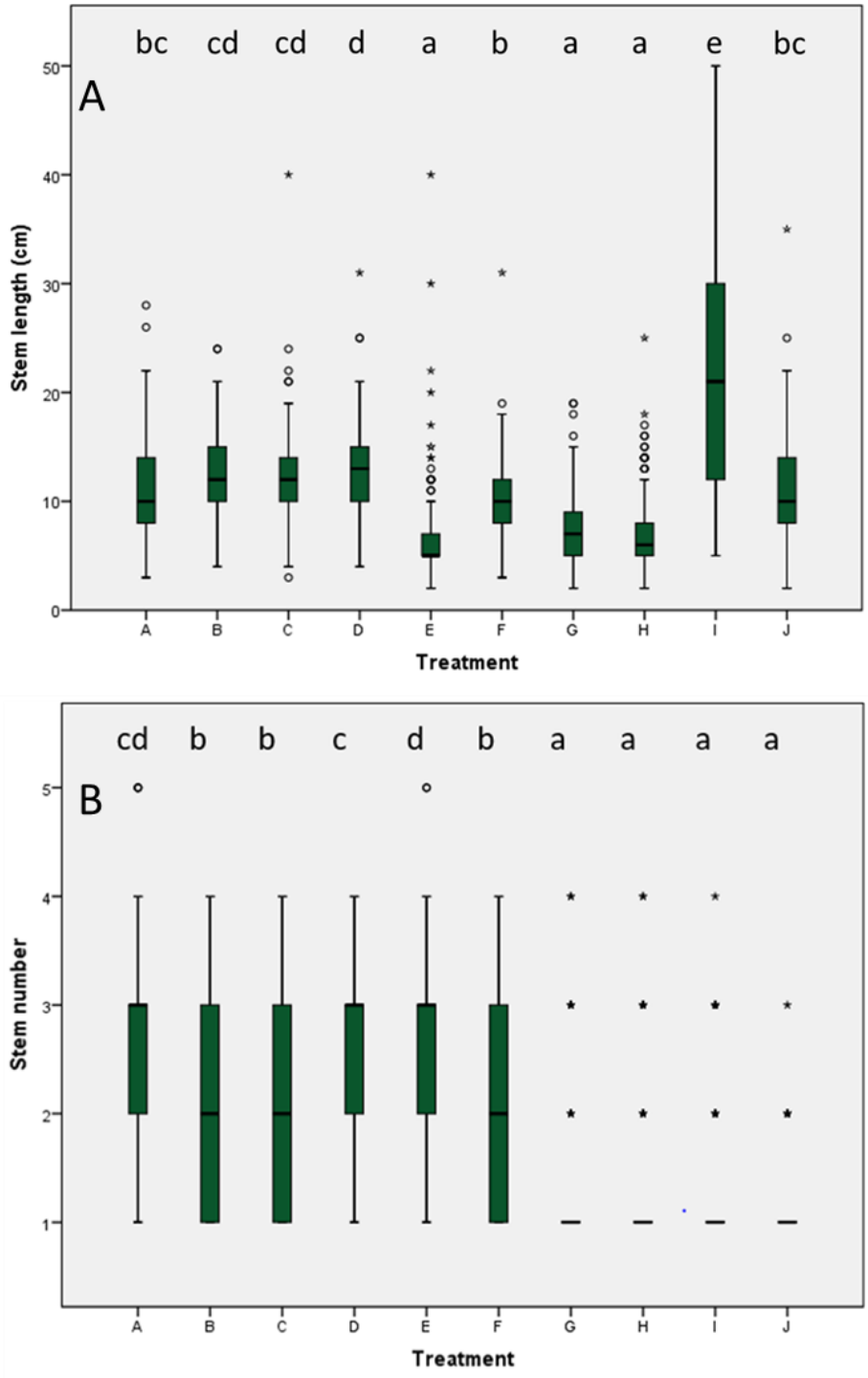


Figure 6.18 Phenotyping results of all plants pre-planting in May that were planted in the trials. Picture A measurement of tallest stem for each plant. Picture B – the stem number of each plant. N = 198 - 200 for treatments A,B,C,D,E,F+ I. N= 124-161 for treatments G,H+J depending on survival. Homogenous subsets are labelled a – e. Circles denote SPSS considered outliers. Asterisks denote extreme outliers.

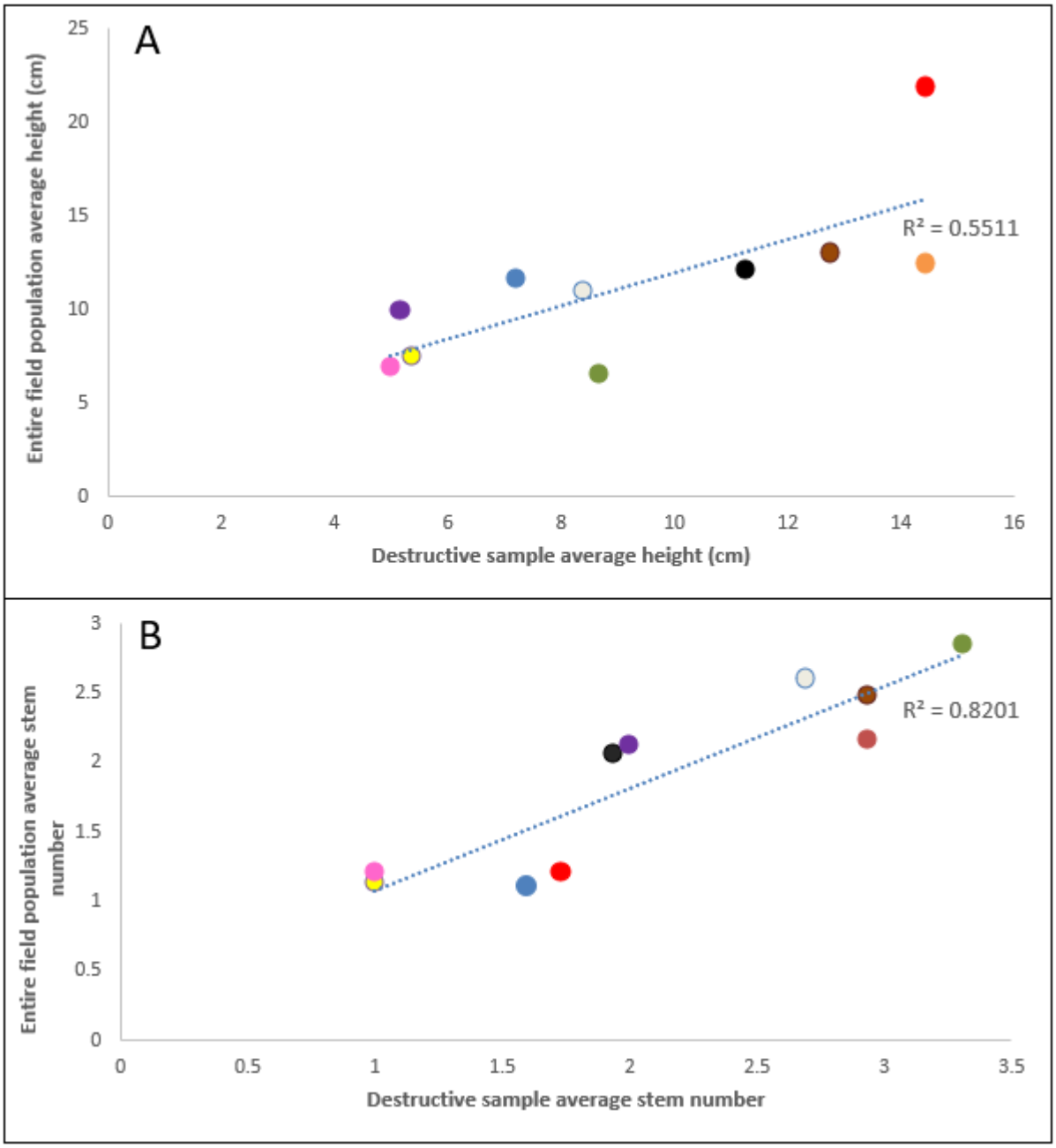


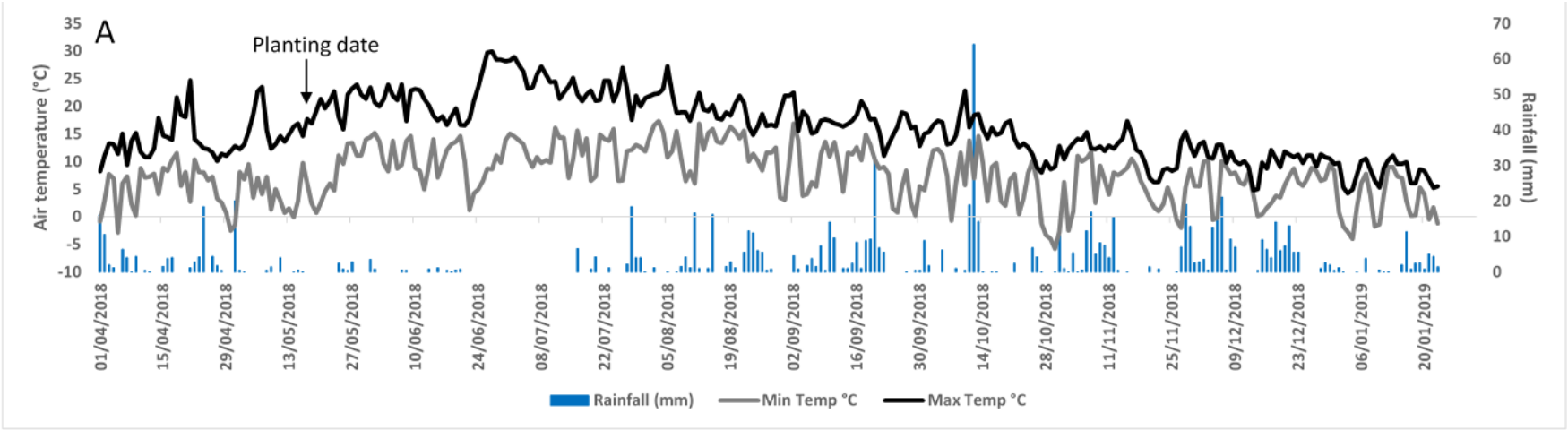
Figure 6.19 Average tallest stem height (A) and average stem number (B) of destructively harvested smaller sample, correlated with whole population results on a per treatment basis. Results are pre – field-planting phenotyping assessments conducted on all plants of ten different treatments in May 2018. Colours are used to match the treatment colour code in the field plan (see appendix).

6.3c Regression and biomass modelling

Stem data (stem length and number of stems) was obtained from all plants individually prior to field planting. Complete biomass data could not be obtained directly for plants in the field trial because this is a destructive assay. A representative subsample of each population was however taken for destructive sampling. Using data from destructively harvested seedlings, which included the same stem length and number data as assessed in the field populations, but additional complete biomass data, a regression model was built to predict biomass for the field populations. The model generated a correlation with above ground biomass and produced a Multiple R^2 value of 0.6483. This model was then applied to the entire seedling dataset, producing a prediction with 65% confidence of reliability, of the likely biomass of a specific field plant. The model was highly significant (p-value: < 2.2e-16), with moderate to high R-squared (0.6423). Predicted belowground biomass was assessed using the same correlations. The stem data correlated with the belowground dry mass of the destructive samples was used to predict the below ground biomass of the field population. The model was highly significant (p-value: < 1.17e-14), with moderate predictive ability R-squared (0.4122).

6.3d Weather conditions at planting

When planting the Aberystwyth trial (Figure 6.20A) conditions were warm and dry with maximum temperatures of approximately 20°C. Between 2 – 4mm of rainfall occurred on two days leading up to planting, but no rainfall is noted for approximately one week post planting. At the Hackthorn site, planting was done under similar temperatures but with a little more rainfall pre and post planting date. Both trials experienced a drought period for much of June and July, requiring supplemental watering.



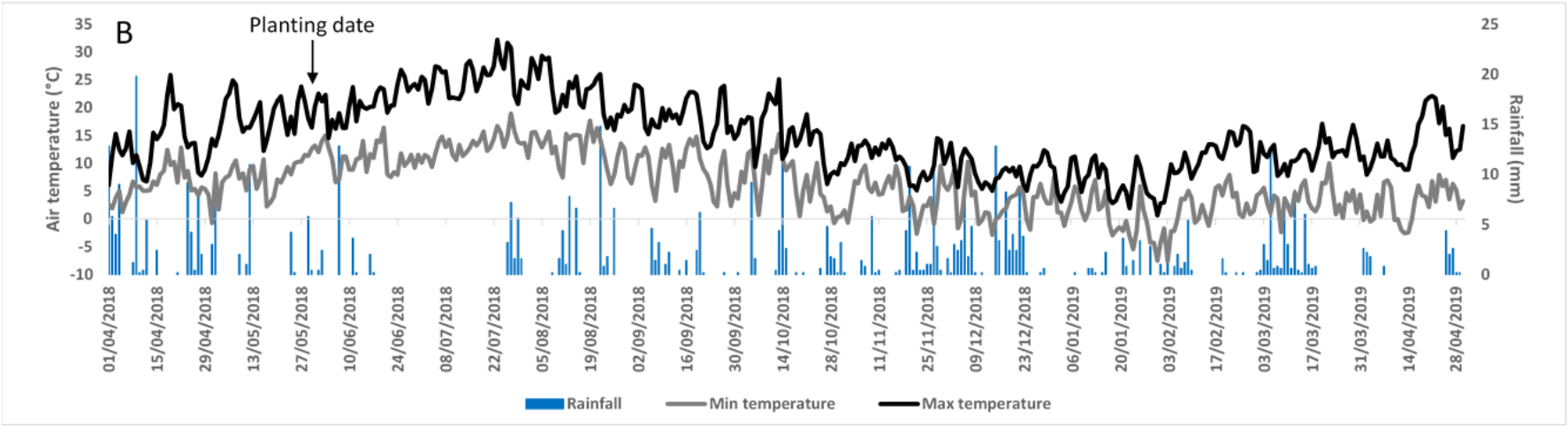


Figure 6.20 Meteorological data from April 2018 – April 2019 for both trials. A – Aberystwyth. B – Hackthorn data. Aberystwyth data only until 20th January 2019, as data was unreliable following this point. Daily rainfall can be seen as blue bars. Maximum air temperature is shown as a black line, and the minimum air temperature shown as a grey line. Aberystwyth planting date is shown on the 18th May 2018. Hackthorn planting date is shown on the 30th May 2019.

6.3e October Phenotyping of stem count and shoot height in all plants from both Aberystwyth and Hackthorn trial in November 2018

There was a significant difference in stem number measured in October between plants growing in Aberystwyth (Figure 6.21B) and in Hackthorn (Figure 6.22B) ($p < 0.01$). Aberystwyth had on average 11 stems fewer per plant than Hackthorn. There was a significant effect of seedling treatment on stem number at Aberystwyth ($p<0.05$), and Tukeys *post hoc* test revealed two homogenous subsets, with some treatments falling into both. Lowest stem counts (subset 'a' alone) included treatment H (cooler temperature and low irradiance) only with an average stem count of 12. Highest stem counts (subset 'b' alone) were seen in the commercial standard treatment (A), high compaction treatment (D) and the seedlings that were cut back pre planting (E), with approximately 17 – 18 stems per plant. All others were placed into both subsets (between 13 – 16 stems). There was no significant difference in stem number between treatments within the Hackthorn trial.

Results of shoot heights were significantly different between sites ($p = 0.012$), with the Hackthorn site (Figure 6.22A) yielding a slightly higher variation in height than Aberystwyth plants (Figure 6.21A). The range of averaged shoot heights from plants at both sites was between approximately 40cm to 52cm. There was no significant effect of seedling treatment on stem height at either site ($p > 0.05$).

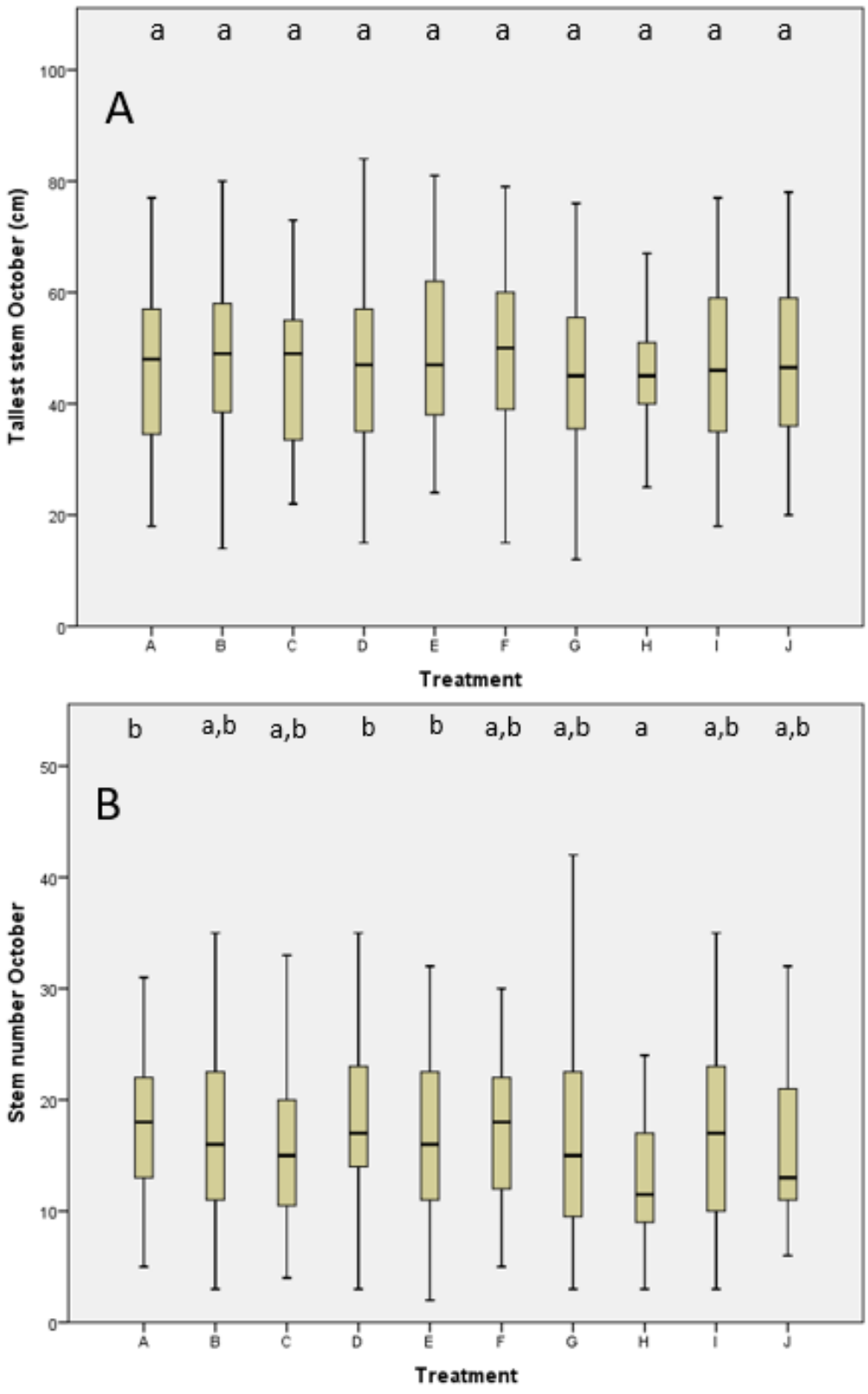


Figure 6.21 Length of tallest stem from all plants per treatment site (A), and number of stems (B) at the Aberystwyth site in Autumn 2018. N variable depending on survival (See table Table 6.2 for exact survival). Homogenous subsets determined by Tukeys post hoc test (lower case letters).

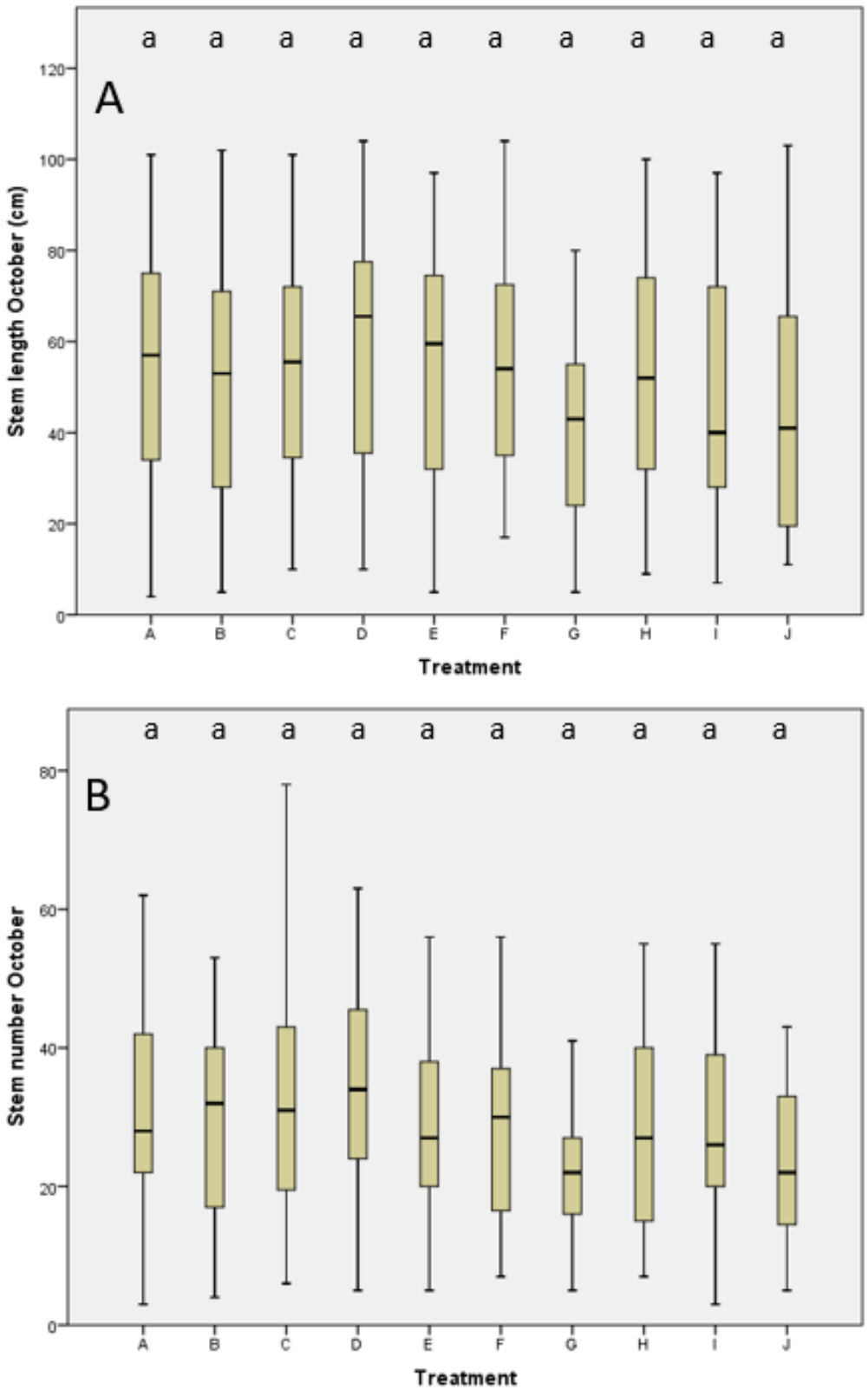


Figure 6.22 Length of tallest stem from all plants per treatment site (A), and number of stems (B) at the Hackthorn site in Autumn 2018. N variable depending on survival (See table Table 6.2 for exact survival). Homogenous subsets determined by Tukeys post hoc test (lower case letters)

6.3f Survival and yield

Under glasshouse conditions temperature appeared to be a critical factor in determining the number of plants surviving treatment. Survival rates were consistently 99 – 100% for all populations grown under warmer conditions, regardless of additional treatments applied (Table 6.2). After transplanting to field conditions, survival rates declined for all populations over the growing season; however, survival by October was still above 70% in most cases, with the exception of treatments G, H & I at the Aberystwyth site, groups that appeared to have slightly improved survival at Hackthorn. Survival over winter was poor for many groups, particularly in the Hackthorn field trial. Plants that originated from the warm glasshouse treatments had a 15-20% higher survival rate in the field than those grown in other treatment environments. Poorest survival rates in greenhouse and in field were seen in cool cabinet conditions, which produced much smaller plants at the seedling stage, and those grown under blue light, which also produced small plants as seedlings. This is consistent between both field trials.

Table 6.2 Survival of *Miscanthus* plants after ten treatments and after treated plants were grown in the field for one growth season and after the subsequent winter period.

Site	Treatment	% survived treatment	% alive in Oct	% harvested March
ABR 71	A	100	77.3	76.1
	B	100	83.1	71.9
	C	100	80.5	68.3
	D	100	77.9	77.9
	E	99	76.9	76.9
	F	100	73.1	73.1
	G	82	67.1	51.4
	H	75	52.3	46.2
	I	98	67.9	57.7
	J	82	80.6	67.7
HCK 35	A	99	77.8	60.6
	B	100	78	62
	C	100	81	60
	D	100	80	62
	E	100	74	49
	F	100	82	51
	G	80	71.3	26.3
	H	77	79.2	40.3
	I	98	72.4	50
	J	67	83.6	28.4

(% survived treatment), percent of planted individuals that survived through the growing season (% alive in October) and the percentage of planted individuals that were alive and harvestable following the winter (% harvested March).

6.3g Post winter harvesting results for both field sites

Biomass dry weight was analysed by two way ANOVA, where treatment and site were considered as independent variables. There was a significant difference in dry weight biomass between the two sites ($p < 0.01$). There was a significant interaction between treatment and site suggesting treatments produced different biomass responses at the two different sites ($p = 0.033$). The treatment effects on biomass were therefore analysed separately for each field trial. There was a significant degree of variation between harvest results obtained from Aberystwyth and those obtained from Hackthorn (Figure 6.23). Results of ANOVA of above ground biomass revealed no significant differences between Aberystwyth groups ($p > 0.05$). Average total above ground dry biomass for each population at the Aberystwyth site (Figure 6.23A) was approximately between 15 and 22g per plant. Lowest averages were seen in the cold cabinet treatment G (approximately 15.1g/plant. The highest biomass average was observed in the later sown population (approximately 22.2g/plant). The amount of plants in each treatment that were alive and large enough to be harvested can be seen next to each population bar in Figure 6.23. The lowest average biomass also had the lowest number of alive and harvestable plants (treatment H), with less than half the survival of several of the other populations (A, B, D & E).

Results were more variable between the Hackthorn site populations (Figure 6.23 B). ANOVA revealed a significant difference of $p = 0.040$ between groups but further post hoc tests did not show significance of $p < 0.05$ for any individual comparisons. Tukeys HSD subset analysis revealed two homogenous subsets 'a' and 'b'. Lowest average biomass occurred in the population of treatment G (approximately 116g/plant). Highest average dry biomass occurred in the highly compacted population (treatment D with approximately 194g/plant). The population with the smallest average biomass per plant (treatment population G) was placed into subset 'a' alone. Treatment D with the highest average biomass was placed into subset 'b' alone. The remaining eight populations fell into both subsets ('ab').

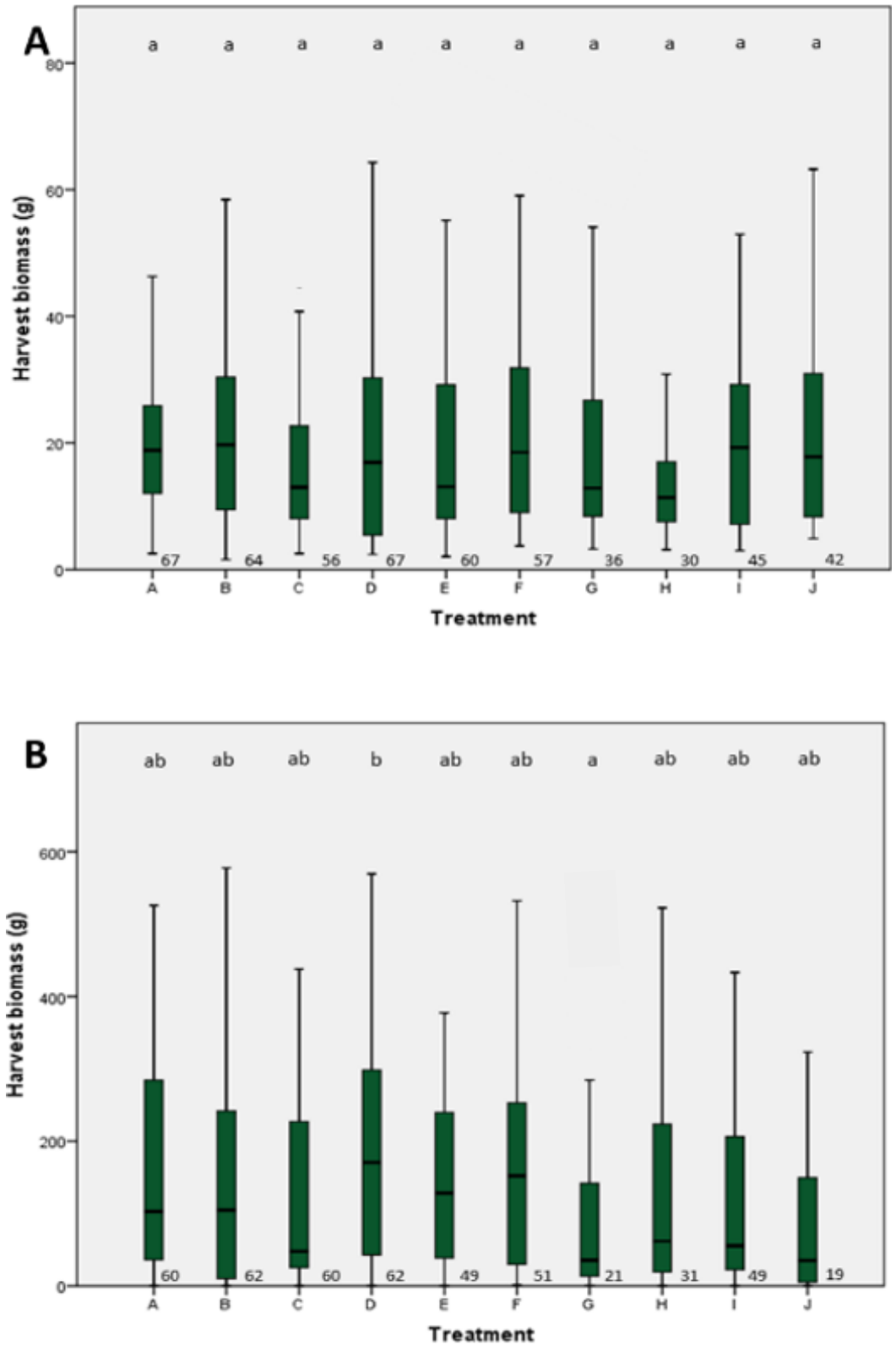


Figure 6.23 Average total plant dry biomass for each population harvested in April 2019. Aberystwyth trial results (A), and Hackthorn results (B). Y-axis scale differs between charts due to large differences in average biomass range between sites. Error bars show ± 1 se with variable N. N per population can be seen at the base of each bar and depicts the amount of plants that were of harvestable size and/or alive at harvest in March 2019. Tukeys HSD homogenous subset allocation is at the top of each chart.

Table 6.3 Comparison of phenotyping result averages between sites for stem number and shoot height of all plants in November 2018, and harvest biomass for all plants from both trials in April 2019 for both sites. Statistical analysis done using Students T test

	HCK	ABR
Stem number	29.6	16
Std deviation	14.3	8.3
P value	$p < 0.01$	
Shoot height (cm)	52.7	47.2
Std deviation	25.6	42
P value	$p = 0.012$	
Plant biomass (g)	149.5	20.4
Std deviation	148.6	15.45
P value	$p < 0.01$	

6.3h Survival and biomass correlation per treatment population

Averaged plant biomass and survival rates were compared between treatments and from each site separately (Figure 6.24). Percentage survival in the field in March was positively correlated with average whole plant biomass at planting (Figure 6.24 charts A and B). The lowest survival rates in the field are correlated with the lower average biomass from glasshouse treatments F, G, H, I and J (Figure 6.24). The treatment populations that had higher overall biomass (A, B, C, D & E) are in the higher survival region of the chart, particularly at the Hackthorn site.

The survival rates in the glasshouse per treatment population were compared against that population's biomass at harvest in March (see Figure 6.24 charts C & D). The correlation R^2 values were 0.2261 and 0.0076 for Aberystwyth and Hackthorn sites respectively. These results were particularly low in the Hackthorn trial suggesting little correlation for this comparison. It can be noted however that the highest average biomass was seen in the population with one of the highest glasshouse survival rates (population D (brown marker)). There is a stronger correlation seen at the Aberystwyth site, particularly seen in treatment H

(pink marker) which had both the lowest glasshouse survival and the lowest average harvest biomass.

Field survival in March and the average biomass produced at harvest are correlated in Figure 6.24 charts E & F. Again, the correlation at the Aberystwyth site (R^2 0.3618) is stronger than that of the Hackthorn site (R^2 0.0738). The lower harvest biomass and lower survival of treatments G & H (yellow and pink markers respectively) encourage a higher correlation value for Aberystwyth, although the majority of other treatments are more closely grouped, with the exception of lower average biomass in treatment C (black marker). At the Hackthorn site, a greater spread of results seen, but treatment G (yellow) remains lowest on all parameters. Other treatments have lower correlation for this comparison.

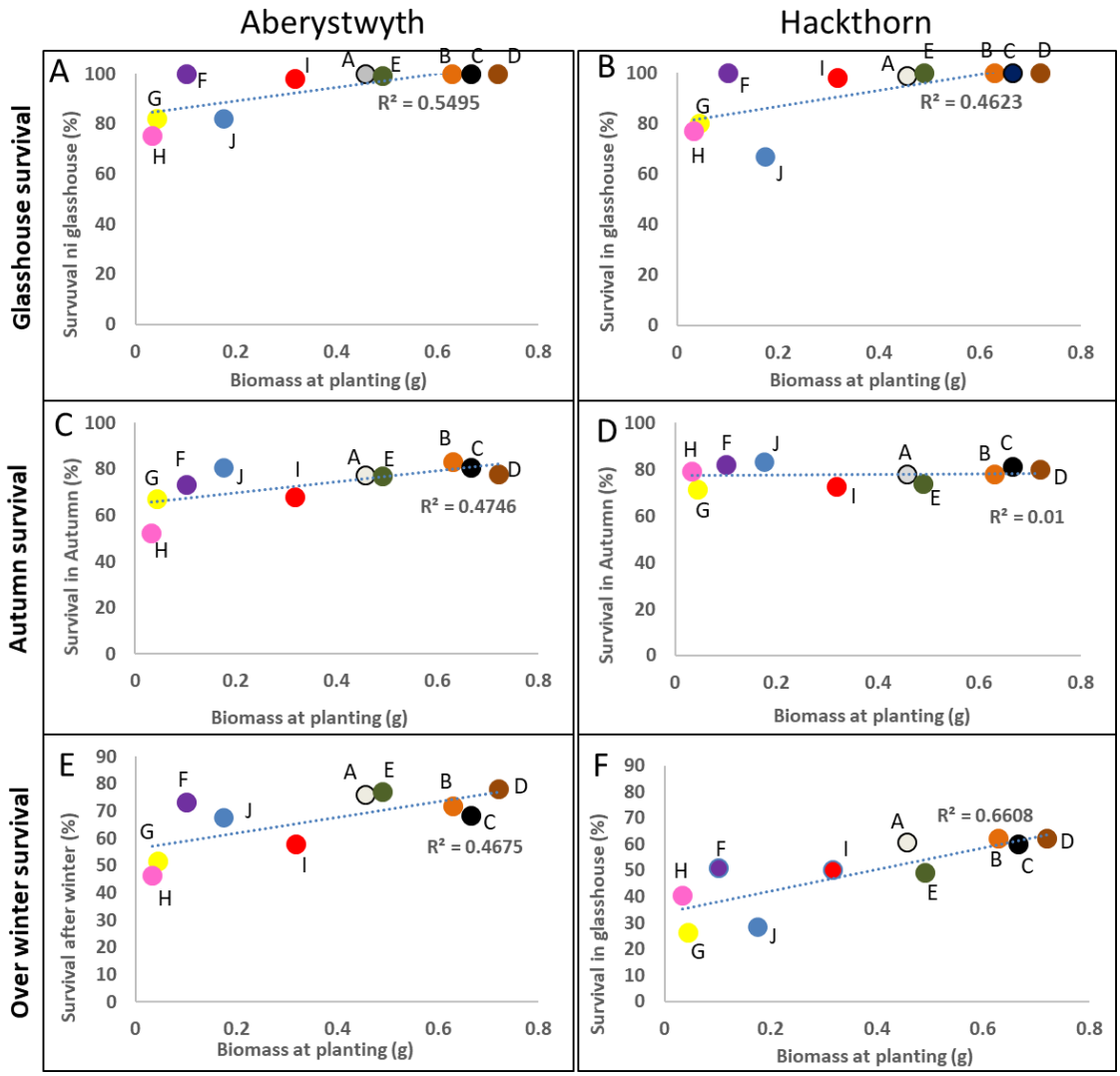


Figure 6.24 Correlations at the population level for survival per field plot. Figures A & B are correlations based on the overall survival percentage for each population at the end of the glasshouse phase against the known average whole plant biomass for that population taken from the representative harvest pre-planting. Figures C & D show correlation of the survival at the end of the first growth season per population, against the known average whole plant biomass for that population taken from the representative harvest pre-planting. Figures E & F show correlation between each population survival and biomass at the after winter against the known average whole plant biomass for that population taken from the representative harvest pre-planting. Colours represent the colour codes for each treatment in the original field plan.

6.3i All harvested plant biomass correlation against known and predicted May biomass

Stem data from individual seedlings after growth in 10 glasshouse treatments (May 2018) was used to predict the biomass of each seedling at planting as described above. The predicted biomass from each plant was compared with harvested biomass from the same plant after one growth year (March 2019). When all plants were used, predicted above and below ground biomass of the seedling at planting did not correlate well with harvested biomass after one growth year at either Aberystwyth or Hackthorn, (Table 4).

Known stem length and number at planting, correlated with final biomass also produced low correlation at Aberystwyth and Hackthorn.

6.3j Representative destructively harvested seedling correlations against March harvest biomass

Results for the remaining significantly different growth parameters seen between populations in May at the population level were correlated against the same population's average harvest biomass in March 2019, for both field sites (Figure 6.25). Average population leaf number in May against harvest biomass for Aberystwyth and Hackthorn showed low correlation; R^2 = 0.0739 and 0.105 respectively (Figure 6.25 A and B). Dry leaf biomass of the leaves in May correlated with harvest biomass was low for Aberystwyth and Hackthorn trials, R^2 = 0.0419 and 0.0247 respectively (Figure 6.25 C and D). Known below ground average biomass for each population in May and the harvest biomass first year field growth was also low for Aberystwyth and Hackthorn; R^2 = 0.0035 and 0.0274 respectively.

A summary of populations, their treatments and their main results can be found in Table 6.5.

Table 4 Correlations on an individual plant level for parameters known or modelled for each plant in May, at each site.

	Aberystwyth	Hackthorn
	Harvest biomass after winter	
Predicted above ground seedling biomass	$R^2 = 0.017$	$R^2 = 0.0128$
Predicted below ground seedling biomass	$R^2 = 0.018$	$R^2 = 0.0189$
Seedling actual height	$R^2 = 0.0123$	$R^2 = 0.009$
Seedling actual stem number	$R^2 = 0.0035$	$R^2 = 0.0274$

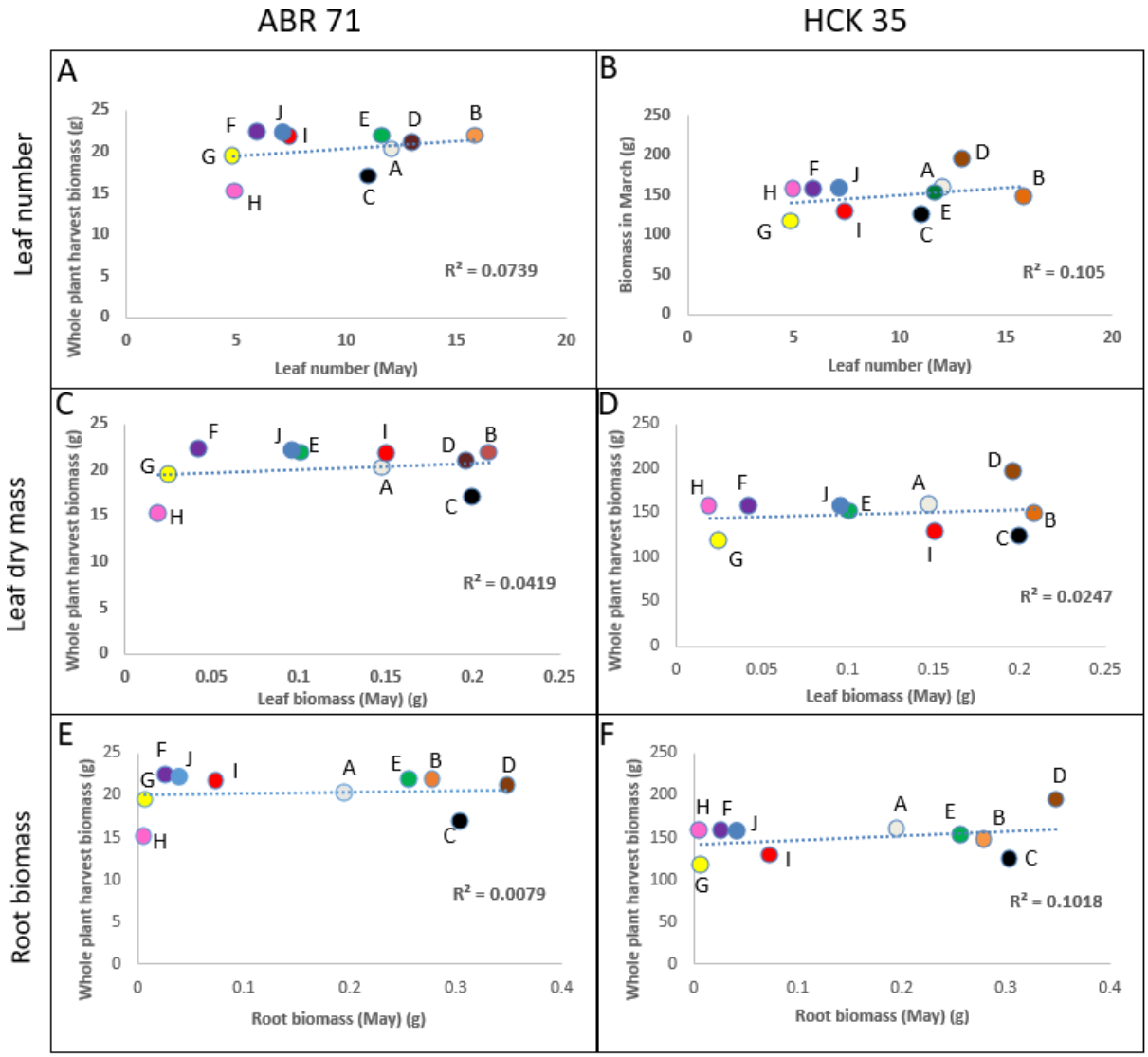


Figure 6.25 Correlations at the population level for growth parameters measured on sacrificed representative plants compared against the same population final harvest biomass. Figures A&B are correlations based on the seedling leaf number for each population, against average whole plant biomass at the end of the first growth year. Figures C & D show leaf dry mass at the seedling stage for each population, against the average biomass at the end of the first growth year. Figures E & F show correlation between average root biomass at the seedling stage and average biomass at the end of the first growth year. Colours represent the colour codes for each treatment in the original field plan.

Table 6.5. Summary table of populations, and treatment description, and subsequent morphological characteristics of the population. Various growth and survival parameters that any particular population falls into are marked with an asterisk. Two highest or lowest average biomass were selected as the selected two were often incredibly close in terms of yield. Highest glasshouse survival has multiple asterisks due to many of the populations having the same (100%) survival.

Treatment ID	Treatment description	Morphological description of resulting population	Two highest average harvest biomass ABR	Two highest average harvest biomass HCK	Two lowest average harvest biomass ABR	Two lowest average harvest biomass HCK	Highest field survival ABR	Highest field survival HCK	Lowest field survival ABR	Lowest field survival HCK	Highest glasshouse survival	Lowest glasshouse survival
A	Standard commercial	Medium whole plant biomass and root biomass. Medium level height, stem number and leaf number		*							*	
B	Standard commercial but lower available PAR	Medium/high whole plant biomass and root biomass. Tall height. Highest leaf mass and leaf number						*			*	
C	Standard commercial but no fertilizer given	High whole plant biomass and root biomass. Medium leaf number and height and number of stems.			*	*					*	
D	Standard commercial but higher soil compaction	Highest whole plant biomass. Highest root biomass. High leaf number, stem number and height.		*			*	*			*	
E	Standard commercial but main stem cut hard back pre-planting	Medium whole plant biomass and root biomass. Highest stem number with low/medium height									*	
F	Standard conditions but 2 weeks younger	Low biomass in all areas. Medium stem number, low height	*								*	
G	Cooler growth cabinet. All else standard	Lowest biomass in all areas. Low height, lowest stem number and leaf number				*				*		
H	Cooler growth cabinet and lower available PAR	Lowest biomass in all areas. Low height, lowest stem number and leaf number			*				*			
I	Red light alone. All else standard	Medium to low whole plant biomass. Low root mass. Medium/low stem and leaf number. Highest stem height										
J	Blue light alone. All else standard	Low biomass in all areas. Medium to low stem number & height	*									*

6.4 Discussion

Hybrids are not genetically uniform

The aims of this trial were to attempt to produce large variation in *Miscanthus* seedling morphology for populations grown under glasshouse conditions and to use simple regression techniques to attempt to understand important indicators of good or bad first year field performance. We hypothesized that larger plants would perform better in terms of biomass and survival, as opposed to smaller plants. The use of controlled environments in a nursery or glasshouse provides an opportunity to change and manipulate growth conditions before transfer to the less controlled field environment.

By manipulating nursery conditions, it was possible to produce morphologically diverse populations of the same *Miscanthus* hybrid, and to then test these morphologies under field conditions.

6.4a Plant growth characteristics coming out of treatment environments

Six of the ten populations were grown under warm conditions in the same glasshouse (18-26°C night/day cycle). These populations typically exhibited the largest biomass, although there were significant differences between them. The first population (A) was the typical commercial standard seedling, grown under standard PAR, nutrient additions and temperature. Plants from this population appeared to fall into the mid-range for many of the biomass parameters measured. In comparison, increased biomass, in terms of stem height, leaf number and root biomass were observed in the second population (B), which was grown under the same conditions in warm temperatures but had lower levels of photosynthetically active radiation from the glasshouse SonT lights. This population had the highest average leaf number when measured as part of the sacrificial assessment, and exhibited longer average main stem length. This could be a result of seedlings trying to maximise light interception, when grown under a lower PAR. Studies using maize suggest that higher temperatures and high photosynthetic photon flux density (PPFD) increase leaf appearance rate, but that leaf elongation was more variable based on PPFD, and decreased mildly under increased PPFD (Bos *et al.*, 2000).

Highest overall biomass was produced in plugs grown in the higher density soil, within the warm environment (treatment D). Crucially, this population also had significantly higher root

biomass than most other populations. Reasons for this are likely numerous. It is probable that higher soil density increased root – soil contact making uptake of nutrients and water easier. It is likely that a greater soil density also had a greater water holding capacity, and greater amount of available nutrients (Alameda and Villar, 2009). Compacted soil is often regarded as a plant stressor, negatively affecting plant growth as a result of decreased rooting due to soil resistance, particularly in small plants (McNearny *et al.*, 2002). However, some studies have shown that moderate soil compaction can have a positive effect on growth. In a study using seedlings of 17 woody plant species, Alameda and Villar (2009) found that 53% of the species exhibited positive growth effects from a moderate increase in soil compaction, 41% of species increased relative growth rate, and 35% the total leaf area. Later studies using tobacco plants also showed that soil compaction positively affected plant performance, but only up to a certain level, after which plant growth declined (Alameda *et al.*, 2012). In *Miscanthus* our results suggest compaction treatment consistently produced plants with the largest overall biomass, with a good source: sink balance, yielding an average root to shoot ratio of one.

The fifth warm compartment treatment (E) involved cutting the main stem of each plant to just below the lowest meristem, approximately one week before the plants were phenotyped prior to planting. This treatment had rapid effects on apical dominance and the normally hormonally regulated responses controlling lateral stem formation, and resulted in the production of new tillers from the base of the plant (McSteen, 2009), producing shorter and bushier plants. It was important to include a higher stem number group of individuals in the assessment. Higher stem numbers in mature plants correlate very strongly with harvest yield (Robson *et al.*, 2019), so it would seem logical therefore to attempt to encourage variation in these traits early on in the plant life cycle.

Lowest overall above ground biomass, height, leaf number and root biomass in the warm environment was seen in the population that was grown two weeks later (Treatment F) than the remaining populations. This developmental delay made a significant difference in terms of morphology at planting, placing the population in the lower subgroups for all parameters measured, indicating the significant impact of plant age on maturity and morphology. This was also noted in chapter 5 where it was observed that plants grown in the glasshouse for

the shortest period of time, appeared to have slightly lower establishment and growth than those left in the glasshouse for longer periods.

Seedling populations G + H were both grown under cooler conditions (12-20°C night/day cycle) with normal or low PAR from SonT lighting. *Miscanthus* utilises C4 photosynthesis and most C4 plants cannot maintain effective photosynthesis under lower temperatures (<15 °C), losing assimilatory capacity when grown under cooler conditions (Wang *et al.*, 2008). However, *Miscanthus* is unusual in exhibiting cold tolerant C4 photosynthesis (Naidu *et al.*, 2003), therefore the effects of cold are likely to be acting by a different mechanism(s). Soil temperature is one of the primary factors affecting plant growth (Alvarez-Uria and Körner, 2007). In Maize plants, low temperatures at the seedling stage are known to retard emergence and vegetative growth (Miedema *et al.*, 1987, Bos *et al.*, 2000). Low temperatures for seedling growth have also been proven to reduce growth variables in Tomato (Melton and Dufault, 1991), and oil-seed rape (Nykiforuk and Johnson-Flanagan, 1999), and reduce root growth in woody plants (Alvarez-Uria and Körner, 2007). In Maize, growth of seedlings at lower temperatures affected chloroplast structure and photosynthetic efficiency, days after the cold treatment had been removed (Sowiński *et al.*, 2005). The difference in PAR in the two cooler treatments (Treatments G and H) made little to no difference to these populations, but the cooler temperatures in general caused a severe arrest of growth. Under higher PAR conditions (Treatment H), it is expected that the plants will have been subject to slightly increased temperature as a result of heat emitted from the closer SonT lights, but this was not enough to encourage productive growth. As a result, both of these populations produced significantly lower total biomass, with shortest average stem lengths, lowest stem numbers, and lowest root mass.

Seedlings grown under red and blue LED lights in growth cabinets produced highly variable biomass. It is known that combinations of red and blue light can provide an effective light source for a large variety of plants (Samuolienė *et al.*, 2010), but these wavelengths are rarely delivered individually. Red light alone here, with no supplemental blue or far-red light produced plants that were particularly notable for their significantly consistent long single stem morphology, although the whole plant biomass was medium to low in comparison to other populations. Below ground biomass did not match the above ground, creating an imbalance in sink/source relationship, and a low root: shoot ratio. This growth response is not

unusual, as red light is highly important for shoot and stem elongation (Schuerger *et al.*, 1997). Blue light is necessary for plant health and effective photosynthetic capacity, the lack of which in treatment I likely affected plant physiology, as although *Miscanthus* grown under red light were tall, the stem had reduced lignification and was thin and fragile. Under blue light growth of above and below ground was significantly reduced, without the red and far-red wavelengths to encourage biomass accumulation. Therefore, the population grown under blue light was, for most growth parameters, placed in the same homogenous subsets as the smallest plants grown under cold conditions.

Survival must also be taken into account when assessing treatments. Reduction in glasshouse survival is costly in terms of seed production and glasshouse space. It also requires that manual gap filling be undertaken prior to planting, as transport of semi filled plug trays is a waste of transport space. Seedlings with smaller average morphology, also had reduced average survival under glasshouse conditions, suggesting that although these treatments created useful experimental variation, they should be avoided in commercial plug growing operations.

Previous studies into the relationship between morphological traits of a plant and the biomass yield in more mature plants are described in Robson *et al.* (2013a) to identify the largest contributing growth trait to overall biomass yield in the plant. Biomass yield is a result of multiple simple traits, combining to produce one complex combination of interactions between all growth parameters. It was decided to attempt to replicate the theory of this assessment on a simple small-scale analysis in seedlings, using the data obtained from the destructively harvested subsamples. From assessment of simple traits in seedlings, leaf number appeared to be the largest correlative factor to overall seedling biomass ($R^2 = 0.8$). Stem number and stem height were comparable at $R^2 = 0.61$ each. This result would likely not be applicable to field harvests, as leaf matter is typically dropped to the ground over winter, and the bulk biomass at harvest, consisting of remaining dry stems.

6.4b Field planting and performance under field conditions

The lack of strong root mass seen in the smaller populations made transplantation into trays and field sites difficult, due to the surrounding soil falling away, and as such these populations were often planted nearly bare rooted, and required a slightly greater amount of time to handle. The tall, thin stemmed population was potentially compromised when mulch film was laid on top of the columns, due to stem snapping. Other than these complications, plant morphology had no other impact on handling and planting method.

When visually inspecting the field plots, it was immediately obvious, that a great variety of morphologies existed within the field plots. It is worth noting that this variation was also obvious in the double, border rows on all sides, which had not been subject to any additional treatments. This is unsurprising, as the seeded hybrids are not clonal, unlike the typical *Miscanthus giganteus* populations, and as such are not genetically uniform, and are likely to exhibit morphological differences regardless of growth conditions. The variation in growth was at its highest within the Hackthorn field trial, due to a significant increase in growth of all planted populations, compared to the Aberystwyth trial, which resulted in greatly increased error margins. The differences in growth and biomass seen between sites are likely a result of field environment, as opposed to glasshouse treatment, as shown in statistical analyses of all field traits measured over the first year. The Aberystwyth site had been less intensively managed prior to planting, and had previously been used as arable grassland before being prepared for the trial, and as such had a much larger latent seed bank present in the soil. The weed issues arose quickly after planting, despite applications of herbicide, and attempts to manage them, and likely had an adverse effect on all seedlings planted. The soil type at Aberystwyth was predominantly low fertility clay loam, with large stones present. At Hackthorn, more intensive crop rotation systems had depleted the soil nutrients somewhat, but it is still described as lime rich, medium fertility grassland with sandy clay loam soil type. The seed bank was reduced, and field trials were managed by specialized labourers to keep on top of weed issues.

Differences in morphology between treatments within field sites were much less conclusive. Results of the phenotyping in the autumn of 2018, and the harvesting of the senesced biomass in early spring 2019, did not produce the same quantity of significant differences and biomass variability between treatments as was seen prior to planting for either field site.

When comparing pre- and post-field planting figures, the obvious divisions seen between the larger biomass populations A – E and the smaller biomass populations F – J had evened out across the populations. There were some remaining trends, whereby the smallest average planted seedlings overall (G & H) did appear to have slightly lower growth parameter averages. Importantly, there were significant differences in survival rates between initial morphologies and treatments at harvest. Pair wise correlations of survival and biomass at planting were the strongest trait comparisons produced overall.

In many *Miscanthus* trials undertaken by the research team at Aberystwyth, phenotyping and/or harvesting at the end of the first growth season are often omitted from analysis, with preference instead given to the third growth season onwards, as this is considered to be the first 'mature year' under the climatic conditions of the UK (Clifton-Brown *et al.*, 2008). Correlations on an individual plant basis for pairwise assessment of the individual plant's biomass after the first year in field, against above and below ground (predicted) values, and stem length and number produced R^2 values less than 0.1 for all comparisons, in both sites. Whilst it can be argued that a positive correlation exists for all of the measured parameters, this result is underwhelming, proving that, within this trial, and with the morphologies measured at least, it is extremely difficult to confidently predict field performance based on seedling phenotype.

Predictions of field survival appear to be more realistic than field biomass when analysing *Miscanthus* seedlings. A study undertaken on five Mediterranean tree species by Tsakaldimi *et al.* (2013), also evaluated nursery characteristics of seedlings, and subsequent field performance. Their results suggested that survival in particular could be predicted from seedling morphological characteristics, but only a selection of the characteristics were good predictors. These included seedling root collar diameter, plant diameter and total dry weight. Diameter and root collar were not measured in this study, however it could be possible to assess stem thickness and base circumference in future assessments. Previous studies using *Miscanthus sinensis* and *Miscanthus sacchariflorus* indicated that plant basal diameter and stem diameter provide important predictors of yield (Davey *et al.*, 2017). However, the data produced from analysis of tree saplings in the study by Tsakaldimi *et al.* (2013), does suggest that larger plants are more likely to survive in the field, a result in agreement with results presented here.

It was hypothesized that larger plants coming out of the glasshouse would be superior field plants over the first year; however, large may be in terms of stem height or total biomass. Taller plants were not necessarily superior plants as was proven by the red light treatment, which produced the tallest seedlings, but an imbalanced shoot:root ratio and lower overall biomass. With some exceptions, such as stem elongation in response to shade signals (Gommers *et al.*, 2013), shoot height is typically correlated with higher leaf number, and it would therefore be expected to have increased photosynthetic capacity and leaf area available for transpiration. Taller seedlings should in theory, have an advantage against weed competition. However as proved here, taller stem does not necessarily equal seedling vigour and maturity, and could be a waste of plant resources, if root biomass accumulation is neglected, and seedlings are cut prior to planting, or are snapped by mulch film cover or the wind. In addition, greater leaf area could increase the risk of desiccation in drier environments, before effective root establishment (Haase, 2008). Smaller morphologies would, it seems, remedy much of these issues, being low to the ground, with reduced transpiration area; however, small plants had reduced vigour and poor physiology, and when combined with small root mass, were less likely to survive and grow well. Studies are in agreement across seedling growth in many woody species including Douglas Fir *Pseudotsuga menziesii (Haase, 2008),* Loblolly pine (South, 2000), (Cork oak *Quercus suber* (Chirino *et al.*, 2008), sugar cane (McIntyre, 1993), that large root morphology with a strongly balanced root: shoot ratio is likely the 'holy grail' of seedling morphology.

Poor performance seen in seedlings can often be a result of transplant shock, weed competition, poor soils or unfavourable site selection and preparation (Pinto *et al.*, 2011). However, issues with seedling quality also play a large part (Jacobs *et al.*, 2012). In a similar study based on establishment of Pine seedlings, South (2000) described a hierarchy model illustrating the importance of factors affecting survival of transplanted pine seedlings, although it can be reasonably applied here also (Figure 6.26). The most important factor in the model was the environment of choice for where the seedlings will be planted. This factor includes a range of variables including soil type, water content at the time of planting, temperatures at establishment, rainfall after planting and other weather conditions (e.g frosts), weed competition and herbivory. The second factor surrounds the handling of the plants prior to planting. This can include machines used, cold storage length and temperature,

depth of planting, and post planting care (for example applications of mulch films). In the third out of four factors we find seedling morphology, including many of the factors assessed here, for example root: shoot ratio, root mass, secondary foliage and seedling height. Fourthly is seedling physiology, which can be influenced by the nursery environment, although is described as being difficult to evaluate, as it is not visually obvious, but can be affected by nursery growth conditions (South, 2000).

Based on the differences seen between sites, it can be safely assumed that environment was indeed one of the biggest growth influences seen over the first year. It is difficult or near impossible to find a homogenous target site in which to plant a test trial such as this. Field sites are extremely heterogenous by their nature, with levels of nitrogen, phosphorous and potassium likely differing vastly across the site. Presence of stones or harder soil clumps could mean some plants cannot extend roots as easily as others. Some areas may have more weed competition than others, while other areas may be more prone to insect or herbivore issues. The levelling out of the extreme morphological differences seen at the seedling stage, and the relatively comparable harvest biomass for each population does suggest that environmental heterogeneity could be of greater importance than initial morphology in terms of growth potential. The morphology and physiology of the seedlings have a potentially greater effect on the survival rate of a population, rather than biomass accumulation over the season. Much consideration should be given to the biomass accumulation potential of a specific genotype over the course of a growth season. Maximum growth rate is correlated with timing and duration of the logarithmic growth phase as reported by Robson *et al.* (2019). It was noted that compensatory interactions are involved in growth rates, for example if a plant had a higher maximal growth rate, then a shorter period of logarithmic growth was observed. This suggests that plants may reach similar levels of biomass accumulation over the growth season by way of different methods, depending on size at planting.

Competition is also a strong factor affecting plant growth in a monoculture such as a *Miscanthus* sward (Weiner, 1985) . During the plug plant phase under glasshouse conditions, it is likely that rates of competition would be low, as plants are grown in identical plug volumes, with the same access to light and water, and would likely not affect each other, especially when small. Therefore, it is probable that growth differences under these conditions stem from other sources, such as the genotypic heterogeneity seen in seeded

plants as discussed earlier, or potentially heterogeneity of resources within the plug. Identical nutrient availability and soil volume within each plug would be impossible to achieve under mass planting conditions, as would assessments of the resources available in each seed, therefore making the optimisation of the external growth conditions even more important. Under field conditions, plants would have to compete for resources, particularly under high densities. Density of planting is not currently in the remit of this project, but has a vital part to play in the homogeneity of *Miscanthus* field plots (Danalatos *et al.*, 2007). Weed competition would likely be more of a factor for smaller plants with reduced lead canopy, further adding to the potential reasons for larger plants producing higher establishment rates.

These results can be described as both positive and negative for the *Miscanthus* plug plant growing regime. On the one hand, it makes it harder to be certain that a larger morphology of seedling will perform well in a variety of soils and environments. On the other hand, it reveals that most seedling types have potential for producing competitive biomass, if conditions are favourable. It is therefore important to select smartly, to combine best environment, seedling type, handling practice and after care for achieving maximal survival. This experiment proved that larger plants, grown under higher soil density, and in warmer conditions produces a strong plant morphology with a good root: shoot ratio and as such these plants tended to have the higher survival both in glasshouse and field, and trended towards larger biomass under field conditions. It is therefore important to aim for this level of seedling maturity. The importance of careful site selection should therefore be heavily considered, potentially placing bigger plants with well-formed roots on the less favourable areas, and in the pursuit of not wasting smaller morphologies, plant them in more productive land, with careful aftercare.

Figure 6.26 Model of importance of four factors affecting the performance of newly transplanted seedlings. Image courtesy of South (2000)

6.5 Conclusion and further comments

Survival rate is one of the highest priority field assessments when planting large numbers of biomass crops such as *Miscanthus*, because of the high costs involved when gaps appear in field trials, in terms of neighbouring plant biomass, gap filling costs and labour, and final harvestable biomass for that plot at the end of the growth season. As such, the survival of a plant in the field is of highest importance. Typically, it will take a *Miscanthus* field three years at minimum to reach maturity and maximal yield. Often by this point, the high heterogeneity within the trial tends to even out, producing an even sward. For non-clonal plug plant hybrids, patchy establishment and variable growth are commonplace, and are currently a serious drawback to the plug planting system. It is not likely that any one aspect of plug plant supply chain and field conditions will fix this issue. All aspects of the methods should be optimized, from genotypic selection and domestication, to nursery practices described here to produce optimal plug plants, and through to site selection and handling.

While there is no 'landslide' victory for any one of the nursery populations produced here, there are definite trends. The compact soil treatment appeared to be consistently highest performing over all parameters measured, both pre and post planting phase. It is likely that

this was due to advanced maturity as a result of a larger root system. Field establishment in many crops may be improved by promoting development of a deep, well-structured root system, which can then facilitate increased water and nutrient uptake, thus promoting above ground growth, and producing a strong seedling (Chirino *et al.*, 2008). Those with smaller average root morphology before planting appeared to have lower survival rates, and lower average biomass, despite the lack of statistical significance.

From this experiment, it is concluded overall that larger plant biomass should be the goal of plug plant production, due to improved rates of survival, and overall biomass under field conditions. The best glasshouse practice for encouraging larger plants, are a combination of high growth temperatures of between 20 – 25 °C , higher compaction within the plug cell, although this should be carefully managed, to prevent compaction being too high, and lighting that encompasses all spectra to meet a plant's needs, and at a level between 100 – 250 µmol m^{-2} s^{-1}. When using SonT lighting, care should be taken that lights are not so low as to produce leaf burning or a reduction in leaf elongation. The optimum plug plant should have a root:shoot ratio as close to one as possible, and so any imbalance in growth of above to below ground should be minimized where possible by applying the techniques above. While these techniques may not guarantee homogenous and maximal first year growth, they go a long way to ensuring that field survival rates are increased, and that a plant has the best chance of competing with weeds and with each other in a field monoculture.

It is likely that further work is needed in this area, to assess parameters of growth that have not been measured here, including leaf area, stem diameter, and numbers of new stem nodes at planting time. It would be highly informative to be able to produce properly replicated block trials of several key seedling phenotypes, across a higher range of environments, with greater in depth knowledge of the environmental sites.

7 Optimising control of *Miscanthus* senescence by experimentation with the phytohormones Methyl Jasmonate and Ethephon, at various concentrations

Figure 7.1 The *Miscanthus* annual cycle. Circled is the area of the cycle that will form the focus of this chapter

7.1 Introduction

Miscanthus is a genus primarily originating from a large range of latitudes, although typically tropical, allowing for good species adaptability to different environments (Clifton-Brown *et al.*, 2015). Introduced from Japan in the 1930s it was grown as an ornamental grass in many areas of Europe (Lewandowski *et al.*, 2000). After the observation in the 1930s that a particular hybrid named *Miscanthus x giganteus* (or *Mxg*), the sterile progeny of a naturally occurring cross between two *Miscanthus* species (*Miscanthus sinensis* and *Miscanthus sacchariflorus*), had exceptionally vigorous growth, interest began to grow in the biomass potential of the crop. This eventually led to a research programme initiated in 1989 to investigate the potential of *Miscanthus* as a biomass crop in Europe (Lewandowski *et al.,* 2000). *Miscanthus* has many characteristics that make it particularly well suited as a biomass

crop including high yields, annual harvests, perenniality, and ability to grow on lower value land reducing the competition with food crops and low fertilizer requirements (Atkinson, 2009, Hastings *et al.*, 2008). The *Miscanthus* crop is traditionally harvested in spring, when nutrients have been remobilized to the below ground rhizome and moisture content is low, and the crop is fully senesced (Purdy *et al.*, 2015, Robson *et al.*, 2012). The rhizome is primarily involved in the storage and translocation of nutrients, and is the plant organ that allows perennial regrowth after survival over winter (Lewandowski *et al.,*2000). After harvest nutrients translocated to the rhizome are available for new spring growth (Clifton-Brown and Lewandowski, 2000).

Senescence is the termination of active plant growth, leading to the subsequent initiation of cell death in the plant and is the final developmental stage of plant cells, tissues, organs, or in the case of monocarpic plants results in death of the entire plant (Distelfeld *et al.*, 2014). This process can be seen particularly in autumn months as many trees turn brown and lose their leaves. Annual senescence of aboveground tissues in perennial plants, particularly in temperate climates, is the key to winter survival and spring regrowth (Boersma *et al.*, 2015). Ultimately, senescence is the process, which can determine the control of photosynthesis from the level of individual photosynthetic organs, to a whole plant basis. The overall function of senescence is therefore to maintain the optimal conditions for plant photosynthesis and survival by way of nutrient use efficiency (Robson *et al.*, 2012). It will achieve this by removing the nutrients given to leaves deemed no longer of photosynthetic benefit, and re-assimilating the nutrients to leaves more likely to achieve good net productivity, for example leaves higher up the canopy (Robson *et al.*, 2012). Once senescence has been initiated the next stage is mobilization of nutrients from the senescing parts to the developing sinks, such as seeds, grains or plant rhizome (Distelfeld *et al.*, 2014). The timing of this process determines where the mineral and nutrient deposits will predominantly be and when (Boersma *et al.*, 2015). In perennials such as *Miscanthus*, the process of senescence also allows for the survival of the crop over winter because the nutrient and photosynthate is remobilized into the storage organs for use in the next growth season.

7.1a Control of senescence

Timing and control of senescence is a difficult trait to study. It is likely to be modified and altered by a number of physiological processes, abiotic stresses and environmental stimuli

(Leopold, 1961). These include metabolite and hormone regulation on a genotype specific basis, external stresses such as drought, biotic stress from pests, and possibly more importantly the changes in the surrounding environment in terms of day length and temperature (Noodén *et al.*, 2004, Jibran *et al.*, 2013, Gregersen *et al.*, 2013).

The majority of crop plants go through three development phases from germination to death or dormancy; an expansion phase, a maturity phase and finally the senescence phase (Munné-Bosch, 2008). In many species the beginning of senescence can be linked to the maturation and development of reproductive organs such as seeds or grains. As a static organism, incapable of relocating itself if conditions are not favourable, plants have developed other ways to attempt to survive adverse environmental conditions. During a growth season, exposure to less favourable external conditions occurs frequently, a phenomenon associated with great losses in productivity (Gregerson *et al.,* 2013). This is frequently known to induce senescence in leaves especially. Induction of senescence can be triggered by plant hormones synthesized during plant stress, including Abscisic acid (ABA), Jasmonic acid (JA, and Salicylic acid (SA) (Peleg and Blumwald, 2011). Shaded and dark environments are an important trigger of senescence of lower leaves in the canopy. This is beneficial to the plant as the leaves lower down the canopy will not be able to perform photosynthesis efficiently, so assimilates and nutrients are re-mobilized to the upper plant parts (Gregerson *et al.,* 2013). Water stress is responsible for a large percentage of crop loss worldwide, leading to the development and selection of more drought hardy genotypes for many crop plants. Prolonged drought stress could be a cause of early leaf senescence, possibly leading to premature whole plant senescence. Higher or lower light and heat intensities can also induce senescence. Plants have therefore developed ways for surviving low winter temperatures. In perennials this can be seen as an annual cycle of active meristematic growth, interspersed with period of dormancy which reduces the negative effects of low temperature, unfavourable temperature or light balance, which could affect photo-respiration, leading to tissue injury or whole plant death (Atkinson *et al.*, 2013).

7.1b *Miscanthus* and overwintering

Sustainability of the *Miscanthus* crop relies upon the plants undergoing effective senescence prior to the winter period. A lack of senescence results in reduced nutrients available for new growth or ultimately complete death due to colder winter temperatures, a concern of great

importance in more temperate climates. It is imperative that plants are harvested when the crop is sufficiently dry and nutrients relocated away from the harvested aerial biomass. If this is not completed properly, adverse effects on transport and thermal conversion efficiency as well as increasing the risk of post-harvest spoilage may occur (Robson *et al.,* 2012). The intricacies of combustion properties and the influence of agronomy on the final product are described thoroughly in a paper by Baxter *et al.* (2014). In addition to testing fertilizer inputs during growth, and their effects on combustion quality, they conclude that later harvested *Miscanthus* samples have improved fuel quality, due to lower nutrient contents. Effective senescence in addition to optimal fertilizer additions is the primary driver for this improved quality. Higher levels of elements such as Cl, S, K and Fe are responsible for increased ash content, which leads to slagging, fouling and corrosion of the combustion system (Baxter *et al.*, 2014). Effective dry down and reduced moisture content is also important for improving the calorific value of the biomass, reducing the risk of microbial breakdown of the biomass when stored in bales, and reduces bale weight (Clifton-Brown *et al.*, 2017)

Due to the genetic basis for the control of senescence in cereal crops, it has been selected as a suitable trait for genetic selection of *Miscanthus* plants, in an attempt to improve the germplasm of commercially used genotypes. The onset and rate of senescence can influence some key agronomic traits including yield by impacting growth duration (Distelfeld *et al.*, 2014). Delayed senescence in 'stay green' varieties may allow for a longer growing season and greater drought resistance, subsequently increasing yield accumulation over the season, but also carries the risks of reduced nutrient use efficiency and crop quality as a result of a non-senesced offtake, due to the issues regarding spoilage and ash content described above (Robson *et al.*, 2012). Earlier senescing genotypes may over winter better and have better quality yield but could result in lower the amount of accumulated biomass yield at harvest.

Ideally harvested *Miscanthus* biomass would have low N and P, which would reduce the need for subsequent fertilizer inputs (Lewandowski and Heinz, 2003). Material should also have low K and Cl for reduction of corrosion risk in boilers and low Si and ash contents (Jensen *et al.*, 2017). Autumn senescence has been proved to reduce all of these variables, and improve overall combustion quality, as well as over winter survival (Mos *et al.*, 2013).

Temperate climates are characterized by a seasonal phase, whereby suboptimal temperatures can restrict and terminate plant growth (Atkinson *et al.*, 2013). The sub-tropical

origins of some *Miscanthus* genotypes means they do not senesce properly and are vulnerable to loss over winter (Scally *et al.*, 2001). However, senescence in *Miscanthus* is not just affected by geographical origin but also appears to be greatly affected by stand age. A study in Iowa in 2009, 2010 and 2011 assessed senescence parameters in first, second and third year stands of *Miscanthus x giganteus* (Boersma *et al.*, 2015) and found that at the end of the growing season, first year plants retained more photosynthesis and leaf N concentration in comparison to the third year stands. This retention of leaf activity after year 1 growth suggests that young plants do not complete senesce and re-assimilate nutrients before the first killing frosts, significantly reducing successful overwinter dry down and thus increasing the chances of plant injury or death to cold temperatures (Boersma *et al.,* 2015). Importantly senescence appeared normal after growth seasons in year 2 and 3. Early studies of overwintering in *Miscanthus x giganteus* across Europe concluded observations of poor overwintering in the first year at some locations in Northern Europe, but better overwintering in other areas was not correlated with extent of early senescence (Clifton-Brown and Lewandowski, 2000). The study proved the importance and potential for identification of genotypes with improved overwinter survival.

Interactions between a species specific developmental programme and external environmental signals are the combination that ultimately determines the onset of leaf senescence (Jibran *et al.*, 2013). It is hypothesised that the developmental programme in a mature *Miscanthus* stand will induce senescence earlier in the season than a first year stand due to more developed rhizome. It is therefore, important to experiment with potential ways to trigger plant senescence in the first growth season, when natural senescence processes appear to be slower. It is hypothesised that once the plants have survived the first winter an earlier senescence, as indicated by Boersma *et al.,* 2015, and improved winter survival in subsequent years will mean no further manipulation will be required and genotypic control of senesce will be sufficient. Manipulating growth conditions is impossible under field conditions but it is practical to apply inducers such as plant growth regulators.

Senescence processes are influenced by several phytohormones, with cytokinins and ethylene having the most well-known roles in the delay or induction of senescence respectively, but other hormones such as Abscisic acid, auxin, jasmonic acid and salicylic acid also affect the process (Schippers *et al.*, 2007). However, it is difficult to establish the

intricacies of how different hormones regulate the onset and progression of leaf senescence (Jibran *et al.*, 2013). In this study, two *Miscanthus* genotypes known to exhibit slow senescence rates during autumn, were treated with foliar hormonal plant growth regulatory treatments of various concentrations of Methyl Jasmonate or ethephon (the liquid form of ethylene) to assess effects, if any, on senescence and late season growth.

Artificial plant growth regulators (PGRs) are synthetic compounds which are used to alter the morphological structure of plants, usually by way of reducing cell elongation and/or altering the rate of cell division. These compounds usually work as antagonists for example of gibberellins and auxins, and have been used by farmers with many different types of crop (Rademacher, 2000). PGRs have a number of practical application uses, such as reducing lodging after high rain or winds, compacting fruit trees or other ornamental species, and encouraging ripening (Rademacher, 2000). Growth regulators are classed into two major groups; ethylene releasing compounds such as ethephon, and inhibitors of gibberellin biosynthesis. Ethylene is a plant hormone known to influence many aspects of plant growth and development, including forms of programmed senescence (Grbić and Bleecker, 1995). Many plant stresses result in increased ethylene synthesis including cold stress which increase levels of 1 aminocyclopropane – 1 carboxylic acid (ACC) synthase activity, which is a precursor of ethylene (Wang, 1989). Increased levels of ethylene are known to induce leaf senescence through chlorophyll degradation. Ethylene is a gaseous hormone but liquid ethephon elicits ethylene related responses such as fruit ripening by metabolizing the active ingredient to ethylene within the plant tissues (Diesburg, 1999).

Jasmonic acid (JA) and the derivative methyl jasmonate (MeJa) are known to promote leaf senescence when applied exogenously (Creelman and Mullet, 1997) and were first identified as senescence promoters in detached oat (*Avena sativa*) leaves in 1980 (Ueda and Kato, 1980). The effects of exogenous application of this hormone have been reported extensively across a range of species, with many positive effects reported in areas such as fruit ripening and abscission (Khan and Singh, 2007, Mukkun and Singh, 2009), flower senescence (Porat *et al.*, 1993) and induced senescence in the flag leaves, and ears of wheat (Beltrano *et al.*, 1998). Exogenous applications of varying concentrations of methyl jasmonate have a regulatory and often inducing effect on senescence in both monocots and dicots, but responses may be species specific (Herrmann *et al.*, 1989).

7.1c Aims and objectives

The aims of this section of the project were to discover a method of inducing senescence in *Miscanthus* that would allow manipulation of senescence timing in field grown crops. This would allow senescence to be induced in plants in more northern latitudes, leading to increased survival and effective overwintering and spring re-growth. Methods of inducing senescence should be scalable and practical under field conditions. Therefore, it was decided to test variable concentrations of the hormones jasmonic acid and ethylene described above, to be applied as a spray to experimental plants grown under polytunnel conditions.

7.2 Experiment 1 – The effects of variable concentrations of exogenously applied Methyl Jasmonate and Ethephon on senescence rates of *Miscanthus* genotype Mx 2468 in 2017

7.2a Methods

The first experiment used clonally propagated two year old accessions of the *Miscanthus* hybrid Mx2468 (GNT13) to test exogenous applications of Methyl Jasmonate and ethephon at a range of concentrations from low to high.

Germplasm and growth conditions

The genotype chosen for this assessment was a hybrid cross of *Miscanthus floridulus* and *Miscanthus sinensis* known within the institute as Mx 2468 or 'GNT13'. This genotype has been trialled in observation nurseries within field trial conditions and has been described as having promising growth parameters but poor ability to senesce in autumn in the UK. Subsequent biomass production from GNT13 was low due to the resulting poor overwinter survival. Established plants were used that were at the start of their second growth season due to a lack of viable new seed for the hybrid at the start of the experiment. The plants were re potted from 3-inch pots into 9-inch pots at the start of the second growth year (2017), with standard John Innes compost, and were grown in a polytunnel for the rest of the growth season under natural day length conditions. Watering was on an as needed basis, which was often daily on warmer days.

Baseline measurements and design

Plants were arranged in 6 replicate blocks containing 9 plants in a 3 x 3 grid, with the nine treatments (eight hormonal variables and one control) assigned to each of the plants at random (Figure 7.2). Plants were placed in the design from early July onwards. From this point, all plants were measured on a fortnightly basis, which continued after the application of hormones. A single tallest stem was chosen from each plant. A label was tied around the fifth leaf from the base of the stem, to signify the experimental stem, the plant ID, and to identify the leaf to aid in further leaf counts. At each measurement time point, the stem was measured for height, from the base of the plant to the newest leaf ligule. On the same stem, all leaves were assigned a number from a senescence score from 1 – 4. Leaves assigned 1 would have 0-10% senescent brown leaf visible. 2 was between 11-50% senescent brown leaf.

A score of 3 was between 51 – 80% senescent brown leaf, and 4 was 80% + almost entirely senesced to completely senesced leaf (Figure 7.3). The youngest fully expanded leaf was assessed using a chlorophyll meter SPAD 502 plus Konica Minolta, Osaka Japan). Assessment of chlorophyll level was taken at the proximal, middle, and distal end of the leaf and the values averaged for each leaf.

Block 1		
Plant 1 Ethephon Dilution 3	Plant 6 MeJa Dilution 2	Plant 7 Ethephon Dilution 1
Plant 2 MeJa Dilution 3	Plant 5 Ethephon Dilution 2	Plant 8 Ethephon Dilution 4
Plant 3 Control	Plant 4 MeJa Dilution 4	Plant 9 MeJa Dilution 1

Block 2		
Plant 1 Ethephon Dilution 1	Plant 6 Ethephon Dilution 4	Plant 7 Ethephon Dilution 2
Plant 2 Control	Plant 5 MeJa Dilution 1	Plant 8 Ethephon Dilution 3
Plant 3 MeJa Dilution 2	Plant 4 MeJa Dilution 4	Plant 9 MeJa Dilution 3

Block 3		
Plant 1 MeJa Dilution 1	Plant 6 MeJa Dilution 2	Plant 7 MeJa Dilution 4
Plant 2 MeJa Dilution 3	Plant 5 Ethephon Dilution 1	Plant 8 Ethephon Dilution 3
Plant 3 Ethephon Dilution 3	Plant 4 Ethephon Dilution 2	Plant 9 Control

Block 4		
Plant 1 Ethephon Dilution 2	Plant 6 MeJa Dilution 4	Plant 7 MeJa Dilution 1
Plant 2 Ethephon Dilution 4	Plant 5 MeJa Dilution 2	Plant 8 Ethephon Dilution 1
Plant 3 Control	Plant 4 Ethephon Dilution 3	Plant 9 MeJa Dilution 3

Block 5		
Plant 1 MeJa Dilution 2	Plant 6 MeJa Dilution 1	Plant 7 MeJa Dilution 4
Plant 2 Control	Plant 5 Ethephon Dilution 1	Plant 8 Ethephon Dilution 4
Plant 3 MeJa Dilution 3	Plant 4 Ethephon Dilution 3	Plant 9 Ethephon Dilution 2

Block 6		
Plant 1 Ethephon Dilution 4	Plant 6 Control	Plant 7 Ethephon Dilution 3
Plant 2 MeJa Dilution 2	Plant 5 Ethephon Dilution 1	Plant 8 MeJa Dilution 4
Plant 3 MeJa Dilution 3	Plant 4 MeJa Dilution 1	Plant 9 Ethephon Dilution 1

Figure 7.2 Polytunnel experimental design for Mx2468 plants in 2017. Six replicate blocks with 9 plants arranged randomly in 3 x 3 grid. Each of the 9 plants was assigned one of the 9 treatments of Ethephon or Methyl Jasmonate (including control treatment with solution lacking active ingredients).

Applications

Plants were sprayed with one of eight treatment applications. Two hormones at four different concentrations each. The hormone ethephon was purchased locally from a farming supplier, under the agricultural name 'Padawan'. This solution was a soluble concentrate formulation containing 480 g/l (39.6% w/w) ethephon, (2- chloroethylphosphonic acid), and is typically used as a growth regulator for use in winter and spring varieties of barely and winter wheat, rye and triticale. The solution was diluted to four concentrations of 2g, 1g, 500mg and 250mg L^{-1} of ethephon. The second hormone was Methyl Jasmonate (MeJa, synonym: 3-Oxo-2-(2-pentenyl)cyclopentaneacetic acid, methyl ester, Methyl 3-oxo-2-(2-pentenyl) cyclopentaneacetate) at 95% concentration (Sigma Aldrich). Four dilutions were prepared of 200, 100, 50 and 25 µM MeJa L^{-1} in water in a fume hood. Each hormone dilution was applied to the plants using an 800ml spray bottle. Plants were sprayed in October and were moved into well-spaced groups of the same treatment before spraying to avoid cross contamination. Full personal protective equipment was worn to apply the treatments. Each plant was sprayed with the solution until leaves were dripping. Plants were then left for 48 hours to allow dissipation of any remaining spray in the air, before being placed back into the experimental design.

Plant harvest

After the winter dormancy period, each plant was harvested for both above and below ground biomass. Plants were removed from their pots and the soil carefully washed off the roots and rhizome. Stems were cut away from the roots with secateurs as close as possible to the base. All above ground material was placed into a clear plastic porous packets and weighed. Below ground material was placed in net sacks and weighed. All samples were then taken to a large drying oven and dried at 105°C until a constant dry weight was reached. All samples were then weighed again and moisture contents calculated.

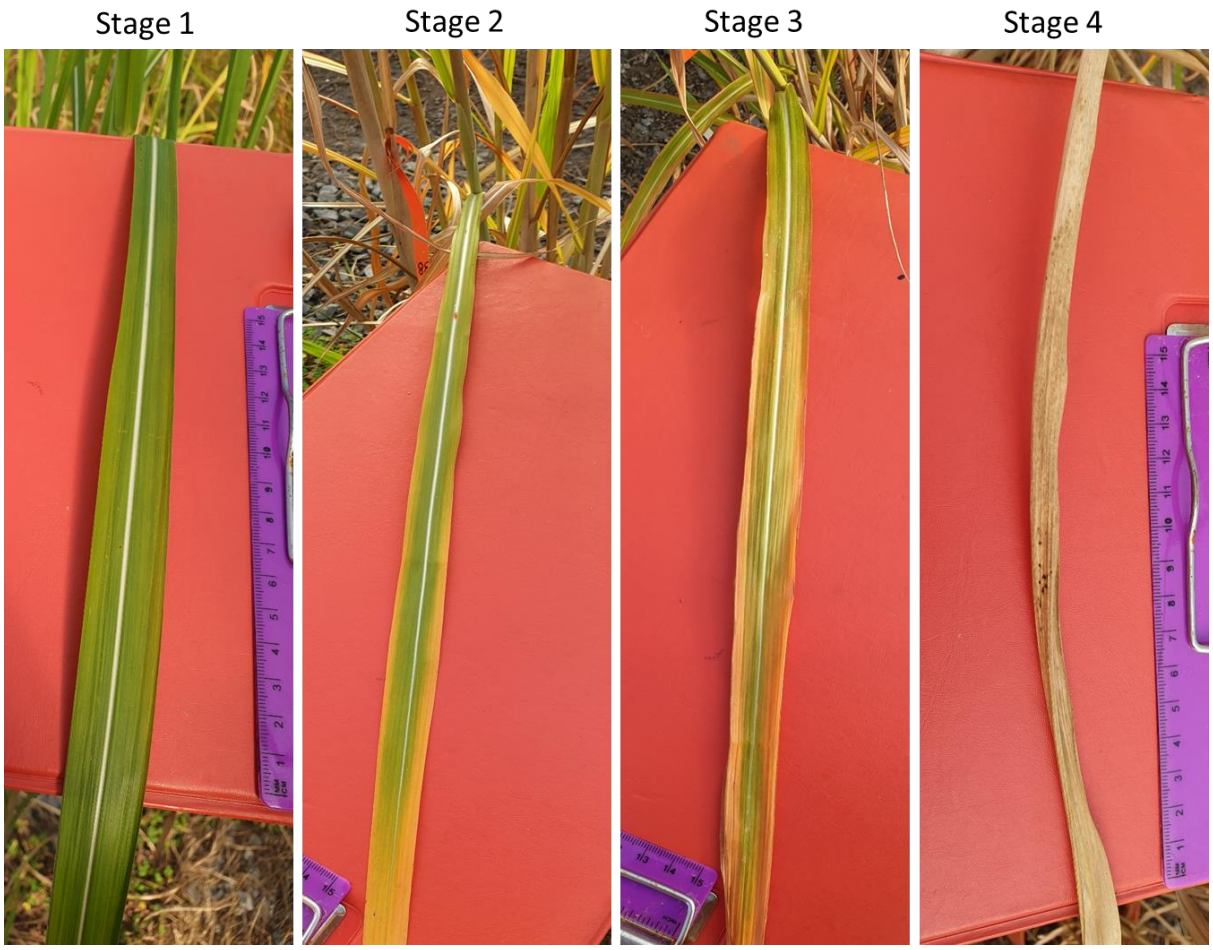


Figure 7.3 Grading of *Miscanthus* leaf senescence stage 1 – 4 shown visually from L – R. Representative leaves for each grade. 1 = 0 – 10% senesced. 2 = 11 – 50% senesced. 3 = 51 – 90% senesced. 4 = 91% + senesced.

7.2b Results - Experiment 1 – effects of four concentrations of Methyl jasmonate and four concentrations of Ethephon on 2-year-old GNT13 hybrids in 2017

Statistical analysis of variation within treatment groups prior to any treatment being applied showed no significant difference in baseline assessments of plant height or SPAD relative chlorophyll content reading in July 2017 ($p>0.05$).

The percentage of totally senesced leaves increased over time but there was no significant difference between treatments for either ethephon treated plants or Methyl Jasmonate (results of ANOVA $p>0.05$ for both hormones) (Figure 7.4 A & B). Percentage of totally senesced leaves increased steadily from between 30-45% for all treatments in mid-July, to 58-72% by the end of August. After application of solutions in October, all treatment groups displayed between 74 – 90% completely senesced leaves, the largest amount seen in the control group with 90% in early November (s.e - 2.9), and the lowest in the third Methyl Jasmonate group with 73% senesced leaf (s.e - 4.27). By the middle of January all groups reach similar averages for all treatments of 89-99% complete senescence.

There was no significant difference in height within ethephon groups or Methyl Jasmonate groups at any time point (ANOVA between groups $p > 0.05$). Height increased slightly over time for all treatments, although more obviously so in the group treated with the highest strength ethephon, reaching a peak of approximately 62 cm on average but with a large error margin (s.e - 7.07). Other groups reach an average maximum height of 47 – 56 cm by the end of 2017, with no significance found between any groups ($p> 0.05$). There were no significant differences for relative chlorophyll content (SPAD) between either ethephon or Methyl jasmonate treatment groups (Results of ANOVA $p > 0.05$) (Figure 7.5 C&D). A typical reduction in greenness over time was observed, from the first assessments in July until the final assessments in early January of 2018, but decrease in chlorophyll content over time was comparable across all treatment groups, the control group remaining consistently lower than all treated groups, although not significantly so. The reductions in green leaf and chlorophyll content over time in the plants can be seen visually in Figure 7.6.

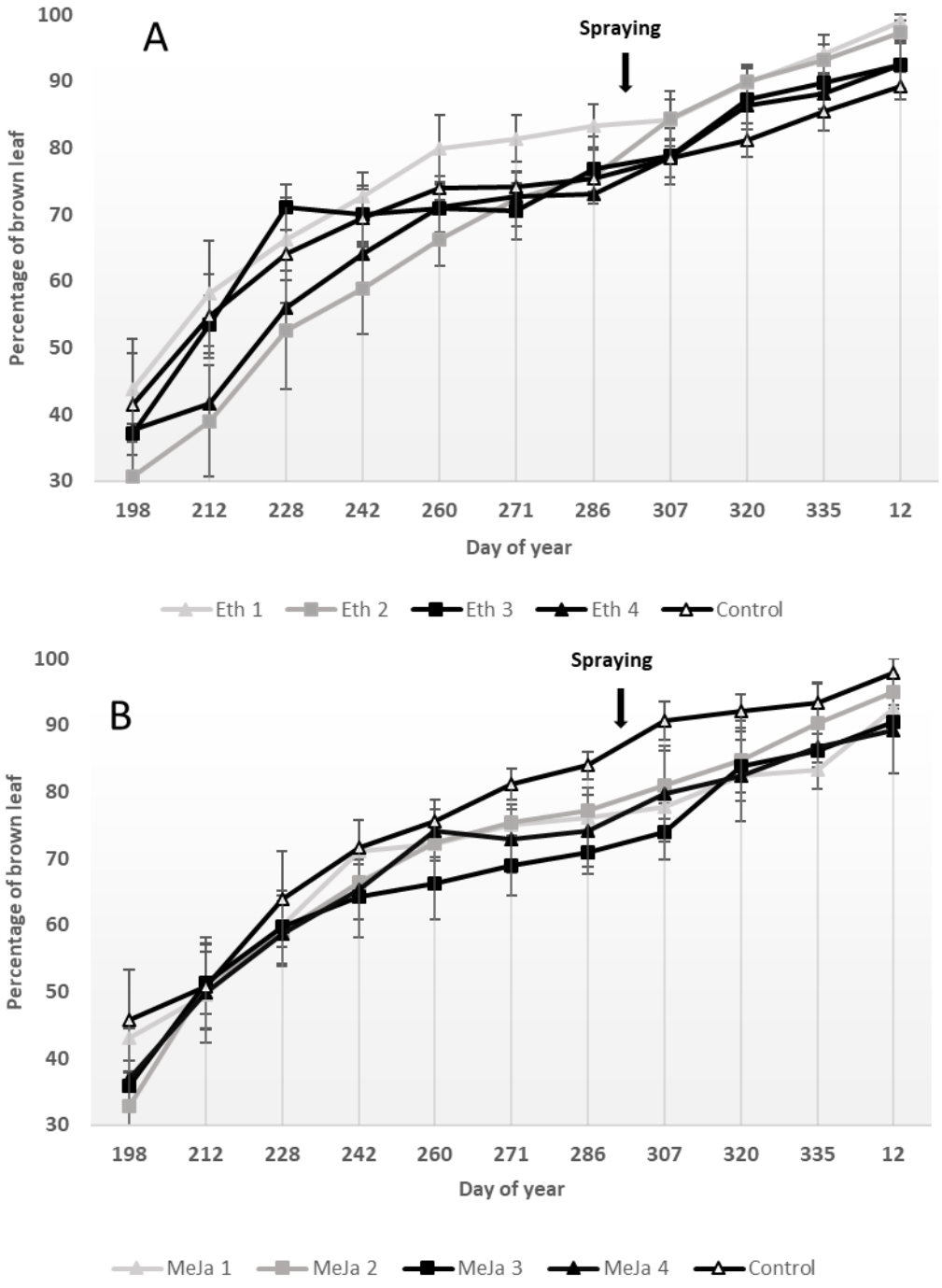


Figure 7.4 Percent of completely senesced leaves over time (labelled as grade 4) for Ethephon (A) and Methyl jasmonate (MeJa) (B). Ethephon applications are labelled 1 – 4 from lowest concentration to highest (250mg/L, 500mg/L, 1000mg/L and 2000mg/L). Methyl jasmonate applications 1 – 4 are from lowest concentration to highest (25µM, 50µM, 100µM and 200µM Methyl jasmonate solution). Lowest concentration for each hormone is shown in grey with grey triangles. Second lowest shown in darker grey with grey squares. Second highest is black with black squares. Highest concentration is black with black triangles. Control is black with open triangles and is the same group for both solutions. Spraying time is shown on all charts, and was done 25th Oct 2017 (day of year 298). For all time points N=6, with StdE.

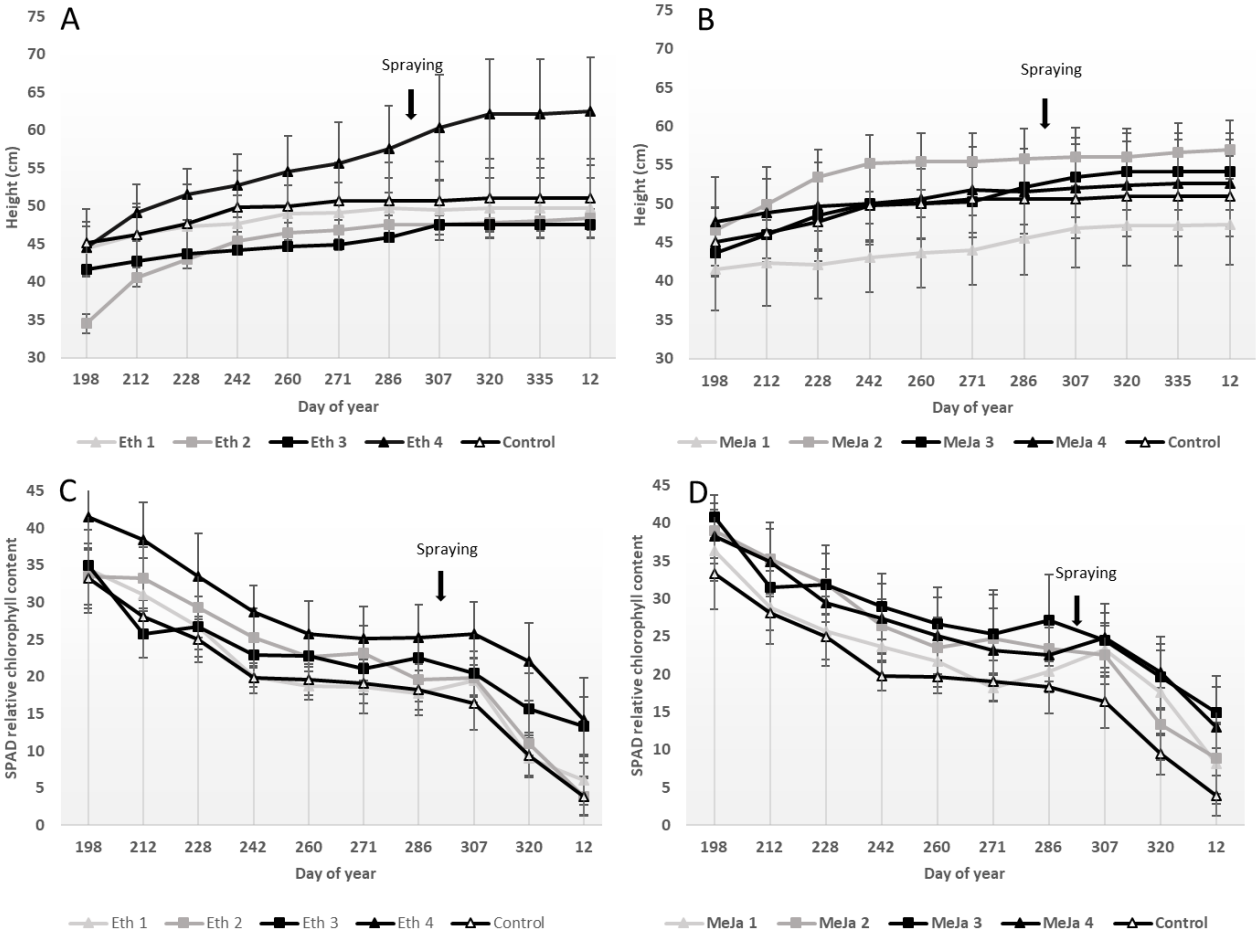


Figure 7.5 Height increase from summer to winter of a selected stem for Ethephon (A) and Methyl jasmonate (B), Average SPAD score over the same time scale for Ethephon (C) and Methyl jasmonate (D). Ethephon applications are labelled 1 – 4 from lowest concentration to highest (250mg/L, 500mg/L, 1000mg/L and 2000mg/L). Methyl jasmonate applications 1 – 4 are from lowest concentration to highest (25µM, 50µM, 100µM and 200µM Methyl jasmonate solution. Lowest concentration for each hormone is shown in grey with grey triangles. Second lowest shown in darker grey with grey squares. Second highest is black with black squares. Highest concentration is black with black triangles. Control is black with open triangles and is the same group for both solutions Spraying time is shown on all charts, and was applied 25th Oct 2017 (day of year 298). At each time point N = 6 with Std E.

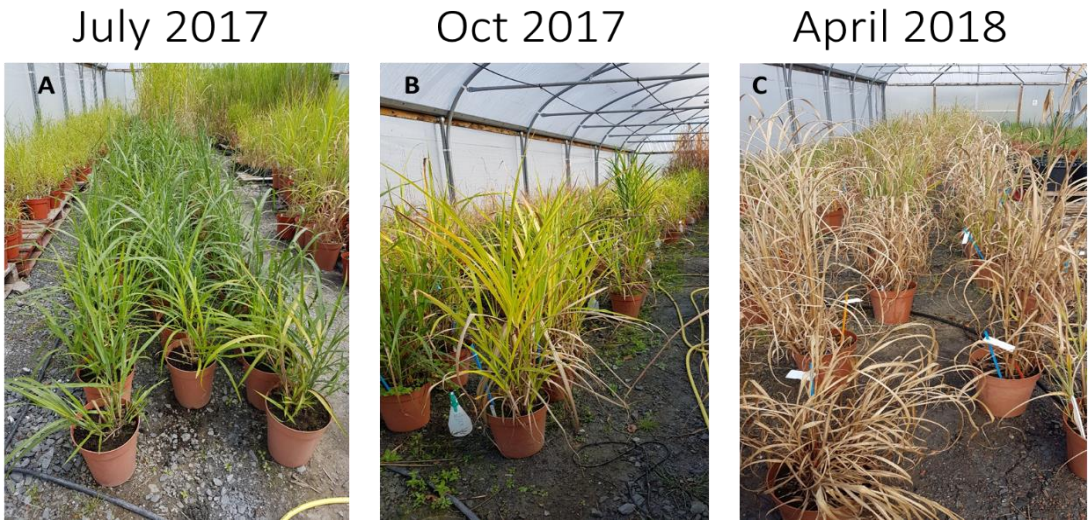


Figure 7.6. Senescence progression of the experimental blocks over 9 months. Photographs of experimental plants taken before treatment in July 2017 (A), 3 days post treatment in Oct 2017 (B), and the next spring (2018) (C).

Harvest of experimental plants in spring 2018

There were no significant differences between the above ground biomass within ethephon or Methyl jasmonate treatment groups ($p > 0.05$ for both) (Figure 7.7A). There was no significant difference in moisture content within thephon or Methyl jasmonate treatment groups ($p > 0.05$ for both) (Figure 7.7C). There was no significant difference in the below ground biomass within ethephon or Methyl jasmonate treatment groups ($p > 0.05$ for both) (Figure 7.7B), or moisture content ($p > 0.05$ for both) (Figure 7.7D).

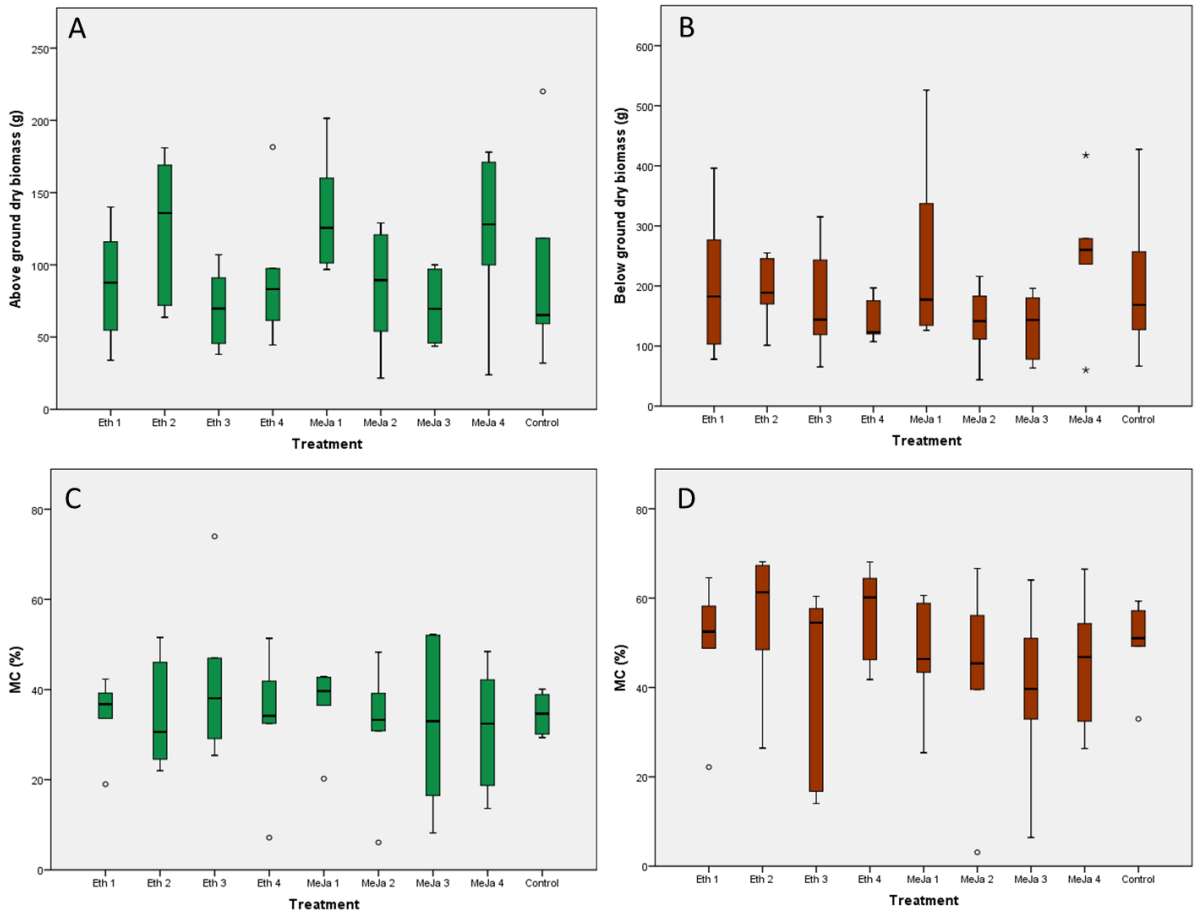


Figure 7.7 Results of above ground biomass (A) and moisture content (C), and below ground biomass (B) and moisture content (D) during the harvest of all experimental plants in April 2018 the spring after treatment in October 2017 with varying concentrations of Ethephon from low to high (Eth 1 – 4) and Methyl Jasmonate low to high (MeJa 1 - 4) with same control group for comparison with both hormones. N = 6 for each result. Circles denote outliers, and asterisks denote extreme outliers.

7.3 Experiment 2 – The effects of exogenous applications of three concentrations of Methyl Jasmonate and Ethephon on the growth and vigour of young seedlings of *Arabidopsis thaliana* type Colombia, and *Miscanthus* GNT14.

Experiment 2 was designed in order to quell doubts about the potential efficacy of the solutions used in experiment 1. It was theorized that the older *Miscanthus* plants used in experiment 1 were likely bolstered by the presence of well-formed rhizome, and as such were unaffected by the exogenous solutions applied. However, it could not be ruled out that the solutions themselves may have been ineffective or the concentrations far too low to have any effect on such large plants. Therefore, it was decided before proceeding to test stronger concentrations on a new population of large *Miscanthus* plants, it would be useful to test the concentrations on young seedlings, which as yet had little to no rhizomatous maturity, and should be susceptible to active ingredients in the solutions. The decision was made to include a population of *Arabidopsis* plants alongside the *Miscanthus* seedlings, due to the popularity of using *Arabidopsis* as a 'model plant'. It was assumed that even if the solutions had no effect on *Miscanthus* species of any age, they would likely produce some effect on *Arabidopsis*, thus helping to reach a conclusion as to whether the solutions were active. *Arabidopsis thaliana* has been the subject of many studies of exogenous hormone application (Chen *et al.*, 2017, Chang and Stadler, 2001, Gazzarrini and McCourt, 2003).

Plant material

Seeds of *Arabidopsis thaliana* type Col 1 and *Miscanthus* genotype GNT14, were sown onto compost in a petri dish and germinated at 25°C in a growth cabinet. After approximately 1 week all seedlings were transplanted using tweezers into square 3" pots into standard John Innes compost. All plants were then placed into a controlled glasshouse environment at approx. 18 – 25°C night/day cycle. Lighting was natural lighting with SonT supplemental glasshouse lights positioned approximately 1.5m above the plants.

Experiment design

To encompass two species and seven treatment variables, a matched pairs block design was chosen, whereby five experimental blocks of seven plants was doubled to encompass the

Miscanthus experimental block, alongside an *Arabidopsis* experimental block. Treatments were randomly assigned to one plant out of seven within the block (Figure 7.8).

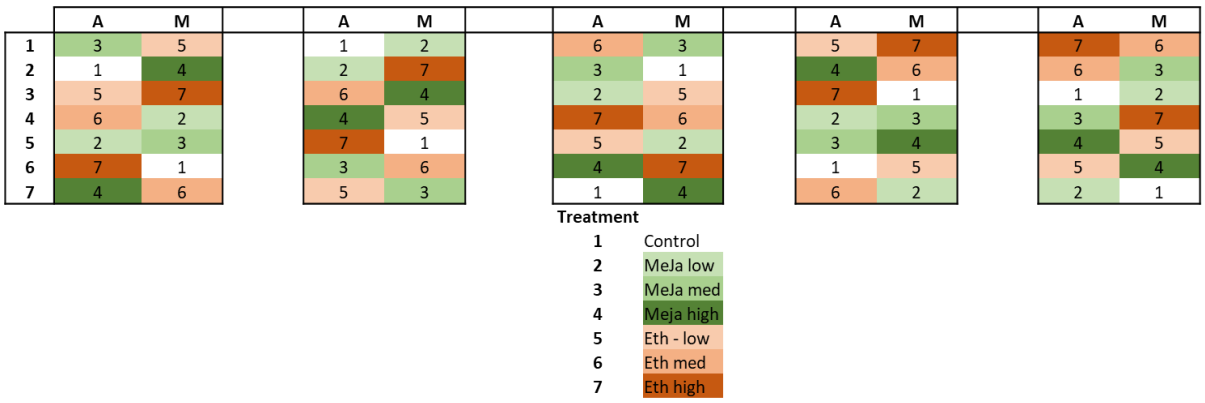


Figure 7.8 Glasshouse design for *Arabidopsis* and *Miscanthus* seedlings treated with one of 6 hormonal applications or a non-treated control. Treatments are colour coded with a key included below. A - *Arabidopsis* column. M - *Miscanthus* column

Plants were sprayed with one of six treatment applications. The same hormones used in experiment 1 were used here but at higher concentrations. The hormone ethephon was diluted from the stock 480g L^{-1} to three concentrations 'low' concentration of 5g L^{-1}, 'medium' concentration of 10g L^{-1}, and 'high' concentration of 20g L^{-1} of ethephon. The second hormone, Methyl Jasmonate (MeJa) was diluted to three concentrations 'low' 200µM L^{-1}, 'medium' 500 µM L^{-1} and 'high' 1000 µM L^{-1} MeJa in water. Once all plants were approximately 3 weeks old, they were taken out of the block design and grouped into treatments in an aerated unused glasshouse environment, with sufficient space between treatment groups to avoid cross contamination. Plants were sprayed until the leaves were dripping, and then left for 48 hours to allow excess vapour to dissipate, before being placed back into the original block design (Figure 7.8).

Plants were measured the day before treatment and then weekly afterwards for 1 month. Measurements for *Miscanthus* seedlings and *Arabidopsis* seedlings differed due to morphological differences. *Miscanthus* seedlings were assessed for height by measuring from the base of the plant, to the newest ligule of the tallest stem. Chlorophyll assessments were taken by using a SPAD meter on the newest fully expanded leaf at three points along the

blade, averaging the results. Stem number was also recorded, adding in any basal node that supported its own leaf. For *Arabidopsis* plants, flowering began sooner than expected. Therefore, measurements were taken once prior to treatment which included rosette radius along the longest angle, and whether flowering was beginning. Photographs were also taken of each plant the day before treatment and 4 days afterwards, using a Canon DSLR camera. Measurements for *Arabidopsis* only continued until a treatment effect was seen, to test the efficacy of the hormonal applications, and ensure the chemicals were active.

7.3a Results of Experiment 2 – testing the efficacy of new solution concentrations of Ethephon and Methyl jasmonate (MeJa) on young *Miscanthus* and *Arabidopsis* seedlings

Assessments of stem number in the *Miscanthus* seedlings showed no significant treatment effect at 7 days post spraying, for any treatment (Figure 7.9C). By 20 days post spraying the highest concentration of ethephon had significantly lower stem number (<0.05) than all other groups with the exception of the medium concentration of ethephon (Figure 7.9B). The lowest concentration of ethephon had significantly higher stem number ($p < 0.05$) than all other treatment groups, with the exception of the control treatment. After a month (Figure 7.9A), no significant differences were seen in the number of stems between treatment groups.

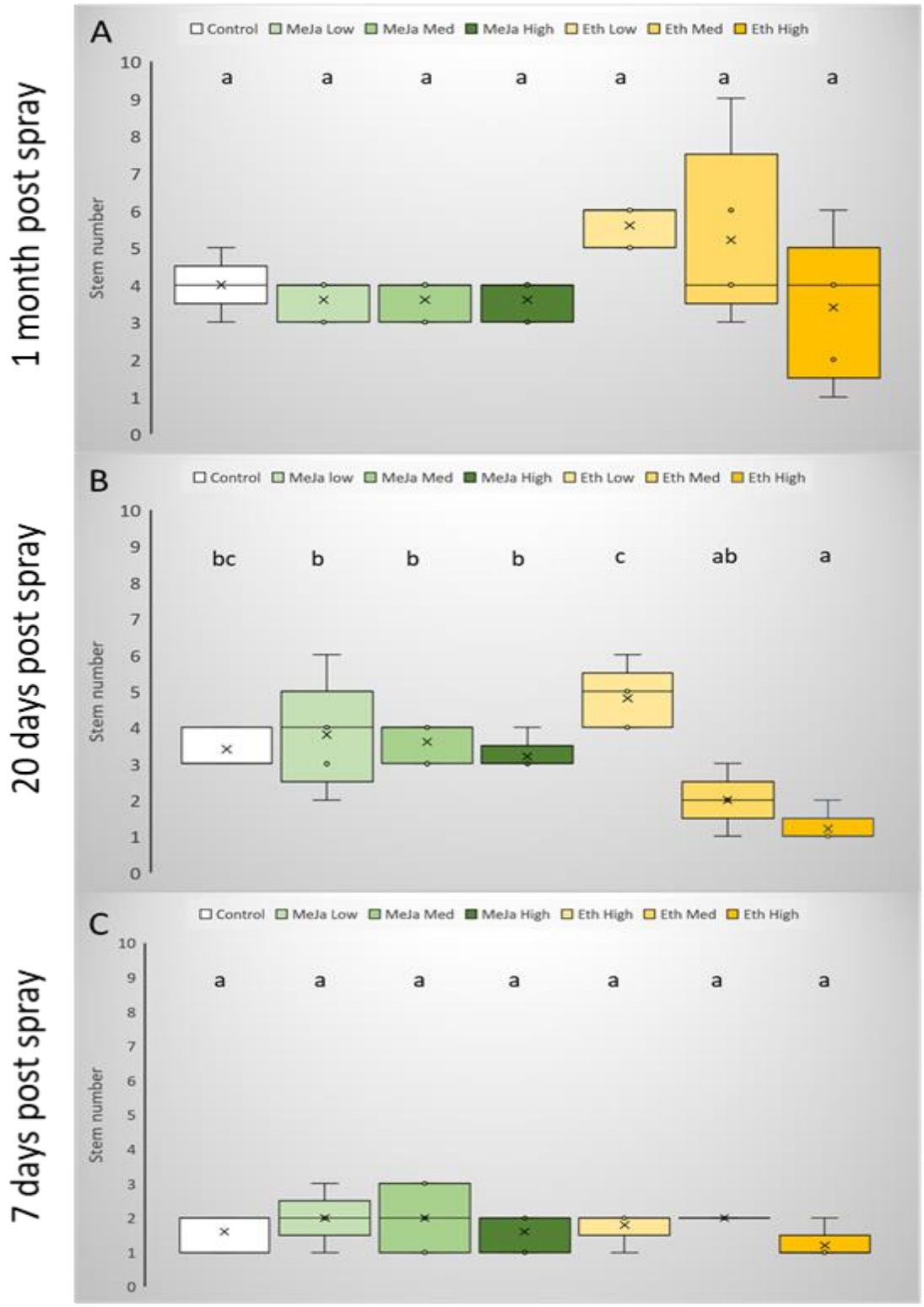


Figure 7.9. Stem number of *Miscanthus* at 7 days post spraying (C), 20 days post spraying (B), and a month post spray (A) with one of six hormonal applications of ethephon low to high (Eth – orange) or Methyl jasmonate low to high (MeJa - green) and control group (white). N = 5, statistical analysis was undertaken using ANOVA, subgroups were assigned using Tukeys HSD post hoc testing.

Plant height assessments in the *Miscanthus* seedlings revealed no significant differences between average plant heights of any groups (Figure 7.10A). After 7 days, the plants treated with ethephon had reduced elongation, with the low and high treatment being significantly shorter than the control group and all MeJa treatments ($p < 0.05$). The medium concentration was also lower than all MeJa groups and the control, but was only significantly lower than the medium MeJa treatment (Figure 7.10B). After 20 days, all ethephon treated plants were significantly shorter than all MeJa groups and the control ($p < 0.05$). There was no significant difference seen between the MeJa and control groups ($p > 0.05$). After 1 month, the significantly shorter stem height average in the ethephon groups remained and post hoc testing placed them into the lowest height subset (a), although the lowest concentration was also in subset b, alongside all MeJa and control groups, with the exception of the highest MeJa concentration, placed into the highest subset 'c' alone.

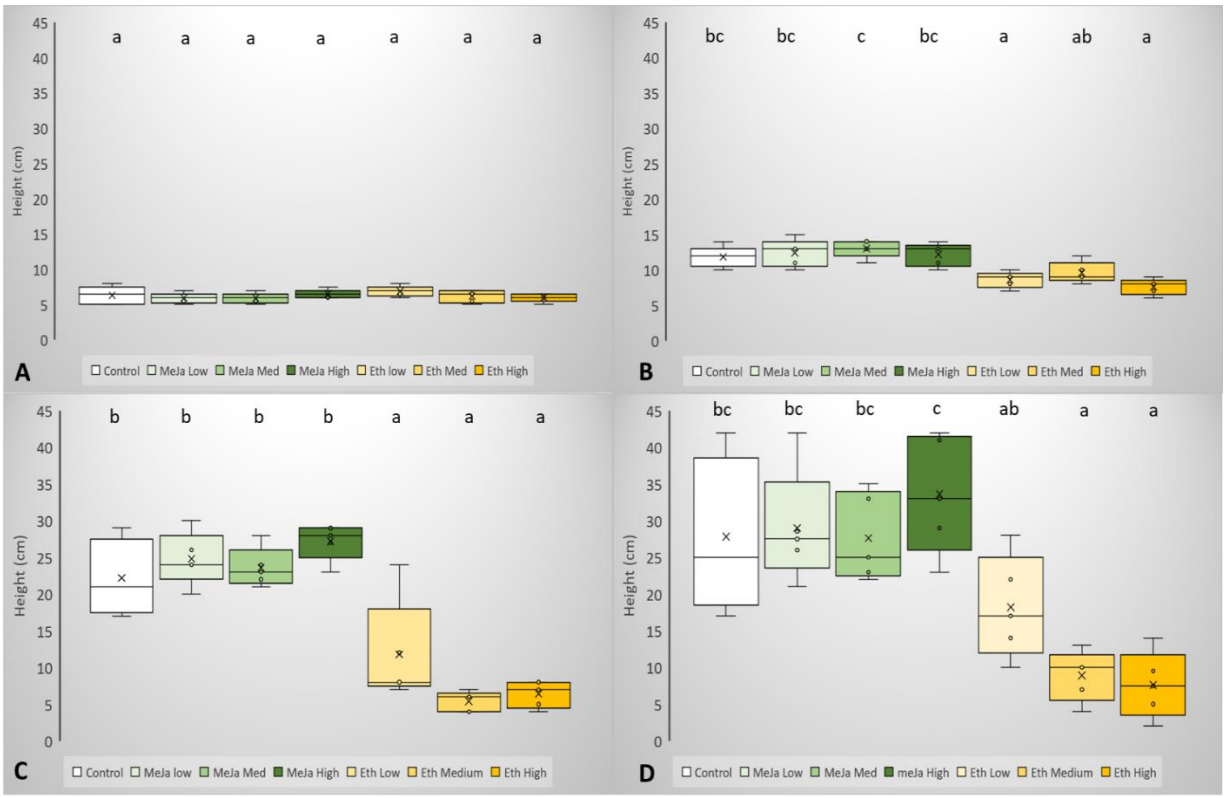


Figure 7.10 Height of tallest stem of *Miscanthus* prior to spraying (A) 7 days post spraying (B), 20 days post spraying (C), and a month post spray (D) with one of six hormonal applications of ethephon low to high (Eth – orange) or Methyl jasmonate low to high (MeJa - green) and control group (white). N = 5, statistical analysis was undertaken using ANOVA, subgroups were assigned using Tukeys HSD post hoc test

Assessment of greenness over all populations was not significantly different prior to treatment ($p > 0.05$) (Figure 7.11A). After 7 days post spraying (Figure 7.11B), the highest ethephon concentration group had significantly lower greenness scores than the lowest ethephon group and medium MeJa group. Variability in greenness scores increased in most groups at 20 days (Figure 7.11C) and a month (Figure 7.11D) post treatment, over most populations and no significant differences were observed.

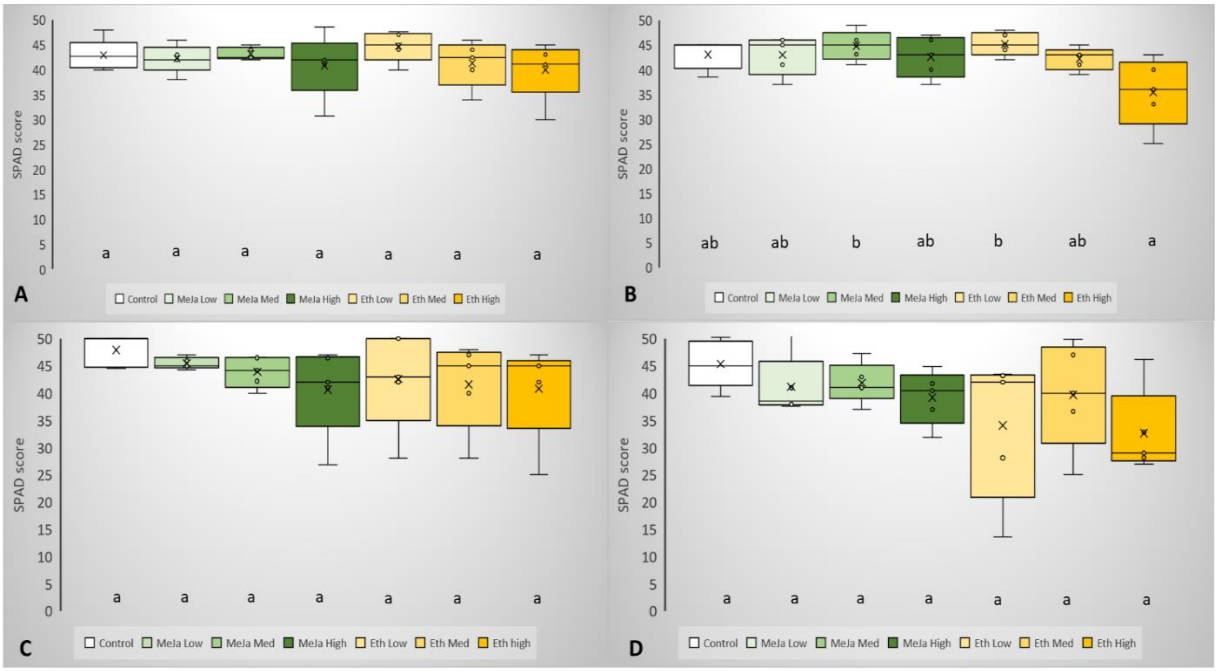


Figure 7.11 Greenness assessment (SPAD)of *Miscanthus* seedlings prior to spraying (A), 7 days post spraying (B), 20 days post spraying (C), and a month post spray (D) with one of six hormonal applications of Ethephon low to high (Eth – orange) or Methyl jasmonate low to high (MeJa - green) and control group (white). N = 5, statistical analysis was undertaken using ANOVA, subgroups were assigned using Tukeys HSD post hoc test

Testing of the same solutions on *Arabidopsis* model plants caused plants treated with all ethephon concentrations to die within 24 – 48 hours of treatment, and as such, assessment was discontinued. There was no significant difference in rosette radius before treatment between populations (Figure 7.12A). 7 days post spraying all ethephon treated plants had died, and no significant difference was noted between the control and MeJa populations (Figure 7.12B). Photographs of experimental plants a few days prior to spraying and again at the same orientation 24 hours later, visually depict the effect that ethephon had, and confirm the efficacy of the solution. There was no visual effect of MeJa on *Arabidopsis* plants (Figure 7.13).

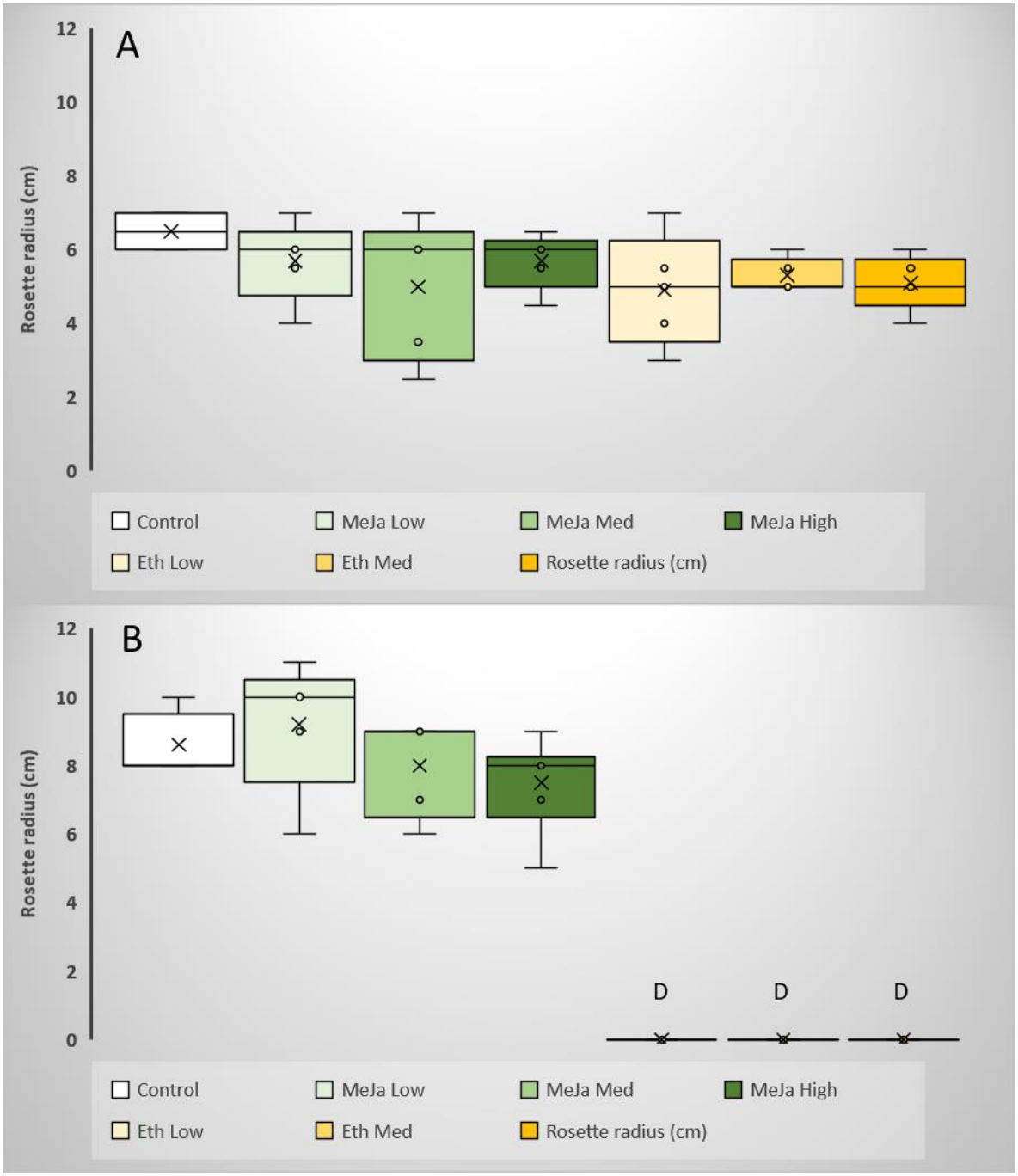


Figure 7.12 *Arabidopsis* rosette radius prior to spraying (A), and 7 days post spraying (B) with one of six hormonal applications of Ethephon low to high (Eth – orange) or Methyl jasmonate low to high (MeJa - green) and control group (white). D denotes dead treatment group. N = 5, statistical analysis was undertaken using ANOVA, subgroups were assigned using Tukeys HSD post hoc test.

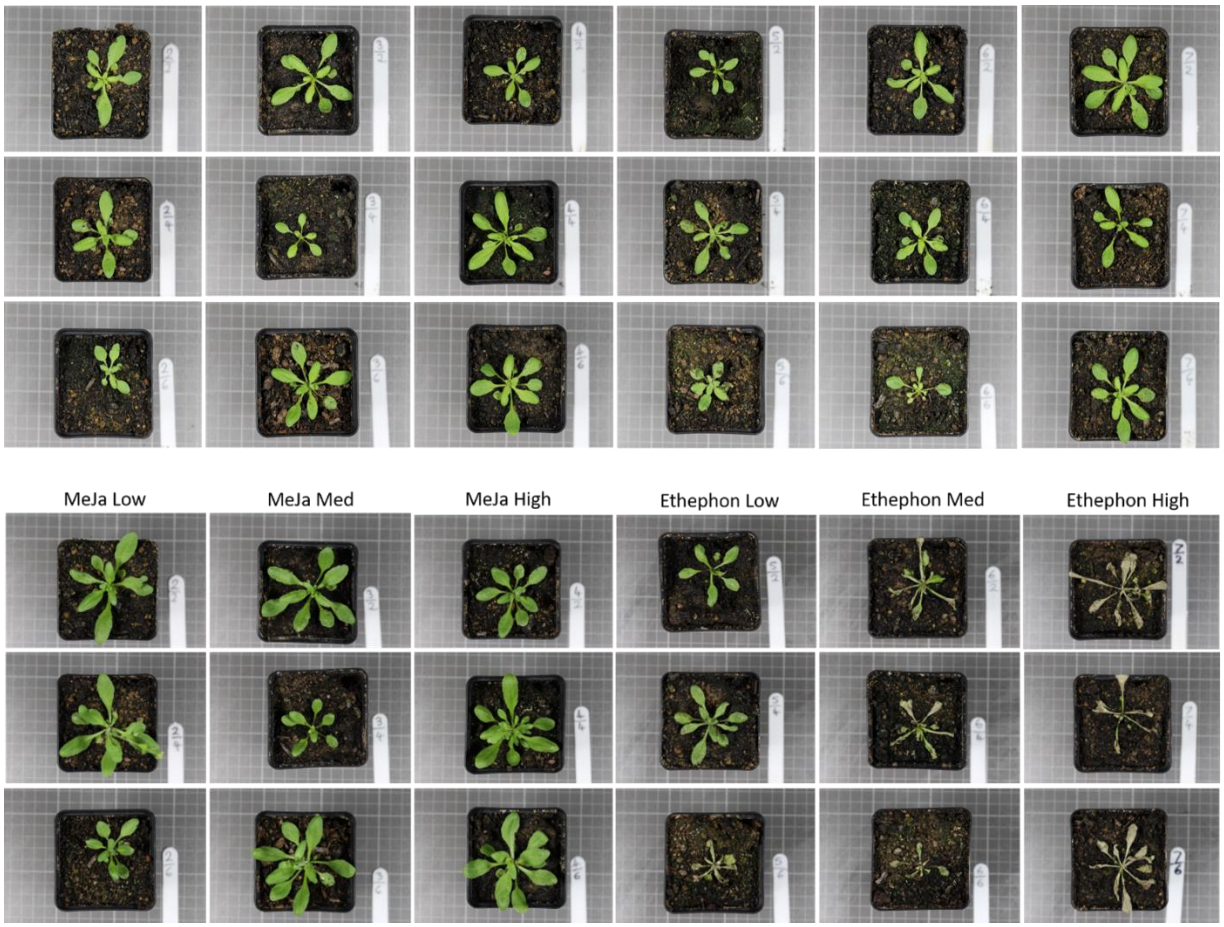


Figure 7.13 *Arabidopsis* plants from rows 2, 4 & 6 from each treatment. Top image shows plants pre-spraying on 31st July 2019. Bottom picture shows the same plants 24 hours post spraying on 3rd of August 2019.

Figure 7.14 *Miscanthus* seedlings a week post treatment with one of 6 foliar applications at concentrations of Methyl Jasmonate (right) at 'low' 200µM/L, 'medium' 500 µM/L and 'high' 1000 µM/L MeJa or Ethephon (left) at 'low' 5g/L, 'medium' 10g/L or 'high' 20g/L. Control plants can be seen below.

7.4 Experiment 3 – The effects of varying concentrations of exogenously applied ethephon treatment to the growth and senescence of 1st year plants of *Miscanthus* genotype GNT14 (Mx 2779).

Based on the results of experiment 2 it was decided to continue with the chosen Ethephon treatments, and use them on more mature first year *Miscanthus* plants to attempt to induce 1st year autumn senescence. The lack of any growth alterations as a result of *Methyl jasmonate* treatment on either *Miscanthus* or *Arabidopsis thaliana* led to a decision to remove this hormone from the final experiment.

7.4a Methods

Miscanthus were grown from seed produce a true year 1 growth phenotype and tested with very high concentrations of ethephon.

Germplasm and experimental design

Seeds of GNT14 from threshing year 2016 were sown on the 10th Jan 2018, onto blue roll in a petri dish, kept damp, and placed in a 25°C growth chamber for a week. After 7 days germinated seedlings were transferred into 3-inch square pots and placed in a heated glasshouse with an 18/25°C 12h night/day cycle. 65 plants were potted on into 9-inch large pots of approximately 7.2L soil volume, on the 22nd April 2018, and grown in a polytunnel under natural light and temperature cycles. The plants were watered as needed but received no additional nutrients. At the end of the summer, plants were arranged into the experimental design (Figure 7.15). 40 of the 65 plants were selected to form the experimental group which was chosen to be as uniform as possible when using non genetically uniform hybrids. Uniformity was based height measurements, with those chosen being as close to the population average height as possible.

The hormonal applications that showed growth effects during experiment 2 were used again on the larger plants. In addition to this, it was decided to experiment with undiluted commercial ethephon. Therefore, the four treatments were a 'low' dose of 5g L^{-1} ethephon, a 'medium' dose of 10g L^{-1} ethephon, a 'high' dose of 20g L^{-1} ethephon and 'maximum' dose of 480g L^{-1} solution plus control treatment lacking ethephon. Due to space confinements in the polytunnel and the size of the plants, each of the five treatments had eight replicates,

arranged in two blocks of twenty plants. Within each block were four rows of five plants, with each of the five plants assigned one of the treatments (Figure 7.15). Plants were spaced close together in double rows, with approximately a metre gap between the double rows for ease of access to each plant, and approximately 70cm gap between columns.

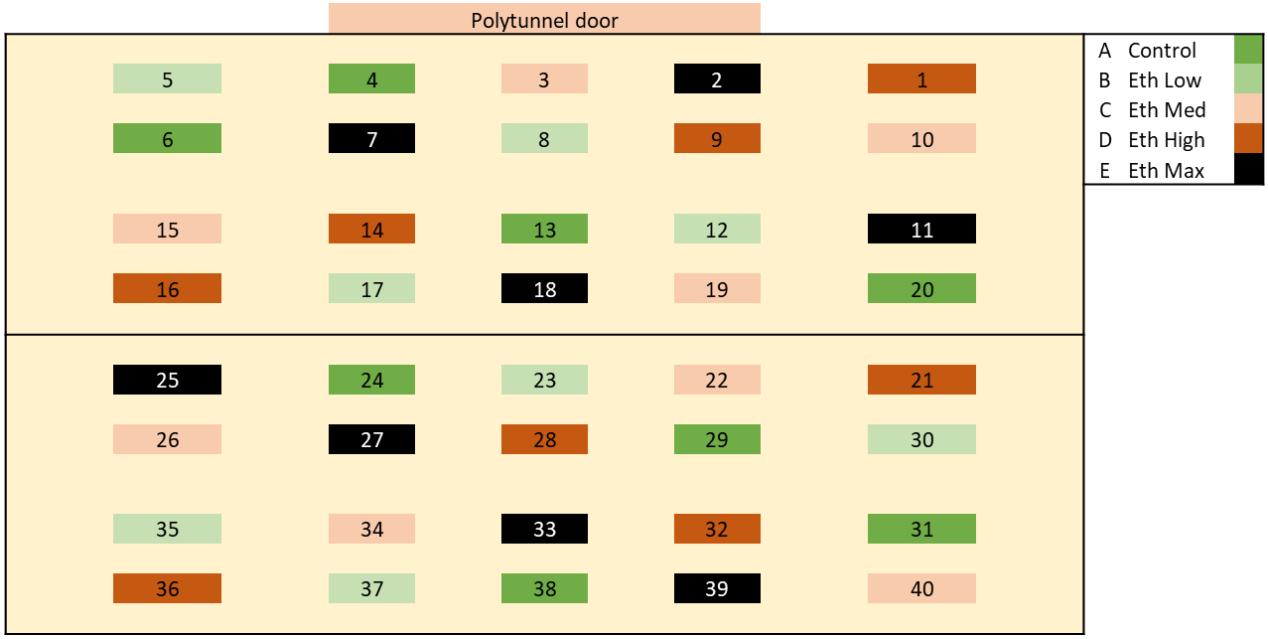


Figure 7.15 Polytunnel design of GNT14 1st year plants, treated with one of four ethephon treatments and a control. Plants 1 – 20 form one replicate block, and plants 21 – 40 form the second. Each treatment is replicated four times within each block. All treatments are colour coded.

Growth and analysis

Prior treatment plants were tested for any pre-existing significant differences that might be a result of variable growth or positional effects in the polytunnel, including height assessment and SPAD relative chlorophyll content of the top ligule leaf. Plants were grouped by choosing 40 plants of similar size, and placing randomly into groups of 8. Once it was established that there were no significant differences prior to any treatment being applied; measurements were taken approximately every 10 days. Measurements commenced in early September and continued after treatments were applied on the 4th of October 2019. Measurements were the same as in Experiment 1. Length of tallest stem, SPAD assessment at three places along the blade of the newest fully expanded leaf, and visual assessment of leaf senescence from 1 – 4. In addition to these, assessment of photosynthetic rate and fluorescence were undertaken approximately 10 days after spraying using a Walz Portable Gas Exchange Fluorescence system Infra-red gas analysis system (Walz, GSF3000 Effektrich, Germany).

Statistical analysis

Data was imported and manipulated using Microsoft Excel 2016. Statistical analysis was done using IBM SPSS statistics Version 21. Data was analysed for normality using Shapiro Wilks, and then for analysis between populations using One Way ANOVA, with Tukeys HSD post hoc tests. Where non-equal variance was detected, an independent samples non-parametric test was performed.

IRGA measurements

Assessments of photosynthetic rate – A (µmol CO_2 m^{-2} s^{-1}) and stress responses in the form of chlorophyll fluorescence (Fv/Fm) were undertaken 2 weeks post treatment on the 16th October 2019, using a Walz GFS3000 Infra-Red Gas Analysis system. The IRGA was set up inside the polytunnel and the leaf to be measured fixed in a 4 x1cm measurement cuvette (Figure 7.16). The cuvette conditions were set as follows: Light source illuminating from above the leaf at 500 µmol $m^{-2}s^{-2}$ PAR; flow rate 700ml min^{-1}; ambient CO_2 level 400ppm; cuvette temperature 25 ^{o}C. The leaf was retained in the chamber until a stable reading was obtained on the IRGA. All the readings were taken between 12:00 and 15:00 on an overcast day to reduce the effects of external environment. A top ligule leaf was selected for each plant from control plants, and high and maximum ethephon plants were tested. The low and medium ethephon treatments were not analysed by IRGA due to time limitations.

Figure 7.16 *Miscanthus* leaf being measured by Walz IRGA on 16th October 2019, 12 days after plants were treated with one of four applications of ethephon

Final assessments

The final assessments of senescence scores, height and greenness were undertaken on the 16th November 2019. In addition to the senesced leaf scores on the selected stem of each plant, an additional entire plant score was given to each experimental plant, based on visual inspection of the amount of senesced above ground biomass based on a 0 – 10 grading system. 0 being no senescent material observed, and 10 being 91 – 100% senesced plant material as described in Robson *et al.,* 2013.

12 days post treatment leaf samples of a top ligule leaf without the midrib were flash frozen in liquid N for sequencing to assess expression of senescence-associated genes (SAGs). Results will be available after project completion.

7.4b Experiment 3 – Results. Effects of four concentrations of ethephon applied to first year GNT14 potted hybrids, on leaf senescence

Growth and leaf senescence progression

There was no treatment effect on extension rate between treated populations at any measured time point ($p > 0.05$) (Figure 7.17A). Relative chlorophyll content (SPAD) scores were non-significant between groups until treatment was applied (Figure 7.17B). On October 12th, a week post treatment, there was a significant difference of $p = 0.010$ between groups, and post hoc tests revealed that the maximum ethephon treatment group had significantly lower greenness scores than both control and the medium ethephon treatment plants ($p < 0.05$). On October 24th, greenness in all populations had declined, with the maximum ethephon treatment being significantly lower than all other treatment groups including control, with the exception of the high concentration. Greenness scores continued to reduce in all treatment groups through November. On November 3rd there was no significant difference between groups as other groups had steadily reduced to a comparably low greenness score with the maximum ethephon treatment. This trend continued until measurements finished on the 16th November 2019.

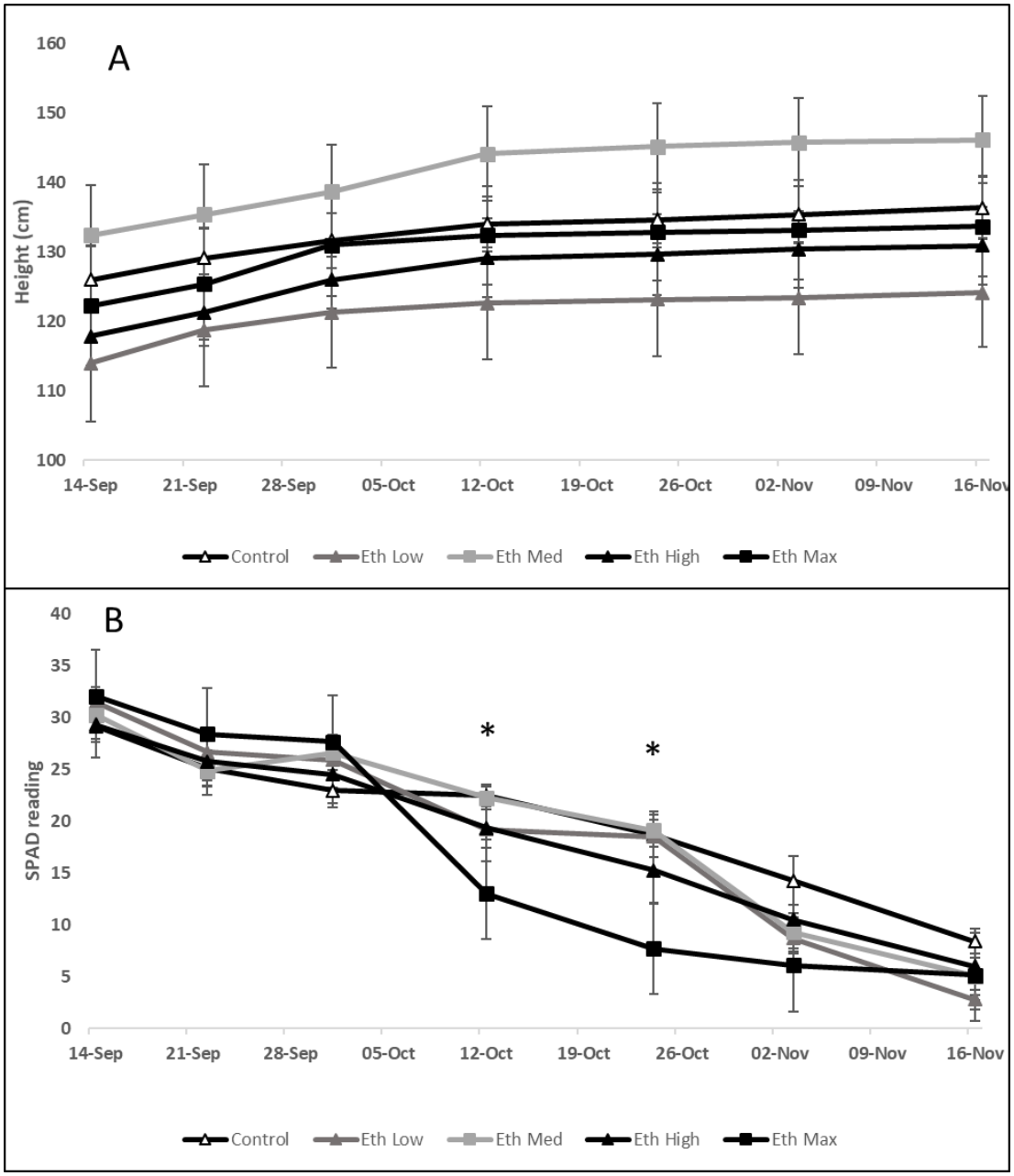


Figure 7.17 Average height over time for each population (A), and average greenness (SPAD) score over the same period of time for each plant in each group. Control shown as a black line with clear triangles; Low Ethephon shown as a grey line with triangles. Ethephon medium shown as grey line with squares. Ethephon High shown as black line with triangles. Ethephon max shown as black line with squares. Measurements started on the 14th September 2019. Treatment was applied on the 4th October 2019. Measurements continued for 6 weeks post treatment. n = 8 ± std e. * indicates a significant difference between groups ($p < 0.05$).

There was a significant difference in percent of senesced leaves between groups prior to starting treatment (Figure 7.18A) ($p < 0.05$). On the 14th September, control plants had on average 56.9% (± 1.7) senesced leaf and other groups had between 42.9 – 45.3 % senesced leaf. This difference continued until the treatment was applied. Measurements taken a week later showed no significant difference between groups as all other treatment groups had increased percentage senesced leaf to a comparable amount each. On the 24th of Oct, 20 days post treatment, there was a significant difference between groups ($p < 0.001$), and post hoc tests revealed the maximum ethephon treatment had a significantly higher senesced leaf average of 88% (± 4.3), than all other groups with between 64 ± 3 to 68 ± 2.1 % senesced leaf. No significant differences were seen during November measurements as other groups reached comparable senescence rates over time. Senescence scores and greenness readings were harder to assess in maximum ethephon treated plants due to slightly differing colouration changes than was typical of normal senescence (Figure 7.19 & Figure 7.20)

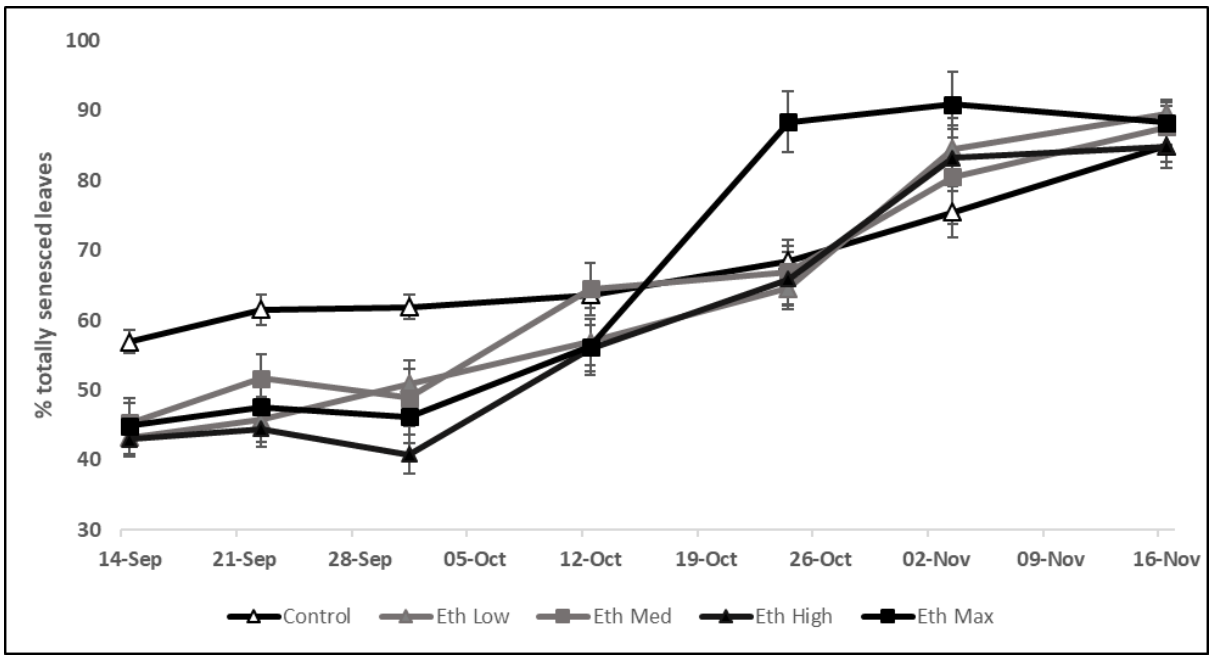


Figure 7.18 Chart A - Percentage of totally senesced leaves (grade 4). Control group is shown as a black line with clear triangles. Ethephon low shown as grey line with triangles. Ethephon medium shown as grey line with squares. Ethephon High shown as black line with triangles. Ethephon max is black line with squares. Measurements started on the 14th September 2019. Treatment was applied on the 4th October 2019. Measurements continued for 6 weeks post treatment. N = 8, ± std e. * = p < 0.05, ** = p< 0.01, *** = p < 0.001.

Figure 7.19 Leaf and plant colour change under typical senescence progress seen in control plants. Leaves and stems go gradually yellow over time and shrivel up. Image taken on October 12th 2019.

Figure 7.20 Leaf and plant colour change in plants treated with maximum concentration of ethephon. Leaves lost green colour but colouration was greyer yellow and more chlorotic, affecting some parts of the leaf and not others. Image taken on October 12th 2019, 8 days post treatment.

Walz GFS3000 IRGA results

Photosynthetic rates in the maximum ethephon treatment were significantly lower than the control group. The high ethephon treatment was not significantly different from either the control or the Maximum ethephon treatment. Photographs of five randomly selected control plants, high ethephon treated plants, and maximum ethephon treated plants can be seen in Figure 7.22.

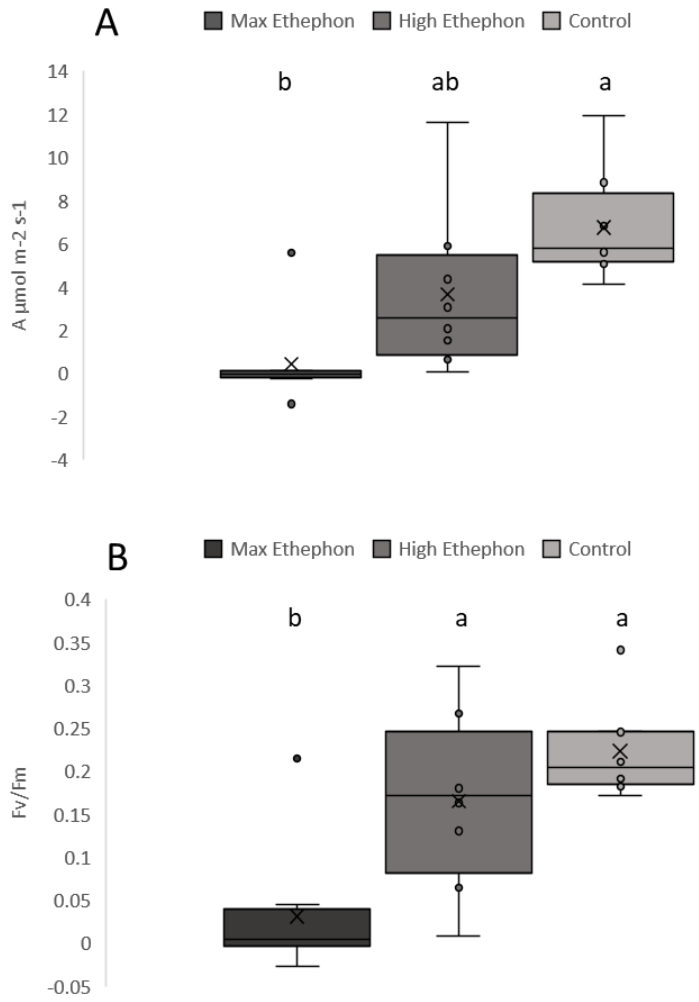


Figure 7.21 Walz IRGA measurements of photosynthetic rate (chart A), and dark-adapted stress response Fv/Fm (chart B). Measurements taken on the control and two highest Ethephon concentrations only. N = 8. Assessments taken under polytunnel conditions approximately 12 days post treatment. Letters above box plots denote Tukeys subset groupings.

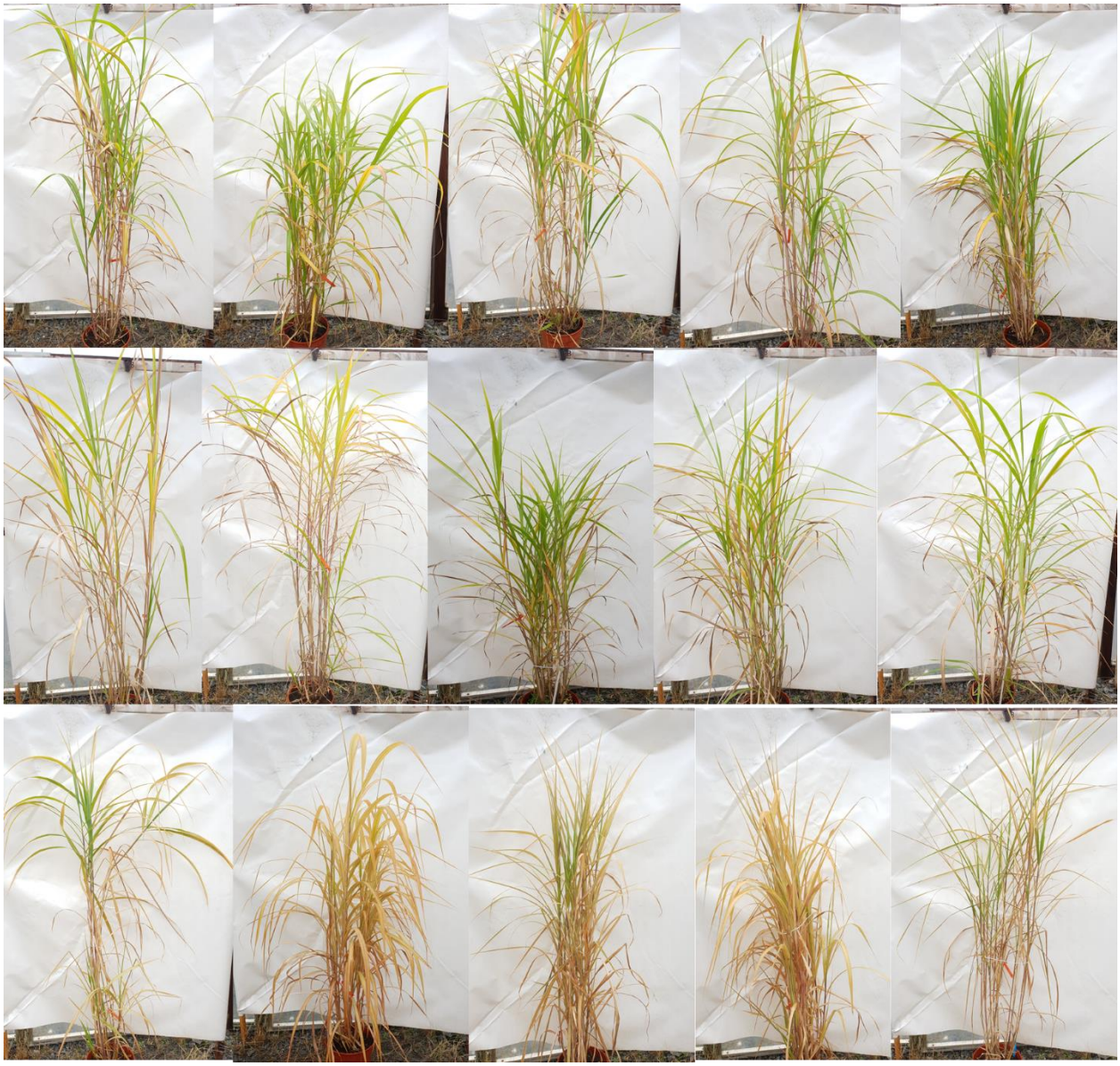


Figure 7.22 Photographs of whole plants, 3 weeks post spraying. Top row photographs of 5 control plants. Middle row photographs of high concentration (20g/L Ethephon). Bottom row photographs of 5 maximum concentration plants (470g/L Ethephon).

The breakdown of percentage of the 1 (<10% senescence) leaves to 4 (totally senesced) leaves over time for each treatment group can be seen averaged as percentage of the entire leaf material on a single followed stem over time in Figure 7.23. The percent of totally senesced leaf increases over a month in all groups including control. The ethephon maximum treatment is the only treatment to have on average no leaves of grade 1 or 2 left on the selected stem by the start of November.

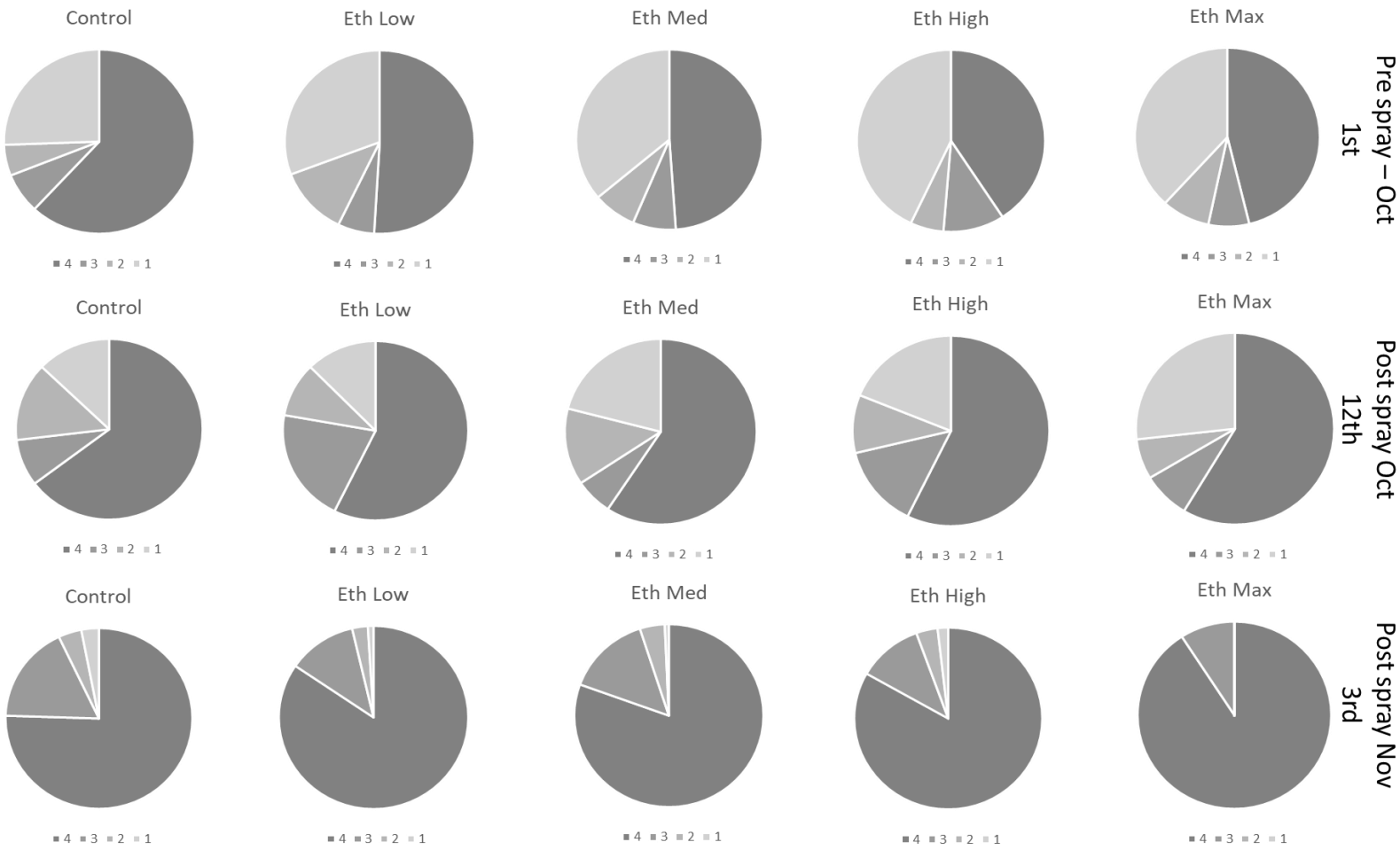


Figure 7.23 Average total leaf colouration percentage breakdown on a selected stem for each treatment. Results are based on a single stem repeatedly measured over time for percent of totally senesced leaves (labelled 4 in dark grey), to the percent of semi senesced labelled 2 & 3 (mid grey colours) and finally percent of green leaf (1) (lightest grey colour). Top row of charts represent the leaf ratio 3 days prior to being treated. The middle charts represent the same stem 8 days post treatment, and the bottom charts show the same plants, a month post treatment. For all assessments N= 8

Whole plant senescence score at the end of the experimental period showed significantly higher scores in the ethephon maximum treated population of on average 81% total senesced material (± 3.9), in comparison to the ethephon high and control populations which averaged 65% (± 3.3) and 63% (± 3.2) total senescence.

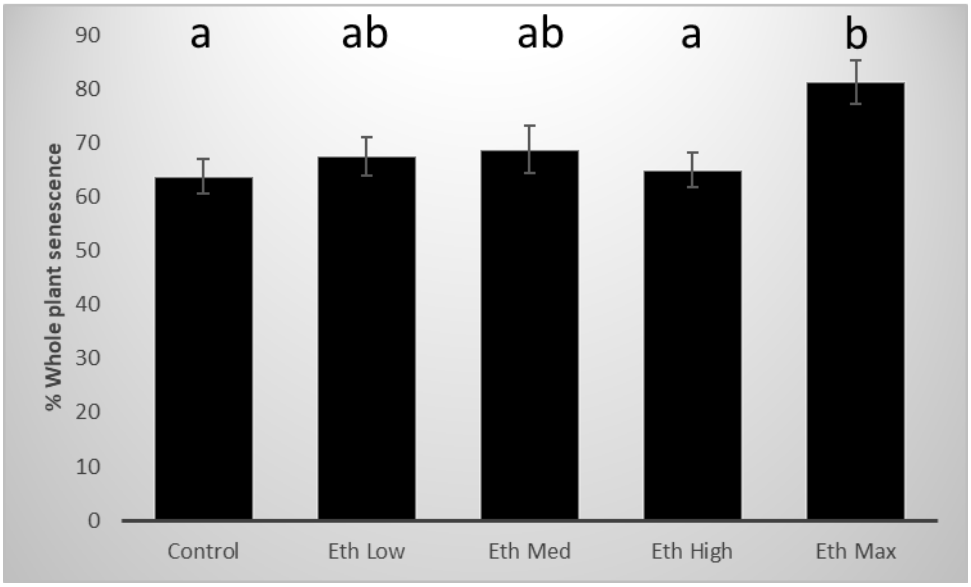


Figure 7.24. Average complete plant senescence score for each treatment group at the end of the measurement period in mid-November 2019. Plants were scored from 0 – 10, 0 being no senesced material, and 10 being 100% senesced material. For each data point N = 8, ± Std Error. Letters denote tukeys subset from ANOVA.

7.5 Discussion

There is significant genetic variation in senescence within *Miscanthus* genotypes; however, if a new seeded hybrid of interest scores highly on all other growth and development characteristics, but exhibits poor overwintering then it would be adventitious to be able to identify ways to induce senescence especially after the first growth year.

Plant growth regulators are a group of synthetic compounds which can be utilized by growers and farmers to elicit morphological changes in plants of interest, by interfering with synthesis of gibberellins and auxins to modify growth characteristics (Rademacher, 2000). Within this study, two hormones were tested at different concentrations, in an attempt to force mature *Miscanthus* to senesce. The hormones chosen were Methyl Jasmonate, a well- known phytohormone expressed in multiple plant stress responses, and ethephon, which is metabolized within plant tissues to ethylene a hormone that promotes senescence.

7.5a Effects of low dose MeJa and ethephon on GNT13 second year hybrids

The literature has a variety of studies using both hormones, on a large array of plant species, and concentrations. Methyl jasmonate was shown to effectively promote senescence in 7 day old zucchini seedlings using a concentration of 100µM (Ananieva *et al.*, 2007). Success using JA was also seen in detached *Arabidopsis* leaves grown on 30µM JA for 12 days, where visible yellowing was observed (He *et al.*, 2002). More recently Chen *et al.* (2017) discovered that MeJa worked as a more intense plant growth inhibiter and senescence promoter when 20µM of MeJa was applied to *Arabidopsis* seedlings (Wild type Col – 0) alongside 100Mm NaCl salt stress treatment. Experiments using ethephon are also widespread. Uses of ethephon on sugarcane plantations were reported to have multiple benefits in a study conducted by Li and Solomon (2003). Benefits included promotion of seed cane sprouting, improved tillering and crucially, advancement in cane maturity, allowing flexible harvest times. The majority of studies based on sugarcane foliar applications of senescence used concentrations in the region of 50mg/L, to 200mg/L. Treatments with ethephon promoted colour change and mature fruit abscission in citrus fruits using concentrations of 400mg/L of ethephon applied by hand sprayer to tree canopy sectors until application run off (Alferez *et al.*, 2006). Results of ethephon and MeJa applications on blueberry harvesting revealed a concentration of 1500g/L or more of ethephon was required to have a significant effect on fruit drop. MeJa applications resulted in leaf yellowing and necrosis of leaf tips and margins, especially at

20mM or greater concentrations (Malladi *et al.*, 2012). These results formed a basis for starting ethephon treatments at 250, 500, 1000 & 2000mg/L and MeJa at 200, 100, 50 and 25 µM. Results of the first attempt to encourage leaf senescence in *Miscanthus* using 2-year-old plants treated with methyl jasmonate and ethylene were inconclusive because plants senesced at similar rates regardless of treatment (Figure 7.4). There were additional confounding factors including variability within the phenotypes of the plants to begin with, despite all being the same age and genotype. This made it difficult to draw conclusive results from height and relative chlorophyll (SPAD) measurements.

Potential reasons why the treatment effects were not significant include that these plants were in their second year and may have more rhizome reserve to buffer against hormone treatments which plants after year 1 growth would lack. It is possible that the solutions applied were too weak to have an effect on plants of such a size as mature *Miscanthus*. There are no known published experiments using MeJa and ethylene on *Miscanthus* plants, so the concentrations applied had to be estimated from experiments in other plant species. It was decided to increase the concentrations compared with published literature; however, the increase may not have been enough to elicit a response. It was also considered that the solutions applied were potentially inactive, this was tested by using the model species *Arabidopsis* in which many published studies were available. A final possibility is that *Miscanthus* plants might not have the pathways required to promote leaf senescence in response to MeJa or ethylene. The latter possibility is unlikely since hormone pathways are well conserved across species (Jing *et al.*, 2003).

7.5b Effects of methyl jasmonate and ethylene hormone treatment on *Arabidopsis* and *Miscanthus* seedlings

Hormones were applied to young *Miscanthus* plants, which had not yet formed any discernible root/rhizome. In addition, the solutions were tested on the model plant *Arabidopsis* (Colombia) in an attempt to gauge the efficacy of the solutions. If MeJa and ethephon had obvious effects upon the model plant *Arabidopsis*, but little to no effect on *Miscanthus* seedlings of similar age, then it could be assumed that there was something within the *Miscanthus* metabolism or genome that makes it unresponsive to hormone treatments.

The effect of the new higher concentrations (5, 10 & 20g L^{-1}) of ethephon on the young plants of *Arabidopsis* Col type in experiment 2 was rapid and fatal after a single dose (Figure 7.13), and in *Miscanthus* seedlings produced higher tillering but reduced height and vigour, dependent on dosage. The necrosis and death seen in the *Arabidopsis* plants showed that there was an active ingredient within the solution, but due to the high concentration probably caused a herbicidal effect. In a study using ethephon on several herbaceous perennials, spraying three times with 1000mg L^{-1} caused necrosis in foliage of one species of bergamot (*Monarda didyma*) (Hayashi *et al.*, 2001). This dosage was lower than that used here, even at the lowest applied concentration.

Ethephon is often used horticulturally to retard stem growth and increase lateral branching (Hayashi *et al.*, 2001), so the result in the *Miscanthus* seedlings can be considered in agreement with other studies. The doses used in Experiment 2 produced increasingly negative effects on plant vigour as the doses increased. Most lateral branching was seen in seedlings treated with the lowest dose of 5g L^{-1} (Figure 7.10), although initially all plants appeared to suffer because of treatment and became limp and weak, with arrested growth (Figure 7.14). None of the plants produced any severe yellowing effects; however, although some reduction in greenness was observed at the highest levels of ethephon treatment (Figure 7.11B). 5, 10 and 20g L^{-1} of ethephon affected *Miscanthus* seedling growth and vigour but appeared to have little effect on colouration and leaf chlorophyll content. Senescence is critically dependent on developmental stage, suggesting it cannot be induced until a certain developmental stage is reached (Jibran *et al.*, 2013) so it is possible that the *Miscanthus* plants were not competent to respond to the hormonal signals.

The Methyl jasmonate treatment produced no discernible differences in either the *Arabidopsis* or *Miscanthus* seedlings (Figure 7.13 & Figure 7.14). The lack of any effect on *Arabidopsis* was surprising and it is unlikely that the ecotype used was insensitive to the hormone so other causes were considered. Many of the studies using MeJa used methods that involved testing using detached leaf and exposure to the hormone for longer periods of time, either by way of multiple treatment events, immersing plants in solution or growing the plants in agar with MeJa (He *et al.*, 2002). Others used concentrations of hormone that were far higher than those used here such as 10, 20 and 30mM as opposed to µM amounts (Malladi *et al.*, 2012). Additionally, some studies used hormones in addition to other test variables

such as darkness treatments, salinity, or in combination with other hormone treatments. More recent studies have discovered that in higher plants, leaf senescence is tightly interconnected with circadian clock (Wang *et al.*, 2018). Zhang *et al.* (2019) report on the existence of what they call the 'evening complex' (EC) in *Arabidopsis* plants. When testing effects of Jasmonates on senescence in *Arabidopsis* plants with and without the EC they found that EC mutants had accelerated leaf senescence when detached leaves were floated in 100µM MeJa under dark conditions. They concluded that molecular mechanisms exist within *Arabidopsis* types, that the circadian clock is a strong regulator of leaf senescence under Jasmonate signalling. Whatever the reason for the lack of response to MeJa, it was decided to focus the treatments on the more active ethephon solutions.

7.5c Ethephon induced senescence in GNT14

Ethephon solutions were tested on mature *Miscanthus* plants at the end of the first growth year in experiment 3. By the time of treatment in October, plants were already beginning to show some signs of senescence and therefore any possibility of lacking competence to respond to inductive signals was less likely. The control group had significantly higher percent of senescent leaves on the chosen stems pre-treatment, despite attempts to homogenize populations. However, following treatment, this significance disappeared, as all treated populations caught up rapidly. The highest rate of senescence increase was in treatments using the maximum concentration of ethephon; plants in this group had the lowest percentage senescence but reached 90% senescence within days of treatment (Figure 7.18). Other treated plants achieved a similar level of senescence but this took several weeks.

The stock solution sold by agricultural suppliers is typically used to be used as a growth regulator on winter and spring varieties of barley and wheat, rye and triticale, as a method of reducing lodging. Instructions for use on Agro base suggest varying the amount depending on the crop type from 0.5L/ha to 1L/ha, with between 200-400 L/ha of water. The effect of treating *Miscanthus* with the maximum concentration was quickly visually apparent as leaves became yellow after 24 – 48 hours. The response did not appear to follow the usual yellowing pathway seen in natural senescence (Figure 7.19). As shown in Figure 7.20, chlorophyll bleaching is evident, but on some leaves, only partly, with green leaf remaining in some areas of the leaf, interspersed with chlorophyll degradation. When visually assessing leaves to give a senescence score the patchy phenotype, along with the grey to yellow colouration of the

affected leaves made it hard to judge what could be classed as true senescence. The gas exchange measurements from plants treated with control solutions and high concentrations of ethephon indicated low levels of photosynthesis was occurring in the hormone treated plants, suggesting treatment had effectively triggered a senescence like processes, but had not killed the plant. The mechanisms by which ethephon and ethylene regulate leaf changes are not fully understood. Progressive leaf yellowing is the most obvious sign of senescence, and the final stage of leaf development, involving the mobilization of nutrients from older plant material, to more useful plant organs such as leaves higher up the canopy, or the rhizome (Koyama *et al.*, 2013). There is little doubt that externally applied ethephon, particularly at large concentrations had a large and rapid effect on the leaves and stems of *Miscanthus* plants. What is harder to conclude is whether the signalling pathways activated by the application, follow similar pathways to what would be seen under true senescence. Despite the large literature base in botanical studies using ethylene, little is known about the intricacies and downstream molecular networks responsible for many of the responses seen in ethylene treated plants (Stepanova and Alonso, 2009). What is even less studied is the potential for ethylene or ethephon as a senescence inducing plant growth regulator in C4 crops such as *Miscanthus*. Biological responses to ethylene depends on tissue sensitivity, maturity, and species sensitivity to hormonal application (Iqbal *et al.*, 2017). The visual signs of senescence include yellowing, chlorophyll degradation and eventual leaf abscission. Yellowing and chlorophyll degradation were obvious in the *Miscanthus* hybrids that were treated with maximum concentration in experiment 3, but not in the low, medium and high concentrations, suggesting *Miscanthus* does have a sensitivity pathway to ethylene, but potentially requires high dosage to activate it. Chlorophyll degradation was obvious across the entire plant after treatment, but assessments at a molecular level are required to be certain about the genes and signalling pathways activated by treatment. Samples of leaf tissue from the control, maximum concentration and high concentration plants were flash frozen and will be analysed for expression of senescence-associated genes, results of which are pending. Current models of the physiological processes of ethylene suggest complex signalling pathways, composed of several phosphorylation cascades, feedback regulated networks and protein and mRNA turnover regulatory modules (Stepanova and Alonso, 2009). Photosynthetic rate decreased significantly alongside the Fv/Fm (Figure 7.21) proving that

systemically, the plants were more stressed, with reduced active photosynthesis, which is a key sign of senescence(Boersma *et al.*, 2015) .

By the time of final assessment (16th November) most of the plants appeared to be forming new basal shoots (Figure 7.25A), including those treated with the maximum concentration. Other observations included remaining green leaves higher up the canopy (Figure 7.25B), and that many leaves still retained the chlorotic appearance with some green areas, possibly where sprayed solution had not been distributed homogenously (Figure 7.25C). The conditions within the polytunnel may be a contributing factor to the formation of new basal shoots as, while it is an adequate intermediate environment between glasshouse and field, plants would still have had some protection from cold and frost events. This suggests that the treatments have not killed the *Miscanthus* plants and that *Miscanthus* does not require a vernalization period to initiate new reproductive growth, and where conditions allow, will keep producing biomass (Kim *et al.*, 2009).

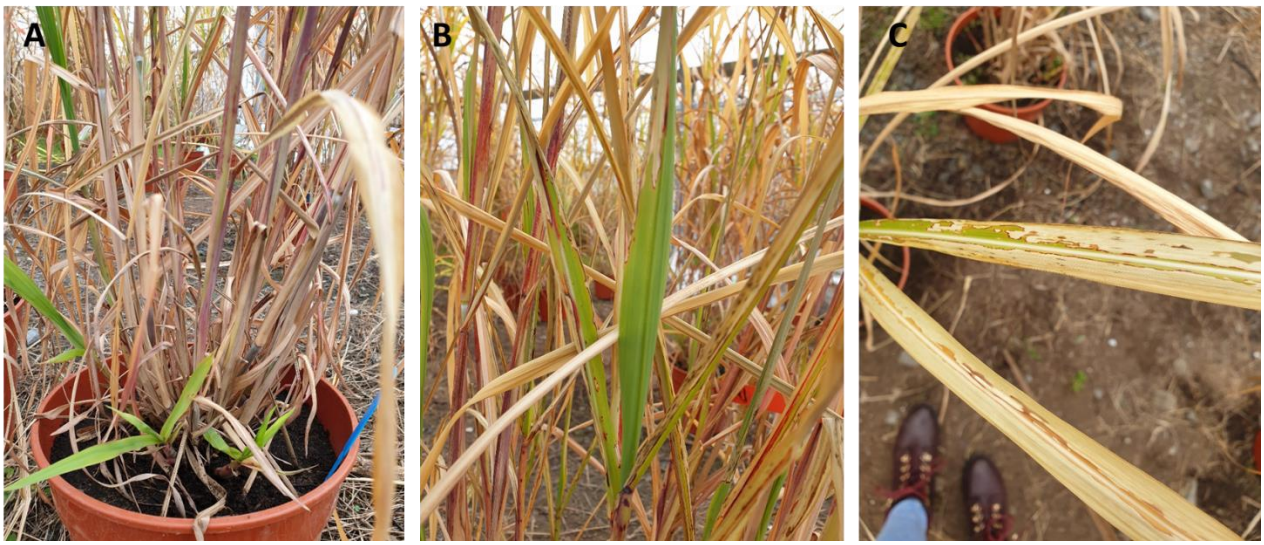


Figure 7.25 Photographs of the maximum concentrated treated plants at the final assessment date on 16th November 2019, 6 weeks post treatment. A – base of plants showing new shoots growing from the rhizome. B – Green leaf left at the top of the stem. C – Remaining chlorotic appearance on treated leaves, interspersed with some green areas

7.5d Conclusion and final remarks

Overall, it can be concluded that ethephon does have the potential to encourage senescence in large *Miscanthus* hybrids, but that dosage needs to be far higher than is typically used in other species in the literature. The maximum concentration used here, of 480g L^{-1} ethephon stock solution straight from the agricultural supplier was the only solution to produce obvious acceleration of leaf senescence but at such high concentrations that it would be expensive to apply to large areas of crop. It was unknown how *Miscanthus* would react to applications of ethephon, or the concentration that would be required to induce senescence signalling pathways. As this was simply an experimental concentration it is probable that much lower concentrations, or treatments of combinations of hormones or surfactants, would achieve similar results and this should be further researched. Methyl jasmonate had no effect on senescence rates in *Miscanthus* at any concentration tested. When tested on model plant *Arabidopsis* thaliana, on which there are many successful studies using the same hormone, again no change was observed in leaf colouration or architecture. Reasons for this remain unclear, but are likely related to concentrations not being sufficient, and/or a requirement for a longer period or multiple periods of treatment to produce an effect. Use of ethephon seems more practical, the compound is already readily available from agricultural supplies, under various names including 'Padawan' and 'Ipanema', and is used regularly on other crops, suggesting the infrastructure for use is already in place across many crop farms. Further assessment into Senescence associated genes (SAGs) from the leaf samples taken from plants after treatment here will improve the conclusions of this study, allowing more definitive answers as to the nature of the senescence activated by ethephon treatment of *Miscanthus*.

8 Overall conclusions and discussion

8.1 Project context and aims

This project was developed as a collaboration between Aberystwyth University and leading bio-energy company Terravesta, to aid in the understanding, uptake and domestication of bioenergy crops of the genus *Miscanthus*. This is an area of high current importance, with research and development being implemented across the globe, with the aims of combatting the largest global threat facing the world today. Atmospheric CO_2 concentrations have risen by 31% since the 18th century as a result of fossil fuel combustion for energy purposes, and vast land use changes for increased agriculture, industry, and other anthropogenic uses (Lal, 2004). This is becoming an increasingly important matter for the global environment and, among other anthropogenic activities is contributing to the increase of freak weather events, global warming and biodiversity loss (IPCC, 2018). Crucially, these issues are now gaining the concern and mitigation measures they deserve as a result of growing awareness both publicly and politically. The remedial actions being taken are widespread and variable, depending on science innovations throughout a plethora of sectors. Progress is slow however, and the impacts of research in these sectors are vital. Biomass production is one such sector, seeking to mitigate the increasing atmospheric carbon concentrations, and reducing them to more manageable levels. Dedicated perennial biomass crops possess growth characteristics and traits that are desirable for sequestering atmospheric carbon and storing it until it can be processed to produce liquid transport fuels, combustion fuel, or other end uses when required (Clifton-Brown *et al.*, 2008). *Miscanthus* species are one of these perennial crops. *Miscanthus* can produce more biomass per hectare than most other energy crops such as Maize (Dohleman and Long, 2009), and as such it is of great importance that research is funded to ensure the full potential of this crop is realisable in a short time frame. While this may sound like the ultimate answer to carbon sequestration and moving toward a carbon neutral economy, the reality of mass uptake of a largely unheard-of biomass crop is fraught with barriers, some of which have been tackled in this project. The development of second-generation energy crops is still in its relative infancy, and as such has not received the breeding and agronomic improvements to the level of many other staple crops. It is also crucial that energy crops do not negatively affect the production of food crops, as intensification of food production is an equally important global issue (Valentine *et al.*, 2012).

As such, compromise has been found in the area of marginal or 'lesser valued' agricultural land that is not profitable for food production, that may be currently unused, or negatively affecting farming income due to the high levels of inputs required. This being said, a recent study by Helliwell (2018), suggests that the scheme that energy crops were aimed at marginal land was acting as a barrier to uptake by farmers, many of whom did not consider their land 'marginal enough'. The study highlights the importance of working closely with farmers and growers, whose cultural values and farm practices, which may have been handed down over generations, may be resistant to a change from the status quo, and a move into a novel crop. This increases the value of developing a product which can be trusted, and will bring in reliable revenue, using known farm practices where possible. This emphasizes the significance of the work, domestication and improvements on *Miscanthus* germplasm by the team at Aberystwyth University, to provide successful methods of establishing *Miscanthus* in the first place, and for businesses such as Terravesta Ltd, to aid farmers in the process, and provide a market to which growers can sell their end product for a competitive price.

These aims formed the basis for the foundations of this project, working with both the breeding team at Aberystwyth, and the business partners, at Terravesta. Because *Miscanthus* cultivation is focussed toward poor quality land, then the crops must be able to thrive and flourish under such conditions. In addition to the successful establishment of a new plantation, it is important that economic return for growers is generated as soon as possible, in order to increase confidence in the crop as a business venture. The perennial nature of the crop also means that successful winter die back and return in spring is the final hurdle for young plants during the first year. Any one of the many potential processes contributing to crop establishment and overwintering can mean the difference between a successful plantation and crop failure. One significant change in recent years has been the change from clonal rhizome planting, to a seeded hybrid system, due to high propagation costs, and low multiplication rates (Clifton-Brown *et al.*, 2017). Adoption of seed propagation has resulted in new challenges but is far more efficient at producing sufficient plants to allow large scale adoption of *Miscanthus* across the significant land areas needed to have the hoped-for global impact. Direct seeding into field trials may be a tried and tested method of planting for many agricultural crops, but due to the small size of *Miscanthus* seed and its high thermal requirements, is not currently a viable option especially in the high latitude, temperate areas

of the globe where *Miscanthus* is being cultivated (Clifton-Brown *et al.*, 2011). As such sowing seeds into plugs under glasshouses conditions provides greater assurance of success while testing of direct seed methods is ongoing. As a relatively new advancement in the *Miscanthus* growing system, there is therefore much scope for improvement of the agronomy of the method of plug plant propagation for *Miscanthus*.

8.2 Plug plant glasshouse establishment improvement techniques

Throughout this project the aim of improving the establishment of *Miscanthus* via plug plants has been a key element and has formed the basis for the majority of the chapters. From germination biology, to glasshouse conditions, field planting, and subsequent crop husbandry, there have been many potential opportunities for experimentation within this project, more than could reasonably be done in the time given. In chapter 3 the establishment of plug plants of an important hybrid 'GNT5' was tested by the application of clear film at the germination phase. This is a method to accelerate growth and seedling establishment typically used to create a microclimate around the crop under field conditions that has been used to great effect in temperate climates (Ashman *et al.*, 2018, O'Loughlin *et al.*, 2017). Mulch film was trialled successfully as an inexpensive way to enhance the temperature and reduce water evapotranspiration under glasshouse conditions. The trial proved that this was an inexpensive method to speed up *Miscanthus* seed germination and accelerate the seedling maturation process, under glasshouse conditions in addition to its more conventional use in the field. Developmentally enhancing seedling growth at germination continued to have a positive effect under field conditions, albeit at a less obvious scale. When selected plants were analysed in detail from two-week-old seedlings until the end of the first year this trial suggested that there may not be a strong correlation between size at planting and the accumulation of biomass at the end of the first growth year. This was not expected and prompted further assessment of the potential factors around seedling growth that are responsible for subsequent biomass accumulation when plants were transferred to the field (chapter 6).

The design of the plug in which *Miscanthus* seedlings were grown in the glasshouse environment was one factor tested (chapter 5). It was demonstrated that module size had a significant impact on seedling development. A small module benefits from more efficient use of glasshouse space, but risks growth problems from root binding and resource depletion

(chapter 5). Larger modules reduce these risks, but cost more in terms of space, growth medium and initial purchase price. Through analysis of four incrementally increasing module sizes it was determined that the then commercial size of 25cm^3 soil volume was not giving plug plants the optimal chance of establishment under the temperate field conditions of places such as the UK. Additionally, the largest module size tested, while providing high establishment and growth rates, was potentially too large for the extent of glasshouse space required, for the number of plugs needed. Comparably good growth and establishment was also observed in plants grown in the second largest module. The majority of the increase in volume was in the depth of the module rather than width, maintaining competitive space requirements with the smaller modules, and as such this plug size was chosen for mass roll out of subsequent trials both within this project, and for the planting of other projects.

Planting conditions during the transfer from glasshouse to field of plug-grown *Miscanthus* were important in growth and establishment. The extent of significant differences in establishment and growth noted when planting date was varied was considerable, with consequences that remained significant in subsequent years. The length of time in plugs is of great importance and can affect success when planted into the field. The extent of root impaction seen in plants kept in small modules for an extended period was a significant factor when choosing the module size (Figure 5.9). The larger modules allowed seedlings to grow well under glasshouse conditions for a longer period where necessary, allowing flexibility in the planting window to allow more weather dependent planting time. Smaller modules used prior to this assessment were more limited, as restriction of roots was a large issue after a shorter glasshouse period. If meteorological conditions were suboptimal by the time that plants were outgrowing the modules then planting would either have to be done under the suboptimal conditions, or postponed, which had a negative effect on small modules noted at later plantings in chapter 5. When taking into account the highly unpredictable meteorological conditions in more temperate regions it makes most sense to encourage planting time flexibility where possible, furthering the decision to move to the larger module.

8.3 Testing seedling morphology

Due to the lower than was expected correlations between large seedlings after growth in the glasshouse, and large plants in the field (chapter 3) a large study was undertaken in 2018 with the aim of encouraging a variety of seedling phenotypes within the same hybrid, and following

the progression of this highly variable population under field conditions. Using the new module size suggested in chapter 6 ten treatments were devised that aimed to alter plant morphology, and the resulting populations were planted into two large field trials at two contrasting sites in the UK. The largest seedlings were produced by increasing soil density within the plug module, and growing seedlings under warm (>25°C) glasshouse conditions. Taller seedlings with reduced overall biomass were produced by controlled environments under red light. A shorter morphology with higher lateral stem density was produced by cutting the main stem. Smaller growth was produced in cooler temperatures (<18°C). The experiment demonstrated that seedling morphology affected two important processes, establishment (survival) rate and biomass accumulation, differently. Higher biomass pants at the plug plant stage with higher root and above ground biomass had significantly better survival rates both under glasshouse and field conditions. Results of the final accumulated biomass yield; however, were less conclusive. Yield was affected to a greater extent by the environment rather than initial plant size in the plug. However, based on the survival rate and the marginally higher likelihood of a strong plant coming from a strong seedling, it was concluded that larger seedlings with a roughly equal root: shoot ratio is the morphotype required at field planting. Much like the effects seen in chapter 5, the results suggested a greater effect of environment during planting, and post planting husbandry on the resulting growth and yield, than the initial seedling morphology. This was an extremely common phenomenon noted during field trials in chapters 3, 5 and 6. While it cannot be understated that seedling morphology is of great importance to establishment rate in particular, a common trend seen throughout assessments of glasshouse to planting in this project is the inability to predict which seedlings would produce the most end of first year biomass. During the first trial, larger seedlings were encouraged by germinating faster under mulch film, which accelerated their growth, but the field phase diluted much of this initial competitive advantage. The large variation in seedling morphology entering the field as a result of the glasshouse treatments in chapter 6, also evened out when yield data was assessed. Similarly, in the testing of plug sizes, the larger plug modules produced significantly larger plants upon exit from glasshouse conditions, but this significance was lost in the harvest data, where the largest differences were seen as a result of planting time and conditions, with the exception of the final planting. Possible reasons for this are likely a result of the newly unlimited supply of necessary growth conditions available to plants once removed from restricted pots and

placed in the field. With no restriction on the amount of rooting space, light, ground water, and nutrient supply all plants are able to maximize their growth rates approximately equally, until a ceiling is reached as a result of genotype or reduction in solar hours. Large plants will likely reach this ceiling quicker than the smaller morphologies, but these will likely catch up when allowed to do so by adequate growth season and thermal time. The later planting differences observed in chapter 5 are proof that when planted later, the amount of growing degree days and thermal time is reduced before autumn reductions in temperature and solar hours occur. This likely has a negative effect on initially smaller morphologies, as they cannot maximise their potential before needing to assimilate all resources to the below ground rhizome for over winter, as is proved by the significant differences in large and small plug modules seen in Figure 5.16.

8.4 Beneficial bacteria and the prospect of biofertilization

One method of growth promotion of increasing interest over many botanical and agricultural sectors, is the addition of natural beneficial endophytes as a method of biofertilization that encourages a move away from energy intensive synthetic fertilizers that can damage ecosystems (Fei *et al.*, 2019). During the start of the *Miscanthus* upscaling technology project in 2016, an exciting opportunity presented itself to work with a company specializing in developing endophyte delivery systems for a range of plant species that could improve many plant traits including biomass accumulation, nitrogen fixation under low nitrogen con ditions, plant stress tolerance and herbivory resistance. This level of plant performance promotion is particularly interesting for biomass crop production, due to the necessity of planting the crops on lower value land, which would likely have low nutrient status, drought or flood issues, and a range of other challenging growth conditions (Schmidt *et al.*, 2018). Aiding sustainable biomass growth using expensive and environmentally damaging products, would be counterproductive because it would negatively impact the positive energy balance achieved by *Miscanthus* cultivation (Felten *et al.*, 2013). Treating *Miscanthus* plantlets with two endophytes, previously isolated from *Miscanthus*, produced mixed results overall. The effects of endophyte treatment appeared to be genotype dependent and dependent on the inoculum size of the endophytes. Some endophyte treatments resulted in significant growth promotion effects on *Miscanthus* plants grown in experimental nitrogen free compost albeit after an initial lag period. However, some endophyte treatments seemed to overwhelm the

developing seedlings killing many. This was a particular problem in genotype GNT14, although compromised seed viability may have contributed significantly to this effect. When treated seedlings were planted into field trials no significant benefits were observed for the most part, on any experimental seeded hybrid. It is likely that this lack of difference was due to the already existing bank of bacteria, which exist in field soils that would have colonized plants quickly, diluting any effects seen from adding endophytes in nitrogen free soil in the glasshouse. There was, however, a consistent improvement seen in *Miscanthus* giganteus plants when the bacteria treated polymer was added to the field soil, a difference that became significant at the third harvest year (2019). This suggests that potentially, clonally propagated plant material is more receptive to the additions of beneficial bacteria, than seeded plants.

It is possible that the field trials were planted on land that could already support good plant growth, as *Miscanthus* plants are favoured for their effective nitrogen use efficiency. There would likely be a stronger effect seen in very low nitrogen marginal soils, suggested by the large growth promotion effect seen in seedlings when grown in N free soils but given endophyte additions (Figure 4.8). Testing the hypothesis that endophyte treatment would improve the performance of *Miscanthus* genotypes suggested there was potential for positive effects on seedlings, but the complex interactions need to be better understood. The effect of endophytes may switch from negative to neutral to positive. Species of bacteria, plant genotype, concentration of bacteria and growth environment interact and require optimisation. In so far as larger *Miscanthus* seedlings at field planting established at higher rates (Chapter 3), if endophyte treatment resulted in larger plants, this would be beneficial. However, the question remains as to whether such bacterial treatments are impactful if *Miscanthus* seeds are cultured in more nutritious media, would the colonisation by endophytes have a significant effect on subsequent field performance and could endophyte treatments impact the interaction of *Miscanthus* within the complex field microbiome. As a long-term prospect of greener innovations for biofertilization this is an interesting area, but this project proved it is unlikely to be implemented in the short term for growth promotion, while the interactions are not fully understood.

8.5 Senescence and overwintering

Successful perennial regrowth of a *Miscanthus* sward under temperate climates depends on the timing and effectiveness of the autumn/winter die back of the above ground biomass (Robson *et al.*, 2012). Translocation of vital nutrients and assimilates away from the leaves and stems and into the rhizome for over winter storage and subsequent spring regrowth is a vital part of perennial survival and contributes to the vigour of the next year's growth. In addition, the drying down of aerial parts over winter is desirable for effective harvesting, transport and combustion efficiency (Jensen *et al.*, 2017). Some more tropicalized *Miscanthus* genotypes often fail to senesce effectively before the first frost episode, which can kill young plants or decrease spring regrowth vigour. Previous unpublished studies using hormones to attempt to encourage senescence in *Miscanthus* had been unsuccessful. In this project, it was proved that lower concentrations of hormones used to initiate leaf senescence in other species, were not active in *Miscanthus* plants with developed rhizome (Figure 7.6). In smaller seedlings, lower doses of ethephon had a marked effect on plant vigour (Figure 7.9); however, in older plants the same doses had little effect (Figure 7.18). The hormone Methyl jasmonate has regularly been reported in the literature as having significant ability to induce leaf yellowing and senescence over a variety of plant species. When applied to *Arabidopsis* and *Miscanthus* seedlings growing in the glasshouse in this project there was no obvious effects upon either species. High concentrations of ethephon had a marked effect on mature *Miscanthus* plants resulting in rapid leaf and stem chlorophyll degradation within 24 – 48 hours of application. The leaf yellowing was visually different from natural senescence observed in other plants and appeared to be patchy across some leaves. More research is required of the regulatory effects that ethephon has on the plant signalling pathways, and how this phenotypical senescence response induced by ethephon compares with natural senescence in terms of remobilisation of nutrients (Distelfeld *et al.*, 2014). It is likely that the response seen in the higher concentration treated populations was a herbicidal and killing treatment, as opposed to the initiation of genetically controlled senescence. Achieving the desired response of a controlled senescence initiating pathway will likely prove be a very complex balance of finding the correct method of treatment, applied at the correct time and at the optimal concentration. Testing of multiple concentrations of ethephon is required as there was a large difference between the two highest concentrations tested in chapter 7, which may reveal different or more physiologically significant senescence responses are

possible at slightly lower treatment concentrations. Molecular analysis is also required to assess the expression of senescence associated genes, and to acquire a better understanding of the required signalling cascade that initiates the autumn senescence process in this perennial crop.

8.6 Final remarks and the future

Improvements along the entire supply chain are aiming to reduce the 'front loaded' initial costs of planting *Miscanthus* commercially. Projects are ongoing to reduce the risks associated with *Miscanthus* and encourage farmers to invest in the crop. Research and innovation such as that which has been developed in this project and throughout the *Miscanthus* team at Aberystwyth are extremely important for the overall uptake of *Miscanthus* in the commercial sector. More funding and collaboration are essential to the future of this research. This project has served as an experimental platform for testing and selection of optimal plug-plant growth conditions during the first year of a plant's life, and as a basis for the testing of methods used to manipulate over wintering timing. With the direct seed sowing innovations as unreliable as they currently are, it is likely the research in this project will be a necessary basis on which to improve the propagation of *Miscanthus* plug plants for several years to come. Discoveries made as a result of this project are already being implemented in large-scale commercial plantings of newer hybrids, including moving to larger plug module sizes and increasing the bulk density of compost within the plug. Experimentation is also starting with microflora by additions of small volumes of target site soil added to the plug medium, to expose plug plants to the microbiota they will experience upon planting into the desired field.

Some elements of this project were restricted in nature by the collaboration with various company partners. Copyright and industry secrets imposed a significant restriction on the write-up of the methodology for chapter 4 in particular. The limited and restricted nature of the product placed a great deal of experimental design in the hands of the commercial partner. In future projects, greater control over experimental design should be undertaken by researchers in order to develop well refined methodology for *Miscanthus* establishment and growth promotion techniques.

8.7 Key Findings

8.7a Optimal plug growth and establishment in the glasshouse

- Germinating *Miscanthus* in plug trays under film significantly increases germination speed and plant growth but this difference did not carry forward into the field.
- Increasing module size from 25cm^3 to 45cm^3 significantly improves plant growth under the glasshouse period, without negatively impacting the costs of nursery growing.
- Marginally increased module size allows for a larger degree of flexibility in length of time necessary under glasshouse before negatively impacting the root: shoot growth
- Growing plugs under high glasshouse temperatures (18 – 26°C) has a positive effect on *Miscanthus* plug plant vigour and produced near 100% glasshouse plug survival
- Growing plugs under cooler temperatures (<15 °C) has a negative impact on the vigour of *Miscanthus* seedlings and survival under glasshouse conditions
- Increasing soil volume by compaction has a positive effect on *Miscanthus* plug plant growth and development, giving a well-balanced root: shoot ratio
- Additions of external fertilizers to well-balanced compost has little effect on growth and vigour of plants
- Cutting of the main stem to below the lowest ligule 7-12 days before planting increases the appearance of new lateral buds and stems
- Additions of nitrogen fixing bacteria under low N conditions can vastly improve the vigour of seedlings but is concentration dependent as small seedlings can easily become overwhelmed by large quantities of beneficial bacteria
- Additions of beneficial bacteria or 'biofertilization' is a promising alternative to synthetic fertilizers but requires a great deal of experimentation, method refining and lab assessment.

8.7b Planting and field performance

- Weather conditions at planting can have a significantly larger effect on growth and establishment than initial plug morphology
- Early planting (March/April) can increase the amount of growing degree days plants can utilize between planting and winter die back, but success is dependent on weather conditions

- Later season planting (May/June) ensures no frost events but can reduce first year growth and yield as a result of reduced growing degree days in free growing field conditions
- Optimal conditions for planting under mulch film are moderate to warmer temperatures between approximately 10 - 20°C with some rainfall prior to planting.
- Temperatures at planting over approximately 25°C and with direct sunlight under dry conditions could increase the risk of desiccating smaller plant morphologies under the mulch film.
- Micro and macro environmental differences can strongly affect seedling growth in the first year, creating larger levels of field variation than initial plug morphology can cause
- Larger plants initially are not necessarily certain to remain larger throughout the growing season, but are however, more likely to survive field planting and have a higher likelihood of producing increased biomass than smaller initial morphologies
- Larger root mass is beneficial under field conditions, and larger plants with a balanced above to below ground ratio should be the goal of glasshouse production
- Additions of beneficial nitrogen fixing bacteria to plants grown in the field had little to no effect on the growth in seeded hybrids in the field; however, a positive effect was shown in *Miscanthus* giganteus when the bacterial polymer was added to the field soil.
- It is likely more positive effects would be observed under very poor nitrogen conditions and heavily depleted soil

8.7c Senescence and overwintering

- Low concentrations of hormones ethephon (250mg – 2g/L) and Methyl jasmonate (25 – 200 µM) had no effect on senescence rate of GNT13
- Higher concentrations of ethephon (5 – 20g/L) had a fatal effect when tested on *Arabidopsis*, and moderately negative effects on the growth and vigour of *Miscanthus* seedlings. Higher concentrations of Methyl jasmonate (200 – 1000 µM) continued to have no obvious effect on either species.
- The same higher concentrations of ethephon had little significant effect on mature *Miscanthus* plants, however tests with the stock concentration of 470g/L ethephon had obvious and rapid effects on leaf colouration

9 References

Alameda, D., Anten, N. P. & Villar, R. 2012. Soil compaction effects on growth and root traits of tobacco depend on light, water regime and mechanical stress. *Soil and Tillage Research,* **120,** 121-129.

Alameda, D. & Villar, R. 2009. Moderate soil compaction: implications on growth and architecture in seedlings of 17 woody plant species. *Soil and Tillage Research,* **103,** 325-331.

Alferez, F., Pozo, L. & Burns, J. K. 2006. Physiological changes associated with senescence and abscission in mature citrus fruit induced by 5-chloro-3-methyl-4-nitro-1H-pyrazole and ethephon application. *Physiologia Plantarum,* **127,** 66-73.

Alvarez-Uria, P. & Körner, C. 2007. Low temperature limits of root growth in deciduous and evergreen temperate tree species. *Functional ecology,* **21,** 211-218.

Ananieva, K., Ananiev, E. D., Mishev, K., Georgieva, K., Malbeck, J., Kamínek, M. & Van Staden, J. 2007. Methyl jasmonate is a more effective senescence-promoting factor in Cucurbita pepo (zucchini) cotyledons when compared with darkness at the early stage of senescence. *Journal of plant physiology,* **164,** 1179-1187.

Anderson, E. K., Lee, D., Allen, D. J. & Voigt, T. B. 2015. Agronomic factors in the establishment of tetraploid seeded Miscanthus × giganteus. *GCB Bioenergy,* **7,** 1075-1083.

Ashman, C., Awty-Carroll, D., Mos, M., Robson, P. & Clifton-Brown, J. 2018. Assessing seed priming, sowing date, and mulch film to improve the germination and survival of direct-sown Miscanthus sinensis in the United Kingdom. *GCB Bioenergy,* **10,** 612-627.

Atkinson, C., Brennan, R. & Jones, H. 2013. Declining chilling and its impact on temperate perennial crops. *Environmental and Experimental Botany,* **91,** 48-62.

Atkinson, C. J. 2009. Establishing perennial grass energy crops in the UK: A review of current propagation options for Miscanthus. *Biomass and bioenergy,* **33,** 752-759.

Atkinson, D. & Porter, J. R. 1996. Temperature, plant development and crop yields. *Trends in Plant Science,* **1,** 119-124.

Awty-Carroll, D., Clifton-Brown, J. & Robson, P. 2018. Using k-NN to analyse images of diverse germination phenotypes and detect single seed germination in Miscanthus sinensis. *Plant methods,* **14,** 5.

Ay, N., Janack, B. & Humbeck, K. 2014. Epigenetic control of plant senescence and linked processes. *Journal of Experimental Botany,* **65,** 3875-3887.

Baxter, X. C., Darvell, L. I., Jones, J. M., Barraclough, T., Yates, N. E. & Shield, I. 2014. Miscanthus combustion properties and variations with Miscanthus agronomy. *Fuel,* **117,** 851-869.

Beekwilder, J., Murphy, B. R., Mathuna, E., Barry, A. & Hodkinson, T. R. 2019. Isolation, diversity and potential use of endophytes in the biomass and bioenergy crop Miscanthus. *Endophytes for a Growing World***,** 188.

BEIS, D. f. 2019. UK Energy Statistics, Q1 2019.

Beltrano, J., Ronco, M. G., Montaldi, E. R. & Carbone, A. 1998. Senescence of Flag Leaves and Ears of Wheat Hastened by Methyl Jasmonate. *Journal of Plant Growth Regulation,* **17,** 53-57.

Bhattacharyya, P. N. & Jha, D. K. 2012. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World Journal of Microbiology and Biotechnology,* **28,** 1327-1350.

Boersma, N. 2013. The influence of propagation method and stand age on Miscanthus x giganteus performance in Iowa, USA.

Boersma, N. N., Dohleman, F. G., Miguez, F. E. & Heaton, E. A. 2015. Autumnal leaf senescence in Miscanthus× giganteus and leaf [N] differ by stand age. *Journal of experimental botany,* **66,** 4395-4401.

Bordiec, S., Paquis, S., Lacroix, H., Dhondt, S., Ait Barka, E., Kauffmann, S., Jeandet, P., Mazeyrat-Gourbeyre, F., Clément, C. & Baillieul, F. 2010. Comparative analysis of defence responses induced by the endophytic plant growth-promoting rhizobacterium Burkholderia phytofirmans strain PsJN and the non-host bacterium Pseudomonas syringae pv. pisi in grapevine cell suspensions. *Journal of experimental botany,* **62,** 595-603.

Bos, H., Tijani-Eniola, H. & Struik, P. 2000. Morphological analysis of leaf growth of maize: responses to temperature and light intensity. *NJAS-Wageningen Journal of Life Sciences,* **48,** 181-198.

Boyer, J. S. 1982. Plant productivity and environment. *Science,* **218,** 443-448.

Bradshaw, C. J. & Brook, B. W. 2014. Human population reduction is not a quick fix for environmental problems. *Proceedings of the National Academy of Sciences,* **111,** 16610-16615.

Brundrett, M. C. 2006. Understanding the roles of multifunctional mycorrhizal and endophytic fungi. *Microbial root endophytes.* Springer.

Campany, C. E., Medlyn, B. E. & Duursma, R. A. 2017. Reduced growth due to belowground sink limitation is not fully explained by reduced photosynthesis. *Tree physiology,* **37,** 1042-1054.

Campbell, N. A., Reece, J. B., Taylor, M. R., Simon, E. J. & Dickey, J. L. 2012. *Biology,* Petaling Jaya, Selangor, Pearson Malaysia Sdn. Bhd.

Carvajal-Muñoz, J. & Carmona-Garcia, C. 2012. Benefits and limitations of biofertilization in agricultural practices. *Livestock Research for Rural Development,* **24,** 1-8.

Chamberlain, S. A., Bronstein, J. L. & Rudgers, J. A. 2014. How context dependent are species interactions? *Ecology letters,* **17,** 881-890.

Chang, C. & Stadler, R. 2001. Ethylene hormone receptor action in Arabidopsis. *Bioessays,* **23,** 619-627.

Chen, Y., Wang, Y., Huang, J., Zheng, C., Cai, C., Wang, Q. & Wu, C.-A. 2017. Salt and methyl jasmonate aggravate growth inhibition and senescence in Arabidopsis seedlings via the JA signaling pathway. *Plant Science,* **261,** 1-9.

Chirino, E., Vilagrosa, A., Hernández, E., Matos, A. & Vallejo, V. 2008. Effects of a deep container on morpho-functional characteristics and root colonization in Quercus suber L. seedlings for reforestation in Mediterranean climate. *Forest Ecology and Management,* **256,** 779-785.

Chory, J., Chatterjee, M., Cook, R., Elich, T., Fankhauser, C., Li, J., Nagpal, P., Neff, M., Pepper, A. & Poole, D. 1996. From seed germination to flowering, light controls plant development via the pigment phytochrome. *Proceedings of the National Academy of Sciences,* **93,** 12066-12071.

Christian, D., Riche, A. & Yates, N. 2008. Growth, yield and mineral content of Miscanthus× giganteus grown as a biofuel for 14 successive harvests. *Industrial crops and products,* **28,** 320-327.

Christian, D., Yates, N. & Riche, A. 2005a. Establishing Miscanthus sinensis from seed using conventional sowing methods. *Industrial Crops and Products,* **21,** 109-111.

Christian, D. G., Yates, N. E. & Riche, A. B. 2005b. Establishing Miscanthus sinensis from seed using conventional sowing methods. *Industrial Crops and Products,* **21,** 109-111.

Clifton-Brown, J. & Lewandowski, I. 2000. Overwintering problems of newly established Miscanthus plantations can be overcome by identifying genotypes with improved rhizome cold tolerance. *The New Phytologist,* **148,** 287-294.

Clifton-Brown, J., Robson, P., Allison, G., Lister, S., Sanderson, R., Hodgson, E., Farrar, K., Hawkins, S., Jensen, E. & Jones, S. 2008. Miscanthus: breeding our way to a better future. *Aspects of Applied Biology,* **90,** 199-206.

Clifton-Brown, J., Robson, P., Sanderson, R., Hastings, A., Valentine, J. & Donnison, I. 2011. Thermal requirements for seed germination in Miscanthus compared with Switchgrass (Panicum virgatum), Reed canary grass (Phalaris arundinaceae), Maize (Zea mays) and perennial ryegrass (Lolium perenne). *GCB Bioenergy,* **3,** 375-386.

Clifton-Brown, J., Schwarz, K.-U., Awty-Carroll, D., Iurato, A., Meyer, H., Greef, J., Gwyn, J., Mos, M., Ashman, C. & Hayes, C. 2019. Breeding Strategies to Improve Miscanthus as a Sustainable Source of Biomass for Bioenergy and Biorenewable Products. *Agronomy,* **9,** 673.

Clifton-Brown, J., Schwarz, K.-U. & Hastings, A. History of the development of Miscanthus as a bioenergy crop: from small beginnings to potential realisation. Biology and Environment: Proceedings of the Royal Irish Academy, 2015. JSTOR, 45-57.

Clifton-Brown, J. C. & Lewandowski, I. 2002. Screening Miscanthus genotypes in field trials to optimise biomass yield and quality in Southern Germany. *European Journal of Agronomy,* **16,** 97-110.

Clifton-Brown, J. C., Neilson, B., Lewandowski, I. & Jones, M. B. 2000. The modelled productivity of Miscanthus×giganteus (GREEF et DEU) in Ireland. *Industrial Crops and Products,* **12,** 97-109.

Clifton-Brown, J., Hastings, A., Mos, M., McCalmont, J. P., Ashman, C., Awty-Carroll, D., Cerazy, J., Chiang, Y. C., Cosentino, S. & Cracroft-Eley, W. 2017. Progress in upscaling Miscanthus biomass production for the European bio-economy with seed-based hybrids. *Gcb Bioenergy,* **9,** 6-17.

Clifton-Brown, J. & Lewandowski, I. 2000. Overwintering problems of newly established Miscanthus plantations can be overcome by identifying genotypes with improved rhizome cold tolerance. *New Phytologist,* **148,** 287-294.

Colchin, M. 2015. Accelerating low carbon energy innovation in the UK. *Energy Technologies Institute*.

Compant, S., Clément, C. & Sessitsch, A. 2010. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. *Soil Biology and Biochemistry,* **42,** 669-678.

Cope-Selby, N., Cookson, A., Squance, M., Donnison, I., Flavell, R. & Farrar, K. 2017. Endophytic bacteria in Miscanthus seed: implications for germination, vertical inheritance of endophytes, plant evolution and breeding. *Gcb Bioenergy,* **9,** 57-77.

Cosentino, S. L., Testa, G., Scordia, D. & Copani, V. 2012. Sowing time and prediction of flowering of different hemp (Cannabis sativa L.) genotypes in southern Europe. *Industrial Crops and Products,* **37,** 20-33.

Creelman, R. A. & Mullet, J. E. 1997. Biosynthesis and action of jasmonates in plants. *Annual review of plant biology,* **48,** 355-381.

Danalatos, N. G., Archontoulis, S. V. & Mitsios, I. 2007. Potential growth and biomass productivity of Miscanthus× giganteus as affected by plant density and N-fertilization in central Greece. *Biomass and Bioenergy,* **31,** 145-152.

Davey, C. L., Robson, P., Hawkins, S., Farrar, K., Clifton-Brown, J. C., Donnison, I. S. & Slavov, G. T. 2017. Genetic relationships between spring emergence, canopy phenology, and biomass yield increase the accuracy of genomic prediction in Miscanthus. *Journal of Experimental Botany,* **68,** 5093-5102.

De Lojo, J., Gandolfo, E., Gómez, D., Feuring, V., Monti, S., Giardina, E., Boschi, C. & Di Benedetto, A. 2017. Root restriction effects on the bedding pot plant Impatiens walleriana. *Journal of Experimental Agriculture International***,** 1-16.

DECC, A. 2012. UK Bioenergy Strategy. *UK Department of Energy and Climate Change*.

Di Benedetto, A. 2011. Root restriction and post-transplant effects for bedding pot plants. *In:* AQUINO, J. C. (ed.) *Ornamental plants: Types, Cultivation and Nutrition.* Nova Science Publishers, Inc.

Di Benedetto, A. & Klasman, R. 2004. The effect of plug cell volume on the post-transplant growth for Impatiens walleriana pot plant. *European Journal of Horticultural Science,* **69,** 82-86.

Diesburg, K. 1999. A new growth regulator for golf course turfgrass. *Golf Course Manage,* **67,** 49-51.

Distelfeld, A., Avni, R. & Fischer, A. M. 2014. Senescence, nutrient remobilization, and yield in wheat and barley. *Journal of experimental botany,* **65,** 3783-3798.

Dohleman, F. G. & Long, S. P. 2009. More Productive Than Maize in the Midwest: How Does Miscanthus Do It? *Plant Physiology,* **150,** 2104.

Dominguez-Lerena, S., Herrero Sierra, N., Carrasco Manzano, I., Ocaña Bueno, L., Peñuelas Rubira, J. L. & Mexal, J. G. 2006. Container characteristics influence Pinus pinea seedling development in the nursery and field. *Forest Ecology and Management,* **221,** 63-71.

Easson & Fearnehough 2000. Effects of plastic mulch, sowing date and cultivar on the yield and maturity of forage maize grown under marginal climatic conditions in Northern Ireland. *Grass and Forage Science,* **55,** 221-231.

Farrar, K., Bryant, D. & Cope-Selby, N. 2014. Understanding and engineering beneficial plant–microbe interactions: plant growth promotion in energy crops. *Plant biotechnology journal,* **12,** 1193-1206.

Fascella, G. & Rouphael, Y. 2017. Influence of container volume and irrigation system on photosynthesis, water productivity and growth of potted Euphorbia× lomi. *Acta Scientiarum Polonorum Hortorum Cultus,* **16,** 163-171.

Fay, P. A. & Schultz, M. J. 2009. Germination, survival, and growth of grass and forb seedlings: effects of soil moisture variability. *Acta Oecologica,* **35,** 679-684.

Fei, H., Crouse, M., Papdopoulos, Y. A. & Vessey, J. K. 2019. Improving biomass yield of giant Miscanthus by application of beneficial soil microbes and a plant biostimulant. *Canadian Journal of Plant Science*.

Felten, D., Fröba, N., Fries, J. & Emmerling, C. 2013. Energy balances and greenhouse gas-mitigation potentials of bioenergy cropping systems (Miscanthus, rapeseed, and maize) based on farming conditions in Western Germany. *Renewable Energy,* **55,** 160-174.

Fleischer, W. E. 1935. The relation between chlorophyll content and rate of photosynthesis. *The Journal of General Physiology,* **18,** 573-597.

Gazzarrini, S. & McCourt, P. 2003. Cross-talk in plant hormone signalling: what Arabidopsis mutants are telling us. *Annals of botany,* **91,** 605-612.

Ghosh, S., Watson, A., Gonzalez-Navarro, O. E., Ramirez-Gonzalez, R. H., Yanes, L., Mendoza-Suárez, M., Simmonds, J., Wells, R., Rayner, T. & Green, P. 2018. Speed breeding in growth chambers and glasshouses for crop breeding and model plant research. *Nature protocols,* **13,** 2944.

Gommers, C. M., Visser, E. J., St Onge, K. R., Voesenek, L. A. & Pierik, R. 2013. Shade tolerance: when growing tall is not an option. *Trends in plant science,* **18,** 65-71.

Grbić, V. & Bleecker, A. B. 1995. Ethylene regulates the timing of leaf senescence in Arabidopsis. *The Plant Journal,* **8,** 595-602.

Gregersen, P. L., Culetic, A., Boschian, L. & Krupinska, K. 2013. Plant senescence and crop productivity. *Plant molecular biology,* **82,** 603-622.

Gross, K. L. 1984. Effects of seed size and growth form on seedling establishment of six monocarpic perennial plants. *The Journal of Ecology***,** 369-387.

Gull, M. & Hafeez, F. Y. 2012. Characterization of siderophore producing bacterial strain Pseudomonas fluorescens Mst 8.2 as plant growth promoting and biocontrol agent in wheat. *African Journal of Microbiology Research,* **6,** 6308-6318.

Haase, D. L. 2008. Understanding forest seedling quality: measurements and interpretation. *Tree Planters' Notes,* **52,** 24-30.

Harris, R. W. 1992. Root-shoot ratios. *Journal of Arboriculture,* **18,** 39-42.

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

Hastings, A., Clifton-Brown, J., Wattenbach, M., Stampfl, P., Mitchell, C. P. & Smith, P. 2008. Potential of Miscanthus grasses to provide energy and hence reduce greenhouse gas emissions. *Agronomy for sustainable development,* **28,** 465-472.

Hastings, A., Mos, M., Yesufu, J. A., McCalmont, J., Schwarz, K., Shafei, R., Ashman, C., Nunn, C., Schuele, H. & Cosentino, S. 2017a. Economic and environmental assessment of seed and rhizome propagated Miscanthus in the UK. *Frontiers in plant science,* **8,** 1058.

Hastings, A., Mos, M., Yesufu, J. A., McCalmont, J., Schwarz, K., Shafei, R., Ashman, C., Nunn, C., Schuele, H., Cosentino, S., Scalici, G., Scordia, D., Wagner, M. & Clifton-Brown, J. 2017b. Economic and Environmental Assessment of Seed and Rhizome Propagated Miscanthus in the UK. *Frontiers in Plant Science,* **8**.

Hayashi, T., Heins, R. D., Cameron, A. C. & Carlson, W. H. 2001. Ethephon influences flowering, height, and branching of several herbaceous perennials. *Scientia Horticulturae,* **91,** 305-324.

He, Y., Fukushige, H., Hildebrand, D. F. & Gan, S. 2002. Evidence supporting a role of jasmonic acid in Arabidopsis leaf senescence. *Plant physiology,* **128,** 876-884.

Heaton, E. A., Long, S. P., Voigt, T. B., Jones, M. B. & Clifton-Brown, J. 2004. Miscanthus for renewable energy generation: European Union experience and projections for Illinois. *Mitigation and Adaptation Strategies for Global Change,* **9,** 433-451.

Helliwell, R. 2018. Where did the marginal land go? Farmers perspectives on marginal land and its implications for adoption of dedicated energy crops. *Energy Policy,* **117,** 166-172.

Herrmann, G., Lehmann, J., Peterson, A., Sembdner, G., Weidhase, R. & Parthier, B. 1989. Species and tissue specificity of jasmonate-induced abundant proteins. *Journal of Plant Physiology,* **134,** 703-709.

Herzog, T. 2009. World greenhouse gas emissions in 2005. *World Resources Institute*.

Ho, M. D., Rosas, J. C., Brown, K. M. & Lynch, J. P. 2005. Root architectural tradeoffs for water and phosphorus acquisition. *Functional plant biology,* **32,** 737-748.

IPCC. 2018. Global Warming of 1.5°C. An IPCC Special Report on the impacts of global warming of 1.5°C above pre-industrial levels and related global greenhouse gas emission pathways, in the context of strengthening the global response to the threat of climate change, sustainable development, and efforts to eradicate poverty. MASSON-DELMOTTE, V., P. ZHAI, H.-O. PÖRTNER, D. ROBERTS, J. SKEA, P.R. SHUKLA, A. PIRANI, W. MOUFOUMA-OKIA, C. PÉAN, R. PIDCOCK, S. CONNORS, J.B.R. MATTHEWS, Y. CHEN, X. ZHOU, M.I. GOMIS, E. LONNOY, T. MAYCOCK, M. TIGNOR, AND T. WATERFIELD (IN PRESS) (ed.)

Iqbal, N., Khan, N. A., Ferrante, A., Trivellini, A., Francini, A. & Khan, M. I. R. 2017. Ethylene Role in Plant Growth, Development and Senescence: Interaction with Other Phytohormones. *Frontiers in Plant Science,* **8**.

Iwasa, Y. & Roughgarden, J. 1984. Shoot/root balance of plants: optimal growth of a system with many vegetative organs. *Theoretical population biology,* **25,** 78-105.

Jacobs, D. F., Goodman, R. C., Gardiner, E. S., Salifu, K. F., Overton, R. P. & Hernandez, G. 2012. Nursery stock quality as an indicator of bottomland hardwood forest restoration success in the Lower Mississippi River Alluvial Valley. *Scandinavian journal of forest research,* **27,** 255-269.

Jaffe, M. J. 1973. Thigmomorphogenesis: the response of plant growth and development to mechanical stimulation. *Planta,* **114,** 143-157.

Jensen, E., Robson, P., Farrar, K., Thomas Jones, S., Clifton-Brown, J., Payne, R. & Donnison, I. 2017. Towards Miscanthus combustion quality improvement: the role of flowering and senescence. *GCB Bioenergy,* **9,** 891-908.

Jeżowski, S. 2008. Yield traits of six clones of Miscanthus in the first 3 years following planting in Poland. *Industrial Crops and Products,* **27,** 65-68.

Jibran, R., Hunter, D. A. & Dijkwel, P. P. 2013. Hormonal regulation of leaf senescence through integration of developmental and stress signals. *Plant Molecular Biology,* **82,** 547-561.

Jing, H.-C., Hille, J. & Dijkwel, P. P. 2003. Ageing in plants: conserved strategies and novel pathways. *Plant Biology,* **5,** 455-464.

Jones, G. A. & Warner, K. J. 2016. The 21st century population-energy-climate nexus. *Energy Policy,* **93,** 206-212.

Jones, M. B., Finnan, J. & Hodkinson, T. R. 2015. Morphological and physiological traits for higher biomass production in perennial rhizomatous grasses grown on marginal land. *GCB Bioenergy,* **7,** 375-385.

Kam, J., Traynor, D., Clifton-Brown, J. C., Purdy, S. J. & McCalmont, J. P. 2020. Miscanthus as Energy Crop and Means of Mitigating Flood. *Frontiers in Water-Energy-Nexus—Nature-Based Solutions, Advanced Technologies and Best Practices for Environmental Sustainability.* Springer.

Keller, H., Pamboukdjian, N., Ponchet, M., Poupet, A., Delon, R., Verrier, J.-L., Roby, D. & Ricci, P. 1999. Pathogen-induced elicitin production in transgenic tobacco generates a hypersensitive response and nonspecific disease resistance. *The Plant Cell,* **11,** 223-235.

Khan, A. & Singh, Z. 2007. Methyl jasmonate promotes fruit ripening and improves fruit quality in Japanese plum. *The Journal of Horticultural Science and Biotechnology,* **82,** 695-706.

Kim, D.-H., Doyle, M. R., Sung, S. & Amasino, R. M. 2009. Vernalization: Winter and the Timing of Flowering in Plants. *Annual Review of Cell and Developmental Biology,* **25,** 277-299.

Kim, S., Lowman, S., Hou, G., Nowak, J., Flinn, B. & Mei, C. 2012. Growth promotion and colonization of switchgrass (Panicum virgatum) cv. Alamo by bacterial endophyte Burkholderia phytofirmans strain PsJN. *Biotechnology for Biofuels,* **5,** 37.

Kloepper, J. W., Leong, J., Teintze, M. & Schroth, M. N. 1980. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. *Nature,* **286,** 885-886.

Koyama, T., Nii, H., Mitsuda, N., Ohta, M., Kitajima, S., Ohme-Takagi, M. & Sato, F. 2013. A Regulatory Cascade Involving Class II ETHYLENE RESPONSE FACTOR Transcriptional Repressors Operates in the Progression of Leaf Senescence. *Plant Physiology,* **162,** 991-1005.

Krzyżak, J., Pogrzeba, M., Rusinowski, S., Clifton-Brown, J., McCalmont, J. P., Kiesel, A., Mangold, A. & Mos, M. 2017. Heavy Metal Uptake by Novel Miscanthus Seed-Based Hybrids Cultivated in Heavy Metal Contaminated Soil. **26,** 121.

Kumar, N. R., Arasu, V. T. & Gunasekaran, P. 2002. Genotyping of antifungal compounds producing plant growth-promoting rhizobacteria, Pseudomonas fluorescens. *Current Science***,** 1463-1466.

Lal, R. 2004. Soil carbon sequestration to mitigate climate change. *Geoderma,* **123,** 1-22.

Lam, E., Kato, N. & Lawton, M. 2001. Programmed cell death, mitochondria and the plant hypersensitive response. *Nature,* **411,** 848.

Lee, Y. K. & Mazmanian, S. K. 2010. Has the Microbiota Played a Critical Role in the Evolution of the Adaptive Immune System? *Science,* **330,** 1768-1773.

Leopold, A. 1961. Senescence in plant development. *Science,* **134,** 1727-1732.

Lewandowski, I., Clifton-Brown, J., Scurlock, J. & Huisman, W. 2000. Miscanthus: European experience with a novel energy crop. *Biomass and Bioenergy,* **19,** 209-227.

Lewandowski, I., Clifton-Brown, J., Trindade, L. M., van der Linden, G. C., Schwarz, K.-U., Müller-Sämann, K., Anisimov, A., Chen, C.-L., Dolstra, O. & Donnison, I. S. 2016. Progress on optimizing miscanthus biomass production for the European bioeconomy: Results of the EU FP7 project OPTIMISC. *Frontiers in plant science,* **7,** 1620.

Lewandowski, I. & Heinz, A. 2003. Delayed harvest of miscanthus—influences on biomass quantity and quality and environmental impacts of energy production. *European Journal of Agronomy,* **19,** 45-63.

Lewandowski, I. & Kicherer, A. 1997. Combustion quality of biomass: practical relevance and experiments to modify the biomass quality of Miscanthus x giganteus. *European Journal of Agronomy,* **6,** 163-177.

Li, Y. & Solomon, S. 2003. Ethephon : A versatile growth regulator for sugar cane industry. *Sugar Tech,* **5,** 213-223.

Lin, K.-H., Huang, M.-Y., Huang, W.-D., Hsu, M.-H., Yang, Z.-W. & Yang, C.-M. 2013. The effects of red, blue, and white light-emitting diodes on the growth, development, and edible quality of hydroponically grown lettuce (Lactuca sativa L. var. capitata). *Scientia Horticulturae,* **150,** 86-91.

Lowe, K., Bowdler, T., Hume, D., Casey, N. & Tapper, B. 2008. The effect of endophyte on the performance of irrigated perennial ryegrasses in subtropical Australia. *Australian Journal of Agricultural Research,* **59,** 567-577.

Lucy, M., Reed, E. & Glick, B. R. 2004. Applications of free living plant growth-promoting rhizobacteria. *Antonie van Leeuwenhoek,* **86,** 1-25.

Malladi, A., Vashisth, T. & Johnson, L. K. 2012. Ethephon and methyl jasmonate affect fruit detachment in rabbiteye and southern highbush blueberry. *HortScience,* **47,** 1745-1749.

Mayerhofer, M. S., Kernaghan, G. & Harper, K. A. 2013. The effects of fungal root endophytes on plant growth: a meta-analysis. *Mycorrhiza,* **23,** 119-128.

McCalmont, J. P., Hastings, A., McNamara, N. P., Richter, G. M., Robson, P., Donnison, I. S. & Clifton-Brown, J. 2015. Environmental costs and benefits of growing Miscanthus for bioenergy in the UK. *GCB Bioenergy*.

McCalmont, J. P., Hastings, A., McNamara, N. P., Richter, G. M., Robson, P., Donnison, I. S. & Clifton-Brown, J. 2017. Environmental costs and benefits of growing Miscanthus for bioenergy in the UK. *Gcb Bioenergy,* **9,** 489-507.

McIntyre, R. Field experiments to test the performance of sugarcane transplants. Proceedings of the South African Sugar Technologists' Association, 1993. 98-101.

McNearny, P., Riley, J. & Wennersten, A. 2002. Trampling increases soil compaction; soil compaction depresses vigor of Andropogon gerardii. *Tillers,* **3,** 25-28.

McSteen, P. 2009. Hormonal Regulation of Branching in Grasses. *Plant Physiology,* **149,** 46-55.

McTague, J. P. & Tinus, R. W. 1996. The effects of seedling quality and forest site weather on field survival of ponderosa pine. *Tree planters notes,* **47,** 16-23.

Mei, C. & Flinn, B. S. 2010. The use of beneficial microbial endophytes for plant biomass and stress tolerance improvement. *Recent Patents on Biotechnology,* **4,** 81-95.

Melton, R. R. & Dufault, R. J. 1991. Tomato seedling growth, earliness, yield, and quality following pretransplant nutritional conditioning and low temperatures. *Journal of the American Society for Horticultural Science,* **116,** 421-425.

Miedema, P., Post, J. & Groot, P. 1987. The effects of low temperature on seedling growth of maize genotypes. Pudoc.

Milborrow, B. 1974. The chemistry and physiology of abscisic acid. *Annual Review of Plant Physiology,* **25,** 259-307.

Mishustin, E. & Naumova, A. 1962. Bacterial fertilizers, their effectiveness and mode of action. *Mikrobiologiya,* **31,** 543-555.

Mos, M., Banks, S. W., Nowakowski, D., Robson, P., Bridgwater, A. & Donnison, I. S. 2013. Impact of Miscanthus x giganteus senescence times on fast pyrolysis bio-oil quality. *Bioresource technology,* **129,** 335-342.

Mukkun, L. & Singh, Z. 2009. Methyl jasmonate plays a role in fruit ripening of 'Pajaro'strawberry through stimulation of ethylene biosynthesis. *Scientia Horticulturae,* **123,** 5-10.

Munné-Bosch, S. 2008. Do perennials really senesce? *Trends in Plant Science,* **13,** 216-220.

Muthukumarasamy, R., Revathi, G. & Loganathan, P. 2002. Effect of inorganic N on the population, in vitro colonization and morphology of Acetobacter diazotrophicus (syn. Gluconacetobacter diazotrophicus). *Plant and Soil,* **243,** 91-102.

Naidu, S. L., Moose, S. P., Al-Shoaibi, A. K., Raines, C. A. & Long, S. P. 2003. Cold tolerance of C4 photosynthesis in Miscanthus× giganteus: adaptation in amounts and sequence of C4 photosynthetic enzymes. *Plant physiology,* **132,** 1688-1697.

NeSmith, D. S. & Duval, J. R. 1998. The effect of container size. *HortTechnology,* **8,** 495-498.

Noodén, L. D., Guiamét, J. J. & John, I. 2004. 15 - Whole Plant Senescence. *In:* NOODÉN, L. D. (ed.) *Plant Cell Death Processes.* San Diego: Academic Press.

Nowak, J., Asiedu, S. K., Bensalim, S., Richards, J., Stewart, A., Smith, C., Stevens, D. & Sturz, A. V. 1998. From laboratory to applications: challenges and progress with in vitro dual cultures of potato and beneficial bacteria. *Plant Cell, Tissue and Organ Culture,* **52,** 97-103.

Nykiforuk, C. L. & Johnson-Flanagan, A. M. 1999. Storage reserve mobilization during low temperature germination and early seedling growth in Brassica napus. *Plant physiology and biochemistry,* **37,** 939-947.

O'Loughlin, J., Finnan, J. & McDonnell, K. 2017. Accelerating early growth in Miscanthus with the application of plastic mulch film. *Biomass and Bioenergy,* **100,** 52-61.

Olle, M. & Viršile, A. 2013. The effects of light-emitting diode lighting on greenhouse plant growth and quality. *Agricultural and food science,* **22,** 223-234.

Pachauri, R. K., Allen, M. R., Barros, V., Broome, J., Cramer, W., Christ, R., Church, J., Clarke, L., Dahe, Q. & Dasgupta, P. 2014. *Climate change 2014: synthesis Report. Contribution of working groups I, II and III to the fifth assessment report of the intergovernmental panel on climate change*, IPCC.

Peleg, Z. & Blumwald, E. 2011. Hormone balance and abiotic stress tolerance in crop plants. *Current opinion in plant biology,* **14,** 290-295.

Pinto, J. R., Marshall, J. D., Dumroese, R. K., Davis, A. S. & Cobos, D. R. 2011. Establishment and growth of container seedlings for reforestation: A function of stocktype and edaphic conditions. *Forest Ecology and Management,* **261,** 1876-1884.

Poorter, H., Bühler, J., van Dusschoten, D., Climent, J. & Postma, J. A. 2012. Pot size matters: a meta-analysis of the effects of rooting volume on plant growth. *Functional Plant Biology,* **39,** 839-850.

Porat, R., Borochov, A. & Halevy, A. H. 1993. Enhancement of petunia and dendrobium flower senescence by jasmonic acid methyl ester is via the promotion of ethylene production. *Plant Growth Regulation,* **13,** 297-301.

Purdy, S. J., Cunniff, J., Maddison, A. L., Jones, L. E., Barraclough, T., Castle, M., Davey, C. L., Jones, C. M., Shield, I. & Gallagher, J. 2015. Seasonal carbohydrate dynamics and climatic regulation of senescence in the perennial grass, Miscanthus. *BioEnergy Research,* **8,** 28-41.

Raaijmakers, J. M. & Weller, D. M. 2001. Exploiting Genotypic Diversity of 2,4-Diacetylphloroglucinol-Producing Pseudomonas spp.: Characterization of Superior Root-Colonizing P. fluorescensStrain Q8r1-96. *Applied and Environmental Microbiology,* **67,** 2545-2554.

Rademacher, W. 2000. GROWTH RETARDANTS: Effects on Gibberellin Biosynthesis and Other Metabolic Pathways. *Annual Review of Plant Physiology and Plant Molecular Biology,* **51,** 501-531.

Reid, W. V., Ali, M. K. & Field, C. B. 2020. The future of bioenergy. *Global Change Biology,* **26,** 274-286.

Robson, P., Jensen, E., Hawkins, S., White, S. R., Kenobi, K., Clifton-Brown, J., Donnison, I. & Farrar, K. 2013a. Accelerating the domestication of a bioenergy crop: identifying and

modelling morphological targets for sustainable yield increase in Miscanthus. *Journal of Experimental Botany,* **64,** 4143-4155.

Robson, P., Mos, M., Clifton-Brown, J. & Donnison, I. 2012. Phenotypic variation in senescence in Miscanthus: towards optimising biomass quality and quantity. *Bioenergy Research,* **5,** 95-105.

Robson, P., Mos, M., Dee, H., Clifton-Brown, J., Donnison, I., Booth, E., Halford, N., Shield, I., Taylor, G. & Turley, D. 2011. Improving bioenergy crop yield and quality through manipulating senescence. *Aspects of Applied Biology***,** 323-332.

Robson, P. R., Donnison, I. S. & Clifton-Brown, J. C. 2019. Stem growth characteristics of high yielding Miscanthus correlate with yield, development and intraspecific competition within plots. *GCB Bioenergy*.

Robson, P. R., Farrar, K., Gay, A. P., Jensen, E. F., Clifton-Brown, J. C. & Donnison, I. S. 2013b. Variation in canopy duration in the perennial biofuel crop Miscanthus reveals complex associations with yield. *Journal of experimental botany***,** ert104.

Rogers, H. H., Prior, S. A., Runion, G. B. & Mitchell, R. J. 1995. Root to shoot ratio of crops as influenced by CO 2. *Plant and soil,* **187,** 229-248.

Rothballer, M., Eckert, B., Schmid, M., Fekete, A., Schloter, M., Lehner, A., Pollmann, S. & Hartmann, A. 2008. Endophytic root colonization of gramineous plants by Herbaspirillum frisingense. *FEMS microbiology ecology,* **66,** 85-95.

Samco 2014. SAMCO Brochure. Limerick, Ireland: Samco Agricultural Manufacturing Ltd.

Samuolienė, G., Brazaitytė, A., Urbonavičiūtė, A., Šabajevienė, G. & Duchovskis, P. 2010. The effect of red and blue light component on the growth and development of frigo strawberries. *Zemdirbyste-Agriculture,* **97,** 99-104.

Sarwat, M., Naqvi, A. R., Ahmad, P., Ashraf, M. & Akram, N. A. 2013. Phytohormones and microRNAs as sensors and regulators of leaf senescence: assigning macro roles to small molecules. *Biotechnology advances,* **31,** 1153-1171.

Scally, L., Waldren, S., Hodkinson, T. & Jones, M. 2001. Morphological and molecular systematics of the genus Miscanthus. *Aspects of Applied Biology***,** 231-237.

Schippers, J. H., Jing, H.-C., Hille, J. & Dijkwel, P. P. 2007. Developmental and hormonal control of leaf senescence. *Senescence processes in plants,* **26,** 145-170.

Schlemper, T. R., van Veen, J. A. & Kuramae, E. E. 2018. Co-variation of bacterial and fungal communities in different sorghum cultivars and growth stages is soil dependent. *Microbial ecology,* **76,** 205-214.

Schmidt, C. S., Mrnka, L., Frantík, T., Lovecká, P. & Vosátka, M. 2018. Plant growth promotion of Miscanthus× giganteus by endophytic bacteria and fungi on non-polluted and polluted soils. *World Journal of Microbiology and Biotechnology,* **34,** 48.

Schuerger, A. C., Brown, C. S. & Stryjewski, E. C. 1997. Anatomical features of pepper plants (Capsicum annuumL.) grown under red light-emitting diodes supplemented with blue or far-red light. *Annals of Botany,* **79,** 273-282.

Schultz, R. & Thompson, J. 1997. Effect of density control and undercutting on root morphology of 1+ 0 bareroot hardwood seedlings: five-year field performance of root-graded stock in the central USA. *New Forests,* **13,** 301-314.

Schwarz, H., Liebhard, P., Ehrendorfer, K. & Ruckenbauer, P. 1994. The effect of fertilization on yield and quality of Miscanthus sinensis 'Giganteus'. *Industrial Crops and Products,* **2,** 153-159.

Sherrington, C., Bartley, J. & Moran, D. 2008. Farm-level constraints on the domestic supply of perennial energy crops in the UK. *Energy Policy,* **36,** 2504-2512.

Shrestha, P., Ibáñez, A. B., Bauer, S., Glassman, S. I., Szaro, T. M., Bruns, T. D. & Taylor, J. W. 2015. Fungi isolated from Miscanthus and sugarcane: biomass conversion, fungal enzymes, and hydrolysis of plant cell wall polymers. *Biotechnology for biofuels,* **8,** 38.

Simon, E., Minchin, A., McMenamin, M. M. & Smith, J. 1976. The low temperature limit for seed germination. *New Phytologist,* **77,** 301-311.

Sladek, B. S., Henry, G. M. & Auld, D. L. 2011. Effect of genotype, planting date, and spacing on zoysiagrass establishment from vegetative plugs. *HortScience,* **46,** 1194-1197.

South, D. B. 2000. Planting morphologically improved pine seedlings to increase survival and growth.

Souza, R. d., Ambrosini, A. & Passaglia, L. M. 2015. Plant growth-promoting bacteria as inoculants in agricultural soils. *Genetics and molecular biology,* **38,** 401-419.

Sowiński, P., Rudzińska-Langwald, A., Adamczyk, J., Kubica, I. & Fronk, J. 2005. Recovery of maize seedling growth, development and photosynthetic efficiency after initial growth at low temperature. *Journal of Plant Physiology,* **162,** 67-80.

Spaepen, S., Vanderleyden, J. & Okon, Y. 2009. Plant growth-promoting actions of rhizobacteria. *Advances in botanical research,* **51,** 283-320.

Stepanova, A. N. & Alonso, J. M. 2009. Ethylene signaling and response: where different regulatory modules meet. *Current Opinion in Plant Biology,* **12,** 548-555.

Straub, D., Rothballer, M., Hartmann, A. & Ludewig, U. 2013. The genome of the endophytic bacterium H. frisingense GSF30T identifies diverse strategies in the Herbaspirillum genus to interact with plants. *Frontiers in microbiology,* **4,** 168.

Sultan, S. E. 2000. Phenotypic plasticity for plant development, function and life history. *Trends in plant science,* **5,** 537-542.

Taur, Y. & Pamer, E. G. 2013. The intestinal microbiota and susceptibility to infection in immunocompromised patients. *Current opinion in infectious diseases,* **26,** 332.

Tejera, M., Boersma, N., Vanloocke, A., Archontoulis, S., Dixon, P., Miguez, F. & Heaton, E. 2019. Multi-year and Multi-site Establishment of the Perennial Biomass Crop Miscanthus× giganteus Using a Staggered Start Design to Elucidate N Response. *BioEnergy Research***,** 1-13.

Tellenbach, C., Grünig, C. R. & Sieber, T. N. 2011. Negative effects on survival and performance of Norway spruce seedlings colonized by dark septate root endophytes are primarily isolate-dependent. *Environmental Microbiology,* **13,** 2508-2517.

Thomas, H. & Ougham, H. 2014. The stay-green trait. *Journal of Experimental Botany,* **65,** 3889-3900.

Tsakaldimi, M., Ganatsas, P. & Jacobs, D. F. 2013. Prediction of planted seedling survival of five Mediterranean species based on initial seedling morphology. *New forests,* **44,** 327-339.

Ueda, J. & Kato, J. 1980. Isolation and Identification of a Senescence-promoting Substance from Wormwood (Artemisia absinthium L.). *Plant Physiology,* **66,** 246-249.

Valentine, J., Clifton-Brown, J., Hastings, A., Robson, P., Allison, G. & Smith, P. 2012. Food vs. fuel: the use of land for lignocellulosic 'next generation'energy crops that minimize competition with primary food production. *Gcb Bioenergy,* **4,** 1-19.

Vessey, J. K. 2003. Plant growth promoting rhizobacteria as biofertilizers. *Plant and soil,* **255,** 571-586.

Wang, C. Y. 1989. Relation of chilling stress to ethylene production. *Low Temperature Stress Physiology in Crops.* CRC Press.

Wang, D., Portis, A. R., Moose, S. P. & Long, S. P. 2008. Cool C4 photosynthesis: pyruvate Pi dikinase expression and activity corresponds to the exceptional cold tolerance of carbon assimilation in Miscanthus× giganteus. *Plant Physiology,* **148,** 557-567.

Wang, Y., Zhang, Y. & Wang, L. 2018. Cross regulatory network between circadian clock and leaf senescence is emerging in higher plants. *Frontiers in plant science,* **9,** 700.

Weiner, J. 1985. Size hierarchies in experimental populations of annual plants. *Ecology,* **66,** 743-752.

Westoby, M., Leishman, M. & Lord, J. 1996. Comparative ecology of seed size and dispersal. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences,* **351,** 1309-1318.

White, A. C., Rogers, A., Rees, M. & Osborne, C. P. 2015. How can we make plants grow faster? A source–sink perspective on growth rate. *Journal of Experimental Botany,* **67,** 31-45.

Witzel, C.-P. & Finger, R. 2016. Economic evaluation of Miscanthus production – A review. *Renewable and Sustainable Energy Reviews,* **53,** 681-696.

Xiang, M., YI, Z., ZHENG, C., XIANG, W. & XIAO, L. 2018. Effect of environmental factors and sowing depth on seed germination and seedling growth of Miscanthus lutarioriparius. *Journal of Hunan Agricultural University (Natural Sciences)***,** 9.

Xue, S., Kalinina, O. & Lewandowski, I. 2015. Present and future options for Miscanthus propagation and establishment. *Renewable and sustainable energy reviews,* **49,** 1233-1246.

Yost, M. A., Randall, B. K., Kitchen, N. R., Heaton, E. A. & Myers, R. L. 2017. Yield potential and nitrogen requirements of Miscanthus× giganteus on eroded soil. *Agronomy Journal,* **109,** 684-695.

Zhang, C., Gao, M., Seitz, N. C., Angel, W., Hallworth, A., Wiratan, L., Darwish, O., Alkharouf, N., Dawit, T. & Lin, D. 2019. LUX ARRHYTHMO mediates crosstalk between the circadian clock and defense in Arabidopsis. *Nature communications,* **10,** 2543.

Zhang, W. & Wen, C.-K. 2010. Preparation of ethylene gas and comparison of ethylene responses induced by ethylene, ACC, and ethephon. *Plant Physiology and Biochemistry,* **48,** 45-53.

Zhu, X.-G., Long, S. P. & Ort, D. R. 2008. What is the maximum efficiency with which photosynthesis can convert solar energy into biomass? *Current Opinion in Biotechnology,* **19,** 153-159.

Zimmermann, J., Styles, D., Hastings, A., Dauber, J. & Jones, M. B. 2014. Assessing the impact of within crop heterogeneity ('patchiness') in young Miscanthus× giganteus fields on economic feasibility and soil carbon sequestration. *Gcb Bioenergy,* **6,** 566-576.

Zub, H., Rambaud, C., Béthencourt, L. & Brancourt-Hulmel, M. 2012. Late emergence and rapid growth maximize the plant development of Miscanthus clones. *BioEnergy Research,* **5,** 841-854.