



UNDERSTANDING
GERMINATION FOR IMPROVED
PROPAGATION AND FIELD
ESTABLISHMENT OF THE
BIOENERGY CROP
MISCANTHUS

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

by

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2017

DECLARATION

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

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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.

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SUMMARY

Miscanthus is an important source of biomass and can be grown on marginal sites so as not to compete with food crops. The crop requires few inputs, is perennial, and yield increases to economic levels over around three years, so *Miscanthus* must be cost effective to establish. Establishment by seed is more cost effective than by rhizome; therefore, improving seed agronomy is vital for the success of the crop. This study contributes an assessment of many previously unknown factors on *Miscanthus* germination. Hormone treatments and cluster sowings are tested, predominantly for direct sowing but also for plug-based establishment. Cluster sowings improved the chance of establishing a plot, some first year competition effects were identified; however, the number of seeds required may not be economic without additional treatments. An optimal set of treatments for successful germination was identified using a Taguchi design experiment. Complex hormone interactions were tested plus effects of light and seed priming. Methodologies and automation of seed germination assessment were developed. The unreliability of manual seed germination scoring was highlighted and germination scoring was only assisted by imaging due to low throughput. A selection of *Miscanthus* genotypes were characterised, for base temperatures of germination and elongation. From this information, a model was parameterised for *Miscanthus* seed germination; this was tested against different sowing methods in field and laboratory conditions and against real data collected from experiments that tested first year agronomic methods, such as sowing time, film, and seed priming. Film was found to have positive temperature effect but mixed effects on germination due to soil water, while priming had little benefit. This model can be used and refined further to test and develop hypotheses for future improvements in seed agronomy.

ACKNOWLEDGMENTS

Thanks to Paul Robson and John Clifton-Brown, for thoughtful support, supervision, and ideas. They made this study possible, with the funding from the BBSRC, GIANT-LINK, and Ceres Inc., particular thanks to Ceres' Richard Flavell FRS for advice and careful questioning of results. Ceres and Terravesta ltd provided opportunities for commercial collaboration, experience, and information sharing during the study. The experiments in this study were made easier with the practical help of the *Miscanthus* breeding team, particularly Chris Ashman and Michal Mos with which much collaboration on field trials was done, also Robin Warren and Chris Glover for laying mulch film in a variety of field conditions. My thanks to Sarah Hawkins, Sue Youell and Marc Loosley for explaining where things were, in the laboratory and in the greenhouse, and how to operate them. Thanks to my fellow PhD students, Marta Malinowska and Evangelia Stavridou for advice on R and statistics, along with much StackOverflow, and recent collaboration with Rebecca Jane Wilson. Some additional thanks goes to MS word™ for much error checking, and to Hadley Wickham for creating his supporting R packages ggplot2 (Wickham, 2009), reshape2 (Wickham, 2007) and dplyr (Wickham & Francois, 2016).

Special thanks go to my partner Katie for much love, support, and proof reading, as well as my parents for listening to years of *Miscanthus* germination problems.

ABBREVIATIONS

ABA	–	Absciscic Acid
BR	–	Brassinosteroid
GA	–	Gibberellic Acid
GI	–	Germination Index
HSB	–	Hue Saturation Brightness
<i>k</i> -NN	–	<i>k</i> -Nearest Neighbour
OEC	–	Overall Evaluation Criteria
PAR	–	Photosynthetically Active Radiation
PCA	–	Principle Components Analysis
PPFD	–	Photosynthetic Photon Flux Density
PPMC	–	Pearson’s Product Moment Correlation
RGB	–	Red Green Blue
RH	–	Relative Humidity
ROC	–	Receiver Operating Characteristic
SDW	–	Sterile Distilled Water
SimPIE	–	SIMulation of PLant Emergence
TiD	–	Time in the Dark

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1 BACKGROUND

1.1 MOTIVATION

Modern civilization requires energy and more of it than at any time in history, a large amount of which is used to grow and transport food (Valentine et al., 2012). The recent reliance on fossil fuels for energy has a number of problems both environmentally, and with the longevity and security of supply and therefore biofuels are becoming an important consideration in securing a sustainable and less environmentally damaging energy supply. Within the UK transport sector the government has committed to EU Directive 2009/28/EC requiring “10% of final energy consumption in the transport sector” to be derived from biofuels by 2020 (DEFRA, 2013), which will continue to be a target until a formal EU exit.

Due to biofuel targets of this kind, biofuel production is important and dependent upon obtaining high yields from specialised crops. Approximately 95% of 2010-11 UK biofuel production was derived from traditional crops repurposed for fuel (DEFRA, 2013). These are the crops UK farmers are most experienced at growing for food; however, being repurposed in this manner is not the most efficient approach for energy production because they utilise prime agricultural land and are in themselves energy intensive in their cultivation and therefore net energy returns are limited. As an example, to meet targets using Sugar Beet or straw would require 10% and 45% of arable land in the UK respectively (Angus et al., 2009). In the UK the net energy balance for Sugar Beet is between

7.3 and 15 (Tzilivakis et al., 2005), compared to *Miscanthus*' net energy balance of between 14 and 20 (Lewandowski et al., 2000). Wheat is currently the most widely grown biofuel accounting for 36% of the current biofuel area (DEFRA, 2013). There is now movement towards a new generation of plants bred specifically for biofuel via their biomass, these are often ligno-cellulose crops (Angus et al., 2009; Valentine et al., 2012) and make more effective use of available land and improve energy ratios for biofuel production. This presents a challenge; while most food crops have been bred for thousands of years and pasture crops bred for centuries if not millennia, the breeding of biofuel crops is still in its comparative infancy. For example research in to the development of *Miscanthus* as a biofuel crop has mostly occurred over the last quarter of a century (Clifton-Brown et al., 2016).

The production of biofuels is becoming more widespread because of the benefits of biofuels over fossil fuels resulting in potential increased pressure on land as governments aim for more energy and food security (Valentine et al., 2012). With requirements for water, food and energy increasing globally in parallel with projected population increase up to 2050 (Godfray et al., 2010), effective use of land and crops is necessary. There are different uses for bioenergy crops including thermal conversion of biomass to replace or supplement coal and fermentation of usually less complex carbohydrates to ethanol to provide liquid biofuel. A variety of crops will be required to provide these various products (Songstad et al., 2010, Chapters 5 & 8). Picking the best crops for bioenergy has proved difficult: "The hunt for carbon neutral energy sources has become one of the primary challenges of the twenty-first century" (Songstad et al., 2010, p. 113). The search for bioenergy crops began in the 1970s following the oil crisis (Heaton et al., 2012), and has continued since. *Miscanthus*, a warm season Asian grass, is a leading contender for biomass production in the UK and

European climate (Hastings et al., 2009b) and could fulfil a significant proportion of the demand for bioenergy. The UK intention is to ramp up biofuel crops to cover 6.5% (350,000 ha) of arable and 'set-aside' land by 2020 (Department of Trade and Industry, 2007). Although this target will probably not be met by 2020 (Ayott & McDermott, 2012), there is 350,000 ha of land in the UK that can be used for biofuels without affecting food production (Lovett et al., 2009). *Miscanthus* had been grown since the 1930s for horticultural reasons (Heaton et al., 2012), it was first grown for biomass in the 1960s in Denmark (Deuter, 2000); and has since been researched as a possible biofuel crop (Hastings et al., 2009b; Heaton, Dohleman, & Long, 2008; Jørgensen & Schwarz, 2000; McCalmont et al., 2015; Nixon & Bullard, 2001).

Miscanthus could be utilised for biomass and/or biofuel production. Currently it is better suited to biomass production, especially because this is also the most commercially accessible process (Robbins et al., 2012): however, in the long term conversion to long chain aliphatic molecules may be favoured because the energy density of biodiesel is higher, which reduces transport costs. Heaton et al. (2012) stated, "using only the same land area currently devoted to producing corn grain ethanol... giant *Miscanthus* could meet biofuel goals without bringing new land into production or displacing food supply". This was in reference to the US market; it also did not include the most likely areas for *Miscanthus* cultivation, which are areas of marginal land. *Miscanthus* is a perennial crop and therefore does not require high levels of fertilizer (Lewandowski et al., 2000), and requires low levels of inputs thus requiring little agricultural energy. The choice of land for energy crops is complicated; for example utilising certain areas of marginal land such as woodland would not be favoured because cultivation of these soils would release significant quantities of soil carbon (Hastings et al., 2009b).

Miscanthus is high yielding crop, producing 36 Mg DM ha⁻¹ yr⁻¹ compared to 12.5 Mg DM ha⁻¹ yr⁻¹ and 19.6 Mg DM ha⁻¹ yr⁻¹ for Switchgrass and corn respectively in US trials (Heaton et al., 2012). When compared with short rotation coppice Willow and Switchgrass, other leading bioenergy crops, *Miscanthus* also outperformed these in the US (Heaton, Voigt, & Long, 2004) as well as in England, Sweden, Denmark, Portugal, and Germany (Clifton-Brown et al., 2001). In the UK *Miscanthus* is predicted to yield an average of 19.35 Mg DM ha⁻¹ yr⁻¹ (Hastings et al., 2009b).

Miscanthus has a wide geographic range and high lignin and cellulose yield, even when grown on marginal land (Heaton et al., 2012). This geographic range and the natural variation in climatic conditions under which *Miscanthus* grows in the wild combined with its wide gene pool provides good potential for breeding improvements (Deuter, 2000). *Miscanthus* utilises C4 photosynthesis, more common in exclusively tropical plants, but unusually for a C4 plant *Miscanthus* is cold tolerant (Naidu et al., 2003). This makes it more attractive as a UK biofuel, potentially combining both the higher yields and water use efficiency associated with C4 photosynthesis with cold or frost tolerance (Jørgensen & Schwarz, 2000).

1.2 CURRENT USAGE

The uptake of new crops is challenging, especially because *Miscanthus* is perennial, yielding over many years. Depending on the definition of a fully established crop, establishment time can be between 2 (Anderson et al., 2010) and 5 years (Lesur et al., 2013). The crop can generate a break-even return after several years, even if it can only achieve 70% of maximum yield in the second year, which is the current target discussed by Clifton-Brown et al. (2016). The

government supported this new crop with a 50% establishment subsidy to help farmers with the high setup costs (DEFRA, 2013) (2007-2013). Once producing consistent high yields *Miscanthus* should continue yielding at this level for 15 or more years (Lewandowski et al., 2000). However, in the USA Arundale et al. (2014) found some evidence of reduced yield after 6 years, they suggest this could be due to nitrogen depletion; however, the yields still remained viable (23.4 Mg DM ha⁻¹ yr⁻¹ down from 29.6 Mg DM ha⁻¹ yr⁻¹ as reported by Heaton et al. (2008)).

Miscanthus produces higher yields than *Panicum virgatum* (Switchgrass) and remains active for longer (Arundale et al., 2014; Heaton et al., 2008; Iqbal et al., 2015). Having higher establishment costs means *Miscanthus* has an even longer economic model than other bioenergy grasses, situated between the economic model for forestry and that of normal annual crops (Scurlock, 1999). McCalmont et al. (2015) suggested that *Miscanthus* when grown on marginal land could act as a break crop in a similar way to 'set-aside' as used in the EU. However, currently *Miscanthus* is not widely grown commercially (DEFRA, 2013), this is mainly due to cost because it is planted as rhizomes that have to be obtained from existing plants (Jørgensen & Schwarz, 2000). Rhizome propagation benefits establishment rates because the rhizomes have a greater carbohydrate store to fuel growth than do seed. Rhizome propagation also improves thermal tolerance because large rhizomes have better cold survivability (Clifton-Brown & Lewandowski, 2000). To utilise properly *Miscanthus* to meet the need for bioenergy, a fundamental hurdle that needs to be overcome is to provide a more cost effective method of crop establishment. As the cost of rhizome planting is comparatively high (Christian, Yates, & Riche, 2005), seed sowing would be the best medium for this with the lowest costs of all the *Miscanthus* production methods (Xue, Kalinina, & Lewandowski, 2015); however,

this requires seed that can germinate reliably and across different temperature ranges. If this is achieved, seed can be produced in large quantities, a potential 1;1,172 upscaling ratio (Xue et al., 2015), as well as improving the economics of transportation and sowing.

1.3 MISCANTHUS FOR BIOFUEL

Miscanthus could eventually be a competitive source of biomass for bioethanol (Boakye-boaten, Kurkalova, & Xiu, 2017); this would be an advantage over food crops as an ethanol source, due to not competing directly with the food market. However, *Miscanthus* is not currently easy to breakdown for fermentation due to the lignin contained in the cell walls (Hodgson et al., 2010; Koçar & Civaş, 2013; Robbins et al., 2012), this restricts *Miscanthus* as a high lignin grass to being used primarily for thermal conversion. *Miscanthus* biomass contains high chlorine and ash content that affects burners (Mościcki et al., 2014; Robbins et al., 2012; Robson et al., 2011), and separate grinders are needed to achieve the correct particle size (Mościcki et al., 2014). Biofuels are normally co-burned with coal or wood, Sami, Annamalai, & Wooldridge (2001) summarise the reasons for this as being due to the availability, and effects of ash and chlorine on the boiler. This is especially the case for power generation, as most solid fuel power installed capacity in UK power stations are coal and those that have been converted to biofuel are normally co-burning (gov.uk, 2015), likely using wood as the main ingredient as it has lower chlorine and ash (Mościcki et al., 2014). Burning involves less processing after harvest than conversion, which means less upfront cost, which could slow uptake of conversion.

1.4 BREEDING *MISCANTHUS*

Miscanthus belongs to the family *Poaceae* and is placed taxonomically between *Saccharum* and *Erianthus* (Cai et al., 2005). There are over 10 known *Miscanthus* species (Pyter et al., 2007) with undoubtedly more still to be discovered (Singh, 2013, p. 233). The main *Miscanthus* species of interest for biomass are: *M. sacchariflorus* which can be diploid or tetraploid depending on origin, and *M. sinensis*, *M. floridulus* and *M. condensatus* which are all usually diploid (Deuter, 2000).

Conventional breeding is the usual way to improve crops and is an important part of *Miscanthus* research (Jørgensen & Schwarz, 2000); although there are other ways to achieve improvements, such as transgenesis or agronomic techniques. Deuter (2000) described *Miscanthus* as being “characterised by high genetic diversity within and between species”; this provides much diversity to breed within a large gene pool (Songstad et al., 2010, Chapter 7). Breeding a new variety produces improvements that can be commercially exploitable; these in turn can be improved on repeatedly over the years to produce more and ever improving varieties. In breeding terms, in comparison to other crops farmed today, Jørgensen & Schwarz (2000) assess bioenergy crop breeding as at the stone age level. However, within wheat domestication, there was a marked change in grain size vs. the wild type 10,000 years ago (Gegas et al., 2010), this suggests that with early breeding techniques and high demand rapid change was possible. Breeding new *Miscanthus* hybrids is the best method of progress toward superior crops; Jensen et al. (2013) stated that “expansion and development of the *Miscanthus* industry requires novel hybrid production through the creation of intra and inter specific hybrids”. Deuter (2000) concurs that hybridising between species often produces plants that are more vigorous.

Whilst *Miscanthus* is self-incompatible it crosses inter and intra specifically to form new and different varieties and hybrids (Deuter, 2000). Interestingly, *Miscanthus* is closely related to Sugarcane; and has been used to breed advances (cold & disease resistance) into Sugarcane; the reverse may be exploited in the future and Sugarcane could be a source of useful traits for improving *Miscanthus* (Heaton et al., 2012, 2008). The variety of frost tolerance found within *Miscanthus* genotypes (Fonteyne et al., 2016), suggests the crop can also be improved for frost tolerance from existing stock.

Miscanthus grows in a varied range of Asian climates (Hodkinson, Renvoize, & Chase, 1997; Slavov et al., 2013), within these climates several species can co-exist. This is useful for breeding, and some species cross-breed naturally (Deuter, 2000) when different species are tolerant of the same climate. This allows different species to be crossbred, either from the same latitude to maintain tolerance of that temperature range as well as between latitudes, in order to broaden the temperature range (Clifton-Brown et al., 2011). Due to overall diversity, breeding resources are plentiful and possibilities for novel breeding of *Miscanthus* are vast. Current breeding systems are focused on producing hybrids and genotypes for use as varieties in industry (Clifton-Brown et al., 2016). Little is known about the heritability of biomass and composition traits (Arnoult & Brancourt-Hulmel, 2015); which may be needed to sustain a pipeline of new seed based varieties each aimed at achieving a better yield than the last, and all likely to outperform *M. x giganteus* (Clifton-Brown et al., 2016).

Flowering is a fundamental part of crossing plants for hybridisation. Jensen et al. (2013) described *M. sacchariflorus* as a perennial grass that naturally hybridizes with *M. sinensis* producing hybrids including *M. x giganteus*. Cross-breeding is complicated by differences in flowering and a lack of understanding of the factors influencing flowering (Jensen et al., 2013). Koornneef et al. (1998)

suggested that flowering in *Miscanthus* is delayed until suitable environmental or biochemical conditions occur. This makes it more difficult to crossbreed in the large quantities required for commercial seed. Flowering also terminates the production of leaves at the stem apex, thereby slowing growth and limiting potential biomass accumulation (Jensen et al., 2013). It could be concluded from this that plants which flower later in the year have more time to accumulate biomass and are therefore preferable. *Miscanthus* species that flower late may be challenging to use for producing seed because late flowering *Miscanthus* plants will be difficult to synchronise for crossing and seed will not have time to develop and ripen before winter.

1.5 IMPORTANT AGRONOMIC TRAITS

After the agronomy of the seed and the initial establishment from planting a seed plug or rhizome, there is a period of establishment over the first two to three years before the plants reach an economically harvestable yield. For UK establishment this means *Miscanthus* will require an ability to grow and thrive through heterotrophic growth after germination (Brunel-Muguet, Aubertot, & Dürr, 2011). In this context there may be factors considered within this project that are more relevant to the period of establishment.

1.5a SENESCENCE

Senescence in different *Miscanthus* genotypes has an impact on the biomass yield and yield quality of the crop (Robson et al., 2011). Early senescence shortens canopy duration which decreases yields; whilst late senescence results in harvested *Miscanthus* having a higher moisture content (Robson et al., 2011). This may lead to higher biomass yields but higher transportation costs from

moving more water, and the moisture content if too high will prevent the crop from being burnt in large-scale power stations. Senescence is also important for establishing long term growth because the plant redistributes nutrients toward storage organs such as the rhizome during senescence; this then allows the plant to establish vigorously the following spring (Robson et al., 2011). While *M. sinensis* has been observed to be able to overwinter in a UK climate (Christian et al., 2005), senescence is especially important in *Miscanthus* in the first year when the rhizomes are not fully developed, and may sometimes not survive overwintering even with well-timed senescence (Clifton-Brown & Lewandowski, 2000). In a 3 year study of senescence in 244 diverse *Miscanthus* genotypes by Robson et al. (2011), the timing of senescence seemed to be influenced by environmental signals, but the order in which different genotypes senesced was broadly consistent between years. In general *M. sinensis* genotypes senesced first and *M. sacchariflorus* genotypes senesced last, implying a genotypic control may exist at the species level (Robson et al., 2011).

1.5b INVASIVENESS

There are various concerns about bioenergy crops, primarily that they tend to be in competition for land with the food supply and therefore may increase food prices (Valentine et al., 2012). There are also concerns over the invasiveness of foreign species that may well be bred for vigour, pest resistance and competitiveness (Barney et al., 2012), such as *Miscanthus*. It should not be assumed that bioenergy crops will be naturally safe or risky (Barney et al., 2012), but they will be grown in massive amounts because bioenergy is all about quantity of biomass. Growing bioenergy crops such as *Miscanthus* on small widely distributed bits of marginal land may also carry more risk.

Most *Miscanthus* grown today for biomass is the sterile triploid *M. x giganteus* (Greef & Deuter, 1993) ex (Hodkinson & Renvoize, 2001). Barney & Ditomaso (2008) stated that *Miscanthus* “poses little threat of escape in the United States”. With the increasing popularity of biofuels and sellers aiming to maximise profits, *Miscanthus* is not always what it is sold as; “Verifying that the material sold is actually *M. x giganteus* is essential to ensure that the sterile triploid hybrid is used and not a fertile variety that could become an invasive liability” (Heaton et al., 2012). The sterility of *M x giganteus* is described as “advantageous because it limits the capacity of *Miscanthus* to spread unintentionally from seed, but it significantly complicates planting of new fields” (Heaton et al., 2012). To which end the European *Miscanthus* improvement project recommends new types of *Miscanthus* be sterile (Scurlock, 1999) although seed propagated varieties of *Miscanthus* have not yet been proven to be invasive.

Currently *Miscanthus* is often grown by farmers as a reliable, profitable crop on marginal land (Farmers Guardian, 2013), to ensure some income if other crops fail. However, bioenergy targets are pushing *Miscanthus* to be grown on significantly more land, heralding a large change in agriculture which will affect nutrients, greenhouse gasses, habitat and biodiversity (Barney et al., 2012). There may however also be benefits to *Miscanthus* being grown. Nixon & Bullard (2001) suggest that the harvesting times of *Miscanthus* are good for grass dwelling birds. Due to this harvest time and lack of chemical input through most of the potentially 20 years or more of cultivation from a single planting, *Miscanthus* can provide many benefits for wildlife conservation over other agricultural land (DEFRA, 2007; Midgley, 2012).

1.5c BIODIVERSITY

The economic models and projections for productively growing *Miscanthus* often focus on growing it on marginal land (de Vries, van de Ven, & van Ittersum, 2014; Liu et al., 2014; Pogson, Hastings, & Smith, 2013). *Miscanthus* can have significant beneficial effects on the environment (Kiesel, Wagner, & Lewandowski, 2016), the effect on biodiversity would depend on where it was planted. The suggested use by bioenergy crops of fallow and 'set-aside' land would be expected to have a negative effect on biodiversity; even if areas with particularly high biodiversity are not counted in the potential land resources (Turley et al., 2010). However, while there have not been many studies particularly on biodiversity in mature *Miscanthus* crops, it does perform better than winter sown cereals particularly by providing a habitat for birds and mammals (Nixon & Bullard, 2001). The clear example of this is due to *Miscanthus* harvest time, it provides winter ground cover and nesting material (McCalmont et al., 2015). However, as Jørgensen (2011) states; *Miscanthus* does not provide a food source for most creatures living in and around it.

The lack of chemical inputs also make *Miscanthus* better than most crop plants for biodiversity (McCalmont et al., 2015). However, these benefits are derived mostly from comparisons with arable agriculture, when compared with biodiversity on marginal land any benefits will depend on what the marginal land is; marginal land tends to be described as unproductive land but Turley et al. (2010), exclude wooded areas, river banks etc. Subsequently which marginal lands are considered suitable for bioenergy crop cultivation will determine the extent of positive or negative environmental impacts (Jørgensen, 2011).

1.6 SEED IMPROVEMENT

Understanding germination in *Miscanthus* is vital for cheap and effective establishment of the crop (Jørgensen & Schwarz, 2000). Establishing *Miscanthus* from seed, especially in the UK environment, may require improvements in a combination of one or more factors such as breeding, sowing time, growing film, and hormone treatments. However, most studies on germination and treatments have been carried out in food crops or using model organisms such as *Arabidopsis*. Most of these organisms are dicots whereas *Miscanthus* is a monocot (Figure 1-1) and the physiological differences in seed between the two groups may result in differences in the effect of treatments when translated to *Miscanthus*.

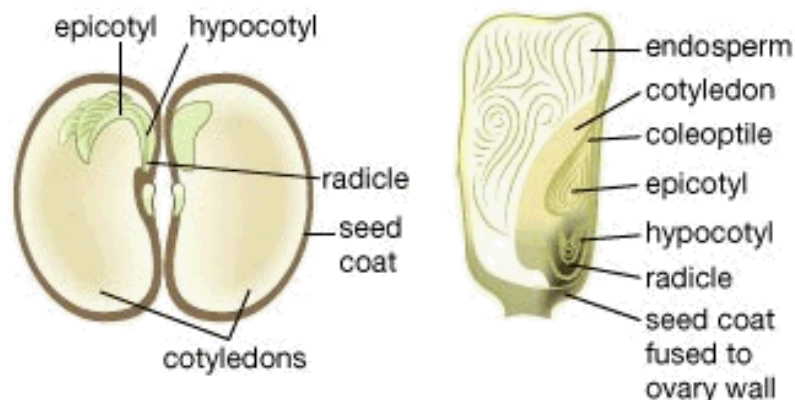


Figure 1-1: Comparison of monocot (Right) and dicot (Left) seed structure (Merriam-Webster, 2006).

Germination is the process by which the seed embryo begins growth and emerges from the seed coat, the seed is normally considered to have germinated at this point (Bewley, 1997a; Sarath et al., 2006, 2007; Xue et al., 2015). The radicle and root emergence occur differently, the radicle via cell elongation but the root via cell division; therefore, a seed can extend a radicle but not go on to produce a viable root (Sarath et al. 2006). This difference has an effect on the

identification of germination, because the extension of a radicle does not indicate viable germination without the appearance of a root. Normal germination in C4 grasses was described by Sarath et al. (2006): “Radicle extension precedes coleoptile emergence”.

The technology currently being pursued as a method of utilising hybrid *Miscanthus* from seed is planting using plugs of soil (Clifton-Brown et al., 2016). By direct sowing of seed the cost is greatly reduced over plugs by saving the cost of the nursery etc. (Anderson et al., 2015). By working to correctly identify and test the germination of *Miscanthus* seed, a better understanding of the crop agronomy can be reached. This study begins in the laboratory and works out to the field, incrementally investigating the germination of *Miscanthus*. This process extended into the effect of hormones, water stress, soil water, and seed pre-treatments. These topics are introduced individually in their respective chapters.

OUTLINE

- Chapter 2 states the techniques used to collect and process the seed for this study, as well as detailing the methods commonly used in this study.
- Chapter 3 outlines the methodology for high throughput image analysis, including automating germination detection and estimating plant biomass. The pros and cons of these methods are discussed in relation to manual methods, along with how these methods affected the other experimental designs in this study.
- Chapter 4 details the main effects of various hormones, pre-treatments, and water stress on *Miscanthus* germination and early growth. This information was used to generate ranges for a Taguchi multi factorial experiment. The results of this are discussed and analysed in relation to the optimal environment and expected interactions.
- Chapter 5 explores using an oversowing technique to solve low field germination and establishment in *Miscanthus*. Oversowing is investigated *in-vitro*, with soil in a controlled environment, and in real field conditions to assess its efficacy.
- Chapter 6 evaluates the effect of agronomic treatments and physical conditions on *Miscanthus* seed, and then uses this information to parameterise and run a computational model of germination, emergence, and early growth.

2 SHARED METHODS

2.1 MAIN MEASUREMENTS

2.1a GERMINATION SCORING

Germination was scored using 1 mm radicle emergence (Bewley, 1997; Ellis et al., 1985), raw scores or percentage/proportion of seed germinated were used.

Germination index (GI) used the (1) equation (Melville et al., 1980 via Ranal & de Santana, 2006) to provide a single score.

$$GI = \sum_{i=1}^n |(D_t - D_i)G_i|/S \quad (1)$$

Where n is the day of final counting; D_t the experiment length; D_i the number of days until day i ; G_i the germination count on day i and S is the total seeds tested (Ranal & de Santana, 2006).

The time for 50% of seeds to germinate was stated occasionally, when germination rates were high enough.

(For methods of automation in germination scoring, see Section 3.1a below.)

It is vital to assess accurately and consistently the germination phenotype after different treatments. In standard temperature testing the most important measurements are measuring time to 50% germination and the total proportion of germination (Covell et al., 1986). This is useful because temperature vs. time to 50% germination is a linear relationship and can be represented using degree

days (Covell et al., 1986). The problem with using the time to 50% germination is that it only records one point during germination (Covell et al., 1986), although it has been shown by Monteith & Squire (1982) that similar linear relationships can be drawn at other germination percentages. Ellis et al. (1987) observed that measuring the time to 50% germination returned an index of the germination rate within a treatment; however, the treatment that achieved maximum final germination percentage could be at a lower overall rate. Also seed lots with maximum germination below 50% but which germinate quickly may well not appear as relevant as seed which reaches 50% germination more slowly (Ellis, Hong, & Roberts, 1985a) making poor seed difficult to assess accurately. A lower time to 10% germination can be used (Ducournau et al., 2005; Hsu, Nelson, & Chow, 1984); however, this will be more sensitive to small fluctuations than time to 50%.

Measuring germination itself is also difficult because despite radicle emergence (sometimes by a defined amount i.e. 1 mm) being a widely used point at which to score germination (Bewley, 1997a; Ellis et al., 1985a), it is not the start of the germination process, only the first externally visible sign (Ellis et al., 1985a). Ellis et al. (1985a) recommends not only scoring at radicle emergence but also confirming seed viability by assessing the seeds at a later point for their ability to survive. At what point during the experiment seeds are definitively scored as germinated will depend on the experiment, but it can be informative to track a final root and stem elongation in germination tests (Aso, 1976). In *Miscanthus*, it would be important to check for effects of seed treatments on subsequent plantlet morphology; to ensure an increase in germination was not at the expense of normal seedling growth.

The experimental design of germination studies is quite varied. Seed banks suggest germinating 200 seeds which are checked 1-3 times per week (Ellis et

al., 1985a). Others designs vary from 4 replicates of 50 recorded at 4 hours intervals (Ellis & Butcher, 1988) to 4 replicates of 100 seeds recorded daily (Sathish, Sundareswaran, & Ganesan, 2011). The choice of method when considering *Miscanthus* will depend upon variation and speed of germination.

Homogeneous (synchronised) seed will germinate within a short space of time but time to germination may be long or short (Ranal & de Santana, 2006). Heterogeneous (unsynchronised) seeds will have a broad range of germination times (Ranal & de Santana, 2006). Being able to determine this range depends on measurement interval; for instance if intervals are short, no two seeds will germinate in the same interval giving an impression of low synchronicity (Ranal & de Santana, 2006). Germination tests are normally run until the germination curve levels off, but it may be necessary to run the experiment for significantly longer to allow all remaining viable seeds to germinate (Ellis et al., 1985a).

Seed vigour is used as a marker of seed quality, but there are many ways of determining it (Brown & Mayert, 1988). A simple index will have drawbacks, yet it is necessary to rank seed groups for vigour (Brown & Mayert, 1988). Past research has focused on time to first germination, ignoring almost all the sample seeds (Ranal & de Santana, 2006). This method can be improved with use of a system of scoring first and last germination; however, this can merely highlight outliers (Ranal & de Santana, 2006). The overall tendency of the group (the median) calculated as 50% germination (discussed above) can be used; however, this is only effective if the distribution is symmetrical (Ranal & de Santana, 2006). Sathish et al. (2011) used the vigour index value, which multiplies the GI by the total seedling number. Other types of scoring include Kotowski's coefficient of velocity which expresses the relationship between the time of germination and the number of seedlings produced (Brown & Mayert, 1988). However, "It is possible for fast- and slow-germinating samples of seed to

have identical values of coefficient of velocity” (Brown & Mayert, 1988). This can happen if the former has a longer lag phase, so that the two share a common average germination time (Brown & Mayert, 1988). Maguire's speed of germination is a time weighted cumulative germination score (Brown & Mayert, 1988). Single scores of germination are clearly useful for ranking seed batches objectively but unfortunately there are issues with this, such as 100% germination on day 10 equalling 10% germination on day 1 (Brown & Mayert, 1988). Overall it is difficult to define germination success using a single score; assumptions about germination are made in order to produce a single numerical value (Brown & Mayert, 1988). One good solution to scoring seed vigour is to use multiple statistics to quantify vigour of the seeds. Comparability of treatments will be essential in *Miscanthus* research; however, multiple values may lead to a more reliable view of the best treatment ranges.

To avoid some of the problems with single metrics discussed above raw germination data is presented where possible. However, GI and time to 50% germination is used when a single point comparison illustrates a key point. GI was chosen because it seems to be an appropriate abstraction of more complex germination data (Walker-Simmons, 1987). Time to 50% germination was found to be a good time metric when compared to mean germination time by Soltani et al. (2015), and was used in the presented study where it could suitably be applied.

2.1b TILLER NUMBER

Tiller number is the total tillers in a plot or cluster of *Miscanthus* plants, unless stated as a number per plant.

Tiller number, often referred to as stem number (Greco et al., 2012; Jensen et al., 2013), is a measure of the number of stems belonging to a plant and is

commonly used as a measure of success particularly with biomass plants including *Miscanthus* (Barney et al., 2012; Heaton et al., 2008). *Miscanthus* seed is sometimes sown in batches and once germinated cannot be distinguished; therefore, in this study tiller number is a measure of the total stems in a plot or row if the number of plants is unknown. Therefore, it describes a total tiller number in an experimental unit not the number of tillers per plant unless otherwise stated.

2.1c HEIGHT

Height was measured as the height of the stem to the youngest ligule (a readily scored point on the stem from which a leaf is subtended).

This is a common way of measuring height in plants and has been previously used with *Miscanthus* (Purdy et al., 2013; Robson et al., 2013; Slavov et al., 2013). This provides a more replicable way of measuring grasses than alternatives considered including measuring to the top of the plant because that could vary with leaf architecture or to the tip of the leaf, which requires finding the longest leaf and extending it upward. Canopy height (the height at which the plant or plot forms a bushy covering of leaves) has been used to define height (Robson et al., 2013; Slavov et al., 2013); however, this was with more mature plants and the *Miscanthus* in this study never reached the level of development for a canopy height measurement to be used.

2.1d STEM ELONGATION

Stem elongation is the distance from the plant base or seed to the tip of the longest leaf.

In seedlings and young plants, elongation has been used (Olsen & Gounder, 2001) as an alternative to height (Section 2.1c above). This is because height to the top ligule is not an accurate description of how big the seedling is. Elonga-

tion is also more comparable when using computer scoring of germination because the computer can also track the furthest plant extension (Wagner et al., 2011). Depending on conditions and seed lot, in a monocot the first leaf could extend centimetres before any discernible stem growth, this might cause growth data to be overlooked at the early stage. Therefore, elongation is the measurement from the seed or base of the plant to the farthest end of leaf or stem and this is only an appropriate measure of plants that are very young. The total elongation will mostly include leaf (Clifton-Brown & Jones, 1997); yet strong correlation between these measures was found in Section 5.3.

2.1e ROOT ELONGATION

This is the length of the longest root fully extended. The root dry weight was also stated where possible to give an indication of root production.

Root measurements were done in the same way as stem elongation, measuring roots from base/seed of the plant to the farthest tip of root (Sarath et al., 2007). This only gives the length of the longest root and thus disproportionately scores plants with one long root over those with a more branched root structure; where possible the dry mass of the roots was also determined to give a measure of total root production. When both are measured Specific Root Elongation (SRL), a measure of the ratio of root elongation to root dry weight (Pérez-Harguindeguy et al., 2013), can be used to provide a single metric for root growth. However, the separate metrics were more appropriate in this study. A more complex measure of root elongation could have been used with the imaging in the 'Physical & Chemical Germination Factors' experiments by using techniques such as RootReader2D developed by Clark et al. (2013) which gives more comprehensive measurements of the root. However, in this study, the plants were not grown

specifically for root imaging and the delicate roots were not suitable for detailed multiple measurements.

2.1f MULCH FILM

Samco Grey mulch film was used to cover seed and seedlings, this film is a 7 μm starch film, which breaks down under UV exposure (Samco Agricultural Manufacturing Ltd, 2014). The film has a 'pinhole 20' aeration that facilitates both ventilation and for the plant to push through the film (Samco Agricultural Manufacturing Ltd, 2014).

2.1g PRIMING

In this study, primed seed refers to SYN55 seed, primed in water until almost chitted, then dried back the original moisture content of the seed; this was done by a commercial company.

Priming was carried out in water by Elsoms Seeds Ltd (Spalding, Lincolnshire, UK). By using an experienced commercial priming company, this was most likely to ensure the treatment was correct and commercially applicable if successful. The seed were tested first for germination time in slowly rotating wet drums, then the drums were used to prime the seed before drying it back to the original dry mass. Elsoms reported that while the priming was successful, the procedure had reduced the germination of the seed batch.

2.2 SEED TYPES

Several types of *Miscanthus* seed were used in this study. Most batches were *M. sinensis* synthetic crosses or *M. sinensis* crossed with *M. sacchariflorus* to give an interspecific hybrid.

SYN55 is a synthetic hybrid of 23 possible parent plants produced in Texas in 2011, all but two (*M. lutarioriparius*) were outstanding *M. sinensis* plants, the resultant seed was primarily diploid. At the beginning of this study (2013), SYN55 was the closest available seed to a commercial variety of *Miscanthus* and it was the most advanced seed available seed in any significant quantity. For this reason, most testing was done on this seed. However, due to its commercial importance, combined with modest seed numbers and tests of the seed at origin that had shown a typical germination rate of ~60%, other seed types were used if more suitable for experimentation.

A larger seed batch of 2013 MX300 was used where better germination was required and where large seed numbers were of importance. This seed batch was a *M. sinensis* × *M. sinensis* open pollinated cross-produced in Wales and processed by the author.

Table 2–1: Table of the *Miscanthus* seed batches used in this study. The primary parent is for synthetic crosses, where it is the species with most plants in the cross.

Seed Name	Cross Type	Parent: female / primary	Parent: male / secondary	Produced	Year
MX300	Open Crossed	<i>Miscanthus Sinensis</i>		Aberystwyth University Wales	2013
SYN55	Synthetic cross	<i>Miscanthus Sinensis</i>	<i>Miscanthus Lutarioriparius</i>	Ceres Inc. Texas	2012
Open Crossed	Open Crossed	<i>Miscanthus Sinensis</i>		Aberystwyth University Wales	
SYN16	Synthetic cross	<i>Miscanthus Sinensis</i>		Ceres Inc. Texas	2010
SYN17	Synthetic cross	<i>Miscanthus Sinensis</i>		Aberystwyth University Wales	2010
SYN56	Synthetic cross	<i>Miscanthus Sacchariflorus</i>		Ceres Inc. Texas	2012
SYN58	Synthetic cross	<i>Miscanthus Sinensis</i>		Ceres Inc. Texas	2011
SYN70	Synthetic cross	<i>Miscanthus Sacchariflorus</i>		Catania	2013
GNT1	Interspecific hybrid	<i>Miscanthus Sinensis</i>	<i>Miscanthus Sacchariflorus</i>	Ceres Inc. Texas	2013
GNT2	Interspecific hybrid	<i>Miscanthus Sinensis</i>	<i>Miscanthus Sacchariflorus</i>	Ceres Inc. Texas	2013
GNT3	Interspecific hybrid	<i>Miscanthus Sacchariflorus</i>	<i>Miscanthus Sinensis</i>	Ceres Inc. Texas	2013
GNT4	Interspecific hybrid	<i>Miscanthus Sinensis</i>	<i>Miscanthus Sacchariflorus</i>	Ceres Inc. Texas	2013
GNT5	Interspecific hybrid	<i>Miscanthus Sacchariflorus</i>	<i>Miscanthus Sinensis</i>	Ceres Inc. Texas	2013
GNT14	Interspecific hybrid	<i>Miscanthus Sinensis</i>	<i>Miscanthus Sacchariflorus</i>	Catania	2014
GNT36	Interspecific hybrid	<i>Miscanthus Sacchariflorus</i>	<i>Miscanthus Sinensis</i>	Ceres Inc. Texas	2013
GNT22	Interspecific hybrid	<i>Miscanthus Sinensis</i>	<i>Miscanthus Sacchariflorus</i>	Catania	2013

Other seed batches (Table 2–1 above) were used for comparison of genetic variation and in some of the preliminary method establishment tests, details of these batches are in Table 2–2 below.

Table 2–2: When each seed batch was threshed, as well as where and when each seed batch is used in this study. Seed were normally threshed the year after they were crossed; this was sometimes done in December the same year.

Seed Name	Year threshed	Experiments	Year used
MX300	2014	Thermal Gradient for Seed Germination, Thermal Gradient for Seedling Stem Elongation, Cluster Sowing to Improve Establishment,	2014, 2015, 2014-2015
SYN55	2013	Multi Genotype, Thermal Gradient for Seed Germination, Dark Burnout, Thermal Gradient for Seedling Stem Elongation, Dark vs. Red Light, Physical & Chemical Germination Factors	2013, 2014, 2014, 2015, 2016, 2014-2016
Open Crossed		Experimenting with Procedures	2013
SYN16	2011	Multi Genotype, Thermal Gradient for Seed Germination	2013, 2014
SYN17	2011	Multi Genotype, Thermal Gradient for Seed Germination	2013, 2014
SYN56	2013	Multi Genotype, Thermal Gradient for Seed Germination	2013, 2014
SYN58	2012	Multi Genotype, Thermal Gradient for Seed Germination	2013, 2014
SYN70	2014	Thermal Gradient for Seed Germination, Dark vs. Red Light	2014, 2016
GNT1	2014	Thermal Gradient for Seed Germination	2014
GNT2	2014	Thermal Gradient for Seed Germination	2014
GNT3	2014	Thermal Gradient for Seed Germination	2014
GNT4	2014	Thermal Gradient for Seed Germination	2014
GNT5	2014	Thermal Gradient for Seed Germination	2014
GNT14	2015	Cold Storage Error! Reference source not found. , Dark vs. Red Light	2015, 2016
GNT36	2014	Thermal Gradient for Seed Germination	2014
GNT22	2014	Thermal Gradient for Seed Germination	2014

Fresh seed batches were also used, particularly in tests involving seed storage or threshing, seed lots were also picked for their commercial importance; lower GNT numbers denote the newest commercially important seed.

2.3 SEED ACQUISITION

It was important to harvest and thresh high quality *Miscanthus* seed. Some of the seed lots referred to in the description (Section 2.2 above) were threshed as part of this study to provide clean uniform *Miscanthus* seed. This seed is suitable for counting germination, manually or automatically. Seed were threshed at either Aberystwyth University or Ceres Inc.'s Texas field labs. Seed threshing methods were developed from personal tuition by a seed specialist while working with the industrial partner Ceres Inc. From this work, a full process description from harvesting to producing clean seed was documented for *Miscanthus*, which is detailed in Sections 2.3a – 2.3f.

2.3a HARVESTING

Panicles (inflorescences) were harvested in late autumn according to when they became ripe (filled out) and dry. Taking a part of the panicle and rubbing it between fingers was used to detect the presence of fertilised seed (Figure 2-1). Then the panicles were cut with ~300 mm of stem attached to allow them to be machine stripped.



Figure 2-1: Testing a piece of panicle for seed, one seed visible at the top of the panicle piece.

2.3b DRYING

Most of the seed lots used in these experiments were dried using a standard time of one to two weeks in a warm, dry room or greenhouse. The methods for drying were tested in a simple un-replicated experiment [Appendix A] this did not find any large changes in breakage rate of seed when varying the standard drying procedure. The proportion of seed germinated had more variation but there was not a trend in conditions or moisture content and germination rate.

2.3c STRIPPING

Miscanthus produces florets in which the seed forms; these panicles are fluffy and light after drying. To thresh heavy seed out of the fluffy heads, first the hard raceme must be removed, because it can break up in the threshing process and form heavy bits that are more difficult to separate from the seed and may clog the thresher. The first stage is stripping the flower heads of the panicle. In this study the florets were stripped either manually (Figure 2-2a & Figure 2-2b) or using a rotary machine fitted with brushes (draught excluders) to strip the fluffy seed heads from the panicles without too much raceme (Figure 2-2c).

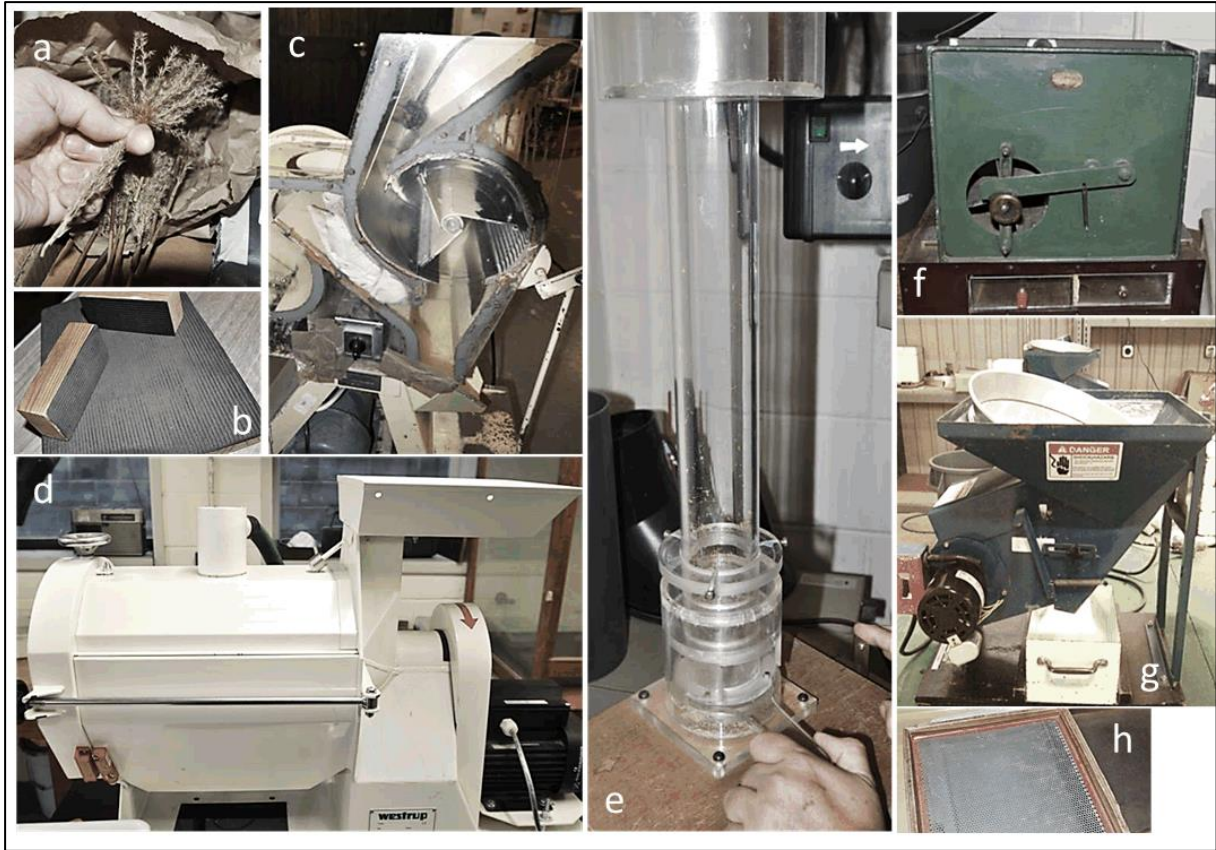


Figure 2-2: Equipment used in the threshing process for Miscanthus seed. a) Hand stripping of florets. b) Grooved rubber pads used for stripping of florets. c) A modified clover seed machine for stripping of heads. d) Westrup (LA-H) threshing machine. e) Vertical blower. f) A Bryan Corcoran horizontal blower. g) The ALMACO blower used in Texas. h) 2 mm cheese sieve.

2.3d THRESHING

Threshing is the removal of the seed from the seed head, this is done using a machine that brushes with stiff bristles against a sieve breaking up the heads and forcing the seed through. Threshing was done using a Westrup (Slagelse, Denmark) LA-H (Figure 2-2d), at 600 rpm with a 2 mm sieve.

Threshing was repeated one or more times until the resultant chaff, seed, and fluff mix was not clumped together. Clumping normally occurs because the fluff from the heads is in long strands (if not broken by the threshing process) and

binds the mix together into a mass. Once un-clumped the mix can be sieved without the seed being stuck above the holes.

The threshing protocol should have been a similar standard to other past seed thrashings; when compared to others, the breakage rate was comparable [see Appendix B for details].

2.3e SIEVING

The loose mix of chaff, seed, and fluff resulting from threshing of *Miscanthus* seed was further partitioned using a 3 mm cheese sieve shown in Figure 2-2h, the lightest 20-40% was discarded. This was to remove the fluff from the broken down seed heads. The sieving was done multiple times until the heaviest and smallest material had been separated. Rejected material was occasionally checked for seed, so the sieving process did not significantly reduce seed yield.

2.3f BLOWING

The remaining mix was blown to remove any lighter material such as husks and fluff and leave the seed. This was done using a combination of bespoke vertical blower (Figure 2-2e) or/and a Bryan Corcoran Ltd (London, UK) horizontal blower (Figure 2-2f) and in Texas a faster blower an ALMACO Air Blast Seed Cleaner (Nevada Iowa, USA) was used (Figure 2-2g). If raceme fragments were left in with the sample it was re-sieved several times through a sieve of 3 mm or less to remove any raceme or fluff that is longer than a seed. After this stage, the clean seed was suitable for commercial sowing and experimentation.

2.4 SEED STERILISATION

Sterilising seed to prevent excessive mould is important for accurate germination testing. The sterilisation should be sufficiently rigorous to remove contamination but not excessively so to avoid adversely affecting germination. The method used was based on Sarath et al. (2006) and Steber and McCourt (2001) with the use of Triton-X for cleaning seeds (Li et al., 2005). Seed were washed for fifteen minutes (with occasional agitation for an even covering) in a 10% bleach solution with 0.1% Triton x-100 added as a surfactant. The seed were rinsed in SDW (sterile distilled water) until the smell of bleach had gone completely (five or more times).

This method was tested for effectiveness on MX300 seed, at 20%, 10%, and 5% household bleach solution plus Triton and compared with a control [Experiment in Appendix C]. The effects of treatment on mould and germination were examined using a one-way ANOVA performed on the 15-day result for mould and the 7-day result for germination. The results indicate that mould decreased significantly between 5% bleach and 10% bleach but not between 10 and 20% bleach; therefore, 10% bleach was selected for all seed sterilisation.

3 IMAGE ANALYSIS

3.1 INTRODUCTION

Use of image analysis techniques has been increasing in the biological sciences. Particularly over the last 20 years computer vision has been adding more impartial measurements to biological studies (Glasbey & Horgan, 1995); it offers improved throughput and unbiased, reduced error results (Wang et al., 2009), but at the expense of real time interaction with samples. The slower setup but faster observations make it ideal for time course studies (French et al., 2009), such as growth or germination. The use of optical data makes image analysis ideal for calculating visual attributes such as plant size non-destructively, as in the case of field or automated glasshouse biomass assessments. This phenotyping technology lags behind that of genotyping technologies; however, it is increasingly being implemented to test or screen genotypes (White et al., 2012).

3.1a GERMINATION

Throughout this study, it was important to count *Miscanthus* seed germination with as much repeatability and reproducibility as possible; this entailed questioning the repeatability of human assessment and investigating computer vision approaches to germination scoring. Measures of germination discussed in Section 2.1a above.

REPEATABILITY OF MANUAL SCORING

Germination of seeds is frequently scored by eye when the radical has visibly emerged (Bewley, 1997a; Ellis et al., 1985a). Although it has been claimed that embryo protrusion can be verified at the same time across many separate researchers (Ranal & de Santana, 2006), human scoring produces some degree of variation. When using small seeds and high numbers of samples, counts will be less repeatable and less true to the unknown correct result. However, in this study the human score was the only reference point available to which the computer score could be compared. Designing a computer system that is able to impartially score germination in a repeatable and reproducible way, would remove unknown variation from the human-based scoring system. This is important when planning experiments around *Miscanthus* to reduce variation when there are many variables.

ADVANTAGES OF A TECHNOLOGICAL APPROACH

A stable computer vision system perfects repeatability but this may be achieved at the expense of trueness, this can be important in many fields especially when human measurement may lack trueness or even be biased (by time of day, repetition, and tiredness). Using photographs or other automatically recorded data for analysis provides two main advantages: firstly, the algorithm can be refined and re-run on the samples at any future time, whereas a protocol update can only be applied to human assessments if the samples still physically exist in the state they were in at the time of the original data collection. Secondly, this allows the results to be directly repeatable, because the images and scripts used for the assessment of germination can be made available, while re-seeing the seeds as a human assessor observed them is impossible. Having all the data recorded digitally may also enable faster data collection, which may be

advantageous by, for example, exposing the samples to non-treatment conditions for shorter times during measurements.

PAST STUDIES

Automated systems such as MARVIN (GTA Sensorik GmbH, n.d.) have frequently been used commercially and academically for the accurate sizing and counting of seeds (Achigan-Dako et al., 2015, 2008; Benor, Fuchs, & Blattner, 2011; Gegas et al., 2010). More recently, mobile applications such as 1KK (WheatGenetics, 2016) have advanced the field of automated seed counting, via imaging groups of seed. However, measuring germination is more complicated because the seeds need to germinate in a medium, and should be observed repeatedly in the same position to monitor the changes.

Computer imaging of seed germination has demonstrated trueness when compared to a human reference in other species such as *Arabidopsis* (Joosen et al., 2010). By thresholding the image to remove the background, then running an analysis on the remaining image objects (these can be in a selected colour range (e.g. RGB)), information about the seed's average shade and perimeter can be determined. These parameters about each object can be collected and analysed simply. These methods have the potential to count laboratory germination rate faster and with greater reproducibility than a human observer does. Joosen et al. (2010) provides a different method of photographing germination where only one final seed image is required and not a comparison with a start photograph. By repeatedly thresholding the same image, a distinction between seed coat and whole seed including any radicle can be found. Using this difference, germination can be scored with a high trueness to a human reference point via analysis of the two images that result from the thresholding (Joosen et al., 2010). This is very useful because the seed often moves when germinating and the image

analysis software has difficulty identifying the same seed on the two different photographs (Joosen et al., 2010).

By using the idea of a ground truth, Ducournau et al. (2004) was able to use ROC (Receiver Operating Characteristic) curves to highlight the best strategy for producing data true to human vision; however, the main unknown is the inaccuracy or bias of human germination scores. The ability to score different seed types depends upon experience and may be affected by mood, time of day and time constraints (Schindelin et al., 2012). To compare the computer's ability directly against that of a human may be unfair because the human is not necessarily an indicator of the real value; yet currently there is no more accurate method of determining the real germination score. Ducournau et al. (2004) used mean time to 50% germination as the primary factor of comparison between the computer and the human analysis. In doing this, a seed-by-seed comparison of germination scoring between people and computers was avoided.

The drawback to this single image photography is that the thresholding process needs to be very precise to achieve two images from one photograph that only differentiate the radicle/hypocotyl (Joosen et al., 2010). To obtain two images that line up well groups such as Wagner et al. (2011) use in house software.

ALTERNATE METHOD

Ideally, the seed dishes would remain stationary and would be photographed *in situ* to capture germination (Ducournau et al., 2004). This method was tested but condensation on the Petri dish lid prevented image analysis. This method was refined to be lidless and use a wick system from a reservoir to prevent drying. It used a blue roll wick coming through a hole in the centre of the Petri dish, to allow time-lapse style photographing of the seed. Whilst this worked, it was unsuitable for the 'Physical & Chemical Germination Factors' (Chapter 4)

because the wick may not have transferred the chemicals up to the Petri dish effectively.

3.1b FIELD BIOMASS

Field biomass is difficult to determine due to a range of problems such as the variable nature of plants and outdoor locations. The background of the image is a problem, because it needs to be distinguishable from the plant, as a minimum this means excluding green from the background, which is difficult in a field and is rendered very difficult on large field plants or tightly spaced plots.

Occlusions, where some of the image is covering other important image data i.e. one leaf obscuring another, can be combated by averaging multiple camera positions (Lou et al., 2014; Malinowska, Donnison, & Robson, 2016). Moving the camera has an effect on perspective and therefore the relative size of objects in the image. This is harder to control in field photography than in laboratory conditions and assessing plants in the field with a camera that is not at a measured height and distance away from the plant could bias results.

Past attempts at biomass assessment of plant areas focused on aerial imaging particularly spectrometry (De Jong, Pebesma, & Lacaze, 2003; Hunt Jr et al., 2005). The green pixel count is a seemingly simple way of calculating green area of the plant from a photo or series of photos. However, there are several ways of achieving a green pixel count. Firstly, colour thresholds for a human predetermined green range (Tomasek et al., 2009) can be used (Figure 3-1b). Secondly, classification of each pixel according to which colour it has more of in RGB space (Malinowska et al., 2016); this may require some adjustment because a pixel with an RGB value of (150,155,150) is not green (as visible in Figure 3-1d). Therefore, some studies set filters first in the RGB channels before calculating green pixels (Rolland et al., 2013). This could also be done using a heuristic

approach such as that used by Paruelo, Lauenroth, & Roset (2000), so as to only select pixels that are distinctly green. Thirdly, it could be done by separating the hue space of a HSB image into n portions, and then classifying one portion as green and selecting that (Figure 3-1c). This method may produce an odd effect because the saturation of the colour makes a large difference to how it looks. Other methods such as Tomasel et al. (2009) separated images into a custom colour space, before calculating pixels that are plant; these methods are more complex but may be required if more simple techniques fail.

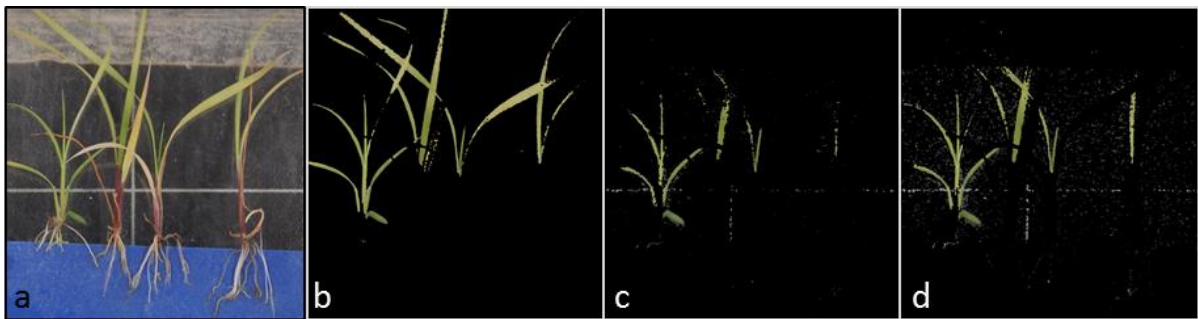


Figure 3-1: Differing green pixel counting methods on original image (a). b is a manual threshold, c is a HSB green selection, and d is a threshold of all pixels that are greener than another colour.

3.2 METHOD DEVELOPMENT

Due to the complexity of experimenting with a range of image analyses techniques, and to convey the process of developing methods using primarily open source tools, this section highlights the steps to developing the methods, and the testing of trueness to human scorers. Then the finalised methods for experimentation are given in Section 3.3 below.

3.2a TOOLS

Common free to access tools were preferentially used for the image analysis in this study to improve possible repeatability and give greater usability if the tools were distributed to other studies.

COMPUTER VISION

The main tools considered for this were OpenCV, Gimp and ImageJ; OpenCV (Kaehler & Bradski, 2014) has the widest range of applications but requires the most expertise to use, because it has no built in user interface and is designed as a platform independent module. As such any user interface would need to be programmed in the language used to interface with OpenCV (probably Python), and this would add complexity to solutions. Gimp (Lecarme & Delvare, 2013) has a comprehensive user interface but automation is difficult because it involves the use of Gimp's macro language, which is a dialect of the Scheme programming language. ImageJ has a lot of community support and add-ons with a functional macro language, and ease for expanding code to other languages. Matlab (MATLAB, 2010) was not considered because it would not be open source. Therefore, FIJI (Schindelin et al., 2012), a distribution of ImageJ (Abràmoff, Magalhães, & Ram, 2004) customised for biological image analysis was chosen. It is based upon the standard but old open source ImageJ program with more modern architecture included (Schindelin et al., 2012). Therefore, FIJI provides the user with tools for the identification and tracking of biologically relevant objects in the images (Schindelin et al., 2012). Being commonly used and open-source it has much more flexibility to be used by others than commercial systems developed for this purpose. Imaging such as this can also be used for seed phenotyping (Wagner et al., 2011); however, there are also off the

shelf methods for doing this e.g. MARVIN (GTA Sensorik GmbH, n.d.) (see Section 3.1a above).

FIJI allows photographs to be batch processed; by using macros, different ways of scoring germination were tested. FIJI has several inbuilt techniques for counting and measuring objects as well as some specific plugins and several of these methods were tested.

MACHINE CATEGORISATION

R and Python were considered. R (R Core Team, 2015) is primarily a statistical language and widely used for data analysis and interpretation. Python (Python Software Foundation, 2012) is a powerful, multipurpose programming language with a wide range of applications including scientific analysis. R was ultimately chosen because there are a plethora of easy to use tools and libraries available for machine learning along with other resources such as online tutorials.

Machine learning algorithms fall into many categories; a major category is machine categorisation, categorising information/data into groups. This technique works by providing training data where the data is already categorised. From this training data, parameters can be extracted for each category provided. The algorithm can then categorise new data according to these parameters. *K*-nearest neighbour (Cover & Hart, 1967) was chosen as a simple machine learning method (Shalev-Shwartz & Ben-David, 2014, Chapter 19) in R with the 'class' package (Venables & Ripley, 2002).

3.2b EXPERIMENTING WITH PROCEDURES

It was necessary to perform pre-testing and development on the image analysis techniques before attempting to apply methods to experiments that were more complex. The stages of testing and refining the automation of germination scoring are outlined below.

COUNTING SEED

An open pollinated, and phenotypically diverse *Miscanthus* seed ('Open Crossed' in Table 2-1) was used for this test. The seed had previously been observed to have a less than 50% germination rate and had a range of seed colour and size that would help test the image analysis software.

A pre-test was completed to check if the 'Computer Vision' techniques (Section 3.2a), under the right selection of variables, could consistently tell the difference between seed and other debris. This was carried out by repeated tests with a human counting the seeds in a Petri dish (Figure 3-2). Images were then manually thresholded and the 'object counter' counts of valid objects obtained. FIJI could identify seed numbers within a ~13% (of the 70 to 120 seed) range of the human scores. While not perfect, for initial testing it demonstrated that it was possible to detect small *Miscanthus* seed with limited processing using FIJI.



Figure 3-2: *Miscanthus* seed in a Petri dish (with a 10p coin for scale) to assess FIJI's ability to detect *Miscanthus* seed, compared to that of a human.

The method could be refined, and cleaner seed would produce counts that are more true to the reference point. This method demonstrated that the camera could produce sufficiently clear images to count the seed automatically. It was concluded that because the seed were large enough to be resolved, it should be possible to resolve changes in the seed as noticeable as those should that occur in germination.

GERMINATION MEDIUM

When testing seed germinated in Petri dishes, a suitable medium needed to be found. Filter paper and Steel Blue Germination Paper (Anchor Paper Co) (hereafter referred to as blue germination paper) have been used widely in other germination studies (Geneve & Kester, 2001; Joosen et al., 2010; Keeley & Fotheringham, 1998; Oluoch & Welbaum, 1996). An agar medium was considered because it can effectively deliver water and chemicals; however, the chance of fungal infection was increased and ripples or bubbles could distort the image analysis.

To test the possible benefits of blue germination paper and filter paper 25 seeds were germinated on both media at 25°C for 5 days. When observing the results of this test, radicals were less distinct on the white filter paper than on the blue germination paper and clear manual thresholding of the resultant image was also more difficult (Figure 3-3). The difference in reproducibility between the computer using blue germination paper and filter paper was compared in a simple test where the computer program was used to examine a manually thresholded image of the seed and test whether the size, perimeter, shade, or position had changed from a starting image. If the seed parameters had changed more than 3 mm for perimeter, 0.4 mm² for area, 0.05 mm³ for volume, or 15.7% lighter for shade, the seed was added to the germination count. Table 3-1 below shows the difference in result between the blue germination paper and white paper was large, even without comparing them to the human scored data.

Table 3-1: Four basic methods of germination detection (perimeter, area, volume, shade) tested to identify any differences in reproducibility. The low levels of germination may make it simpler for the human scorers and more difficult for the FLJI script, the error is the absolute deviation from the human score.

Paper	Human Score	Computer Scores of germination					
		PC1	PC2	PC3	PC4	PC Mean	PC Error
White	3	10	13	17	6	11.5	8.5
White	4	7	13	16	1	9.25	5.25
Blue	4	3	8	2	6	4.75	.75
Blue	5	11	7	8	2	7.0	2.0

In addition, on the white filter paper it was more difficult to identify the parts of the seedling and there was more of an effect from water reflections than on the blue germination paper (see Figure 3-3, upper row second image and fourth image)); this affected the speed and repeatability of manual germination counting.



Figure 3-3: Seed on white filter paper (left) and blue germination paper (right) both germinated and un-germinated. Included below each image is an example of thresholding to remove the background (this was manually applied for an optimal result).

An added advantage of the blue germination paper was that in later studies using fluorescence to determine seedling viability the blue paper did not fluoresce when carrying out chlorophyll fluorescence imaging, whereas the white filter paper did (Section 4.2c below). For these reasons blue germination paper

was determined to be the most appropriate medium for experiments involving seed germination imaging, and was used unless otherwise specified.

COUNTING GERMINATION

It was necessary to test the protocol for counting germination so it could be refined for a real test of trueness to the human reference point. The initial setup consisted of a Nikon D90 DSLR camera set at a fixed distance away from the sample on a tripod stand. This was placed at the same distance for all photographs to ensure seed size was not changing due to perspective. The seeds ('Open Crossed' in Table 2-1) were kept at 25°C under red light which was previously reported as an optimal growing condition (Aso, 1976). To prevent exposure to broad-spectrum daylight during counting, a set of 200 seeds was divided onto eight separate dishes so one could be removed each day from the red light and counted as aliquots. Two extra dishes of 25 seed ran without aliquoting through the whole experiment; this continuous set tested the software's ability to track the seeds repeatedly. This may be important because the germination process can cause the seeds to move. Manual counts and photographs were taken every 12 hours, starting 48 hours into the experiment, until no new germination occurred for 24 hours; the dishes were also photographed initially before placing into the controlled environment. Each dish was photographed separately resulting in 15 start pictures plus an image of each aliquot as it was removed, and daily photographs of non-aliquoted dishes, totalling in 57 images.

The human observer scored germination as the point at which the radical had emerged by more than 1 mm from the seed coat (Bewley, 1997a; Ellis et al., 1985a) (as in explained in Section 2.1a above). FIJI was also adjusted to attempt a similar level of precision, using the photographs in 8-bit grayscale. At the start

of the experiment, FIJI was used to count the seeds and measure the initial perimeter, area, and shade of each seed; this could then be compared to the first image taken [macrocode Appendix D]. With FIJI's 'particle analysis' tool, the seed count could be accurately established and all relevant measurements could be collected for all seeds in an image at once. However, the disadvantage of this was if two seeds touched at any point during the experiment they would be counted as one object. The determination of germination from the volume, area, perimeter, and shade results was done using Microsoft Excel; these scorings were done by removing the first result from later results to calculate a difference; this value would then be recorded as germination if it were over a set tolerance. However, dividing this result by the original, to give a percentage, would be a simpler, more transferable result, yet when attempted it underestimated the germination; this was probably due to the seed identification problems discussed below. This process was also performed on two thresholds of the same image, one thresholding for the seed only just removing the background.

Manual counts showed low germination in all dishes. The total mean final germination was 10.6%; therefore, the time to 50% germination could not be determined. Germination in the aliquoted seed lots was low enough that it was difficult to see a trend in germination over time.

Germination results for FIJI plotted in Excel did not correlate well with the manual counts of germination (Figure 3-4). The amount of seeds germinated each time could be varied by altering the change required in a parameter for a seed to be scored as germinated e.g. the perimeter of a seed needs to change by 3 mm. This was set manually to calibrate the level of each parameter; as is evident from Figure 3-4, no adjustment could bring the parameter estimates in line with the human score.

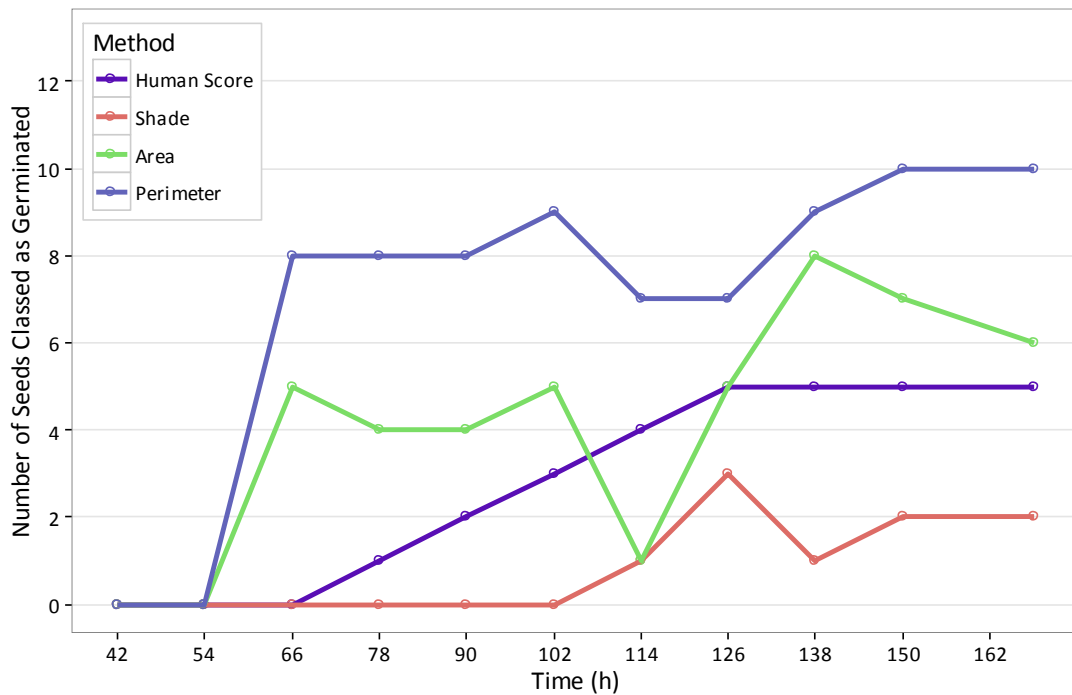


Figure 3-4: Three parameters of germination (shade, area, and perimeter) compared to manual counts (by eye) for germination. Each parameter was manually calibrated to produce a result closest to the human observer, shown in purple.

Although there was clearly inconsistency between germination scores in the initial testing (Figure 3-4), germination was still so low that a true level of reproducibility could not be reliably quantified, particularly if there was any error in the human score. Shade forms the closest line, which is between two and four seeds lower than the humans' scores. Much of the disparity was due to fungal growth or the fact that small rotational movements in the plate led to different seeds being compared (Figure 3-5), which due to the size differences between *Miscanthus* seed could allow seed to be scored as germinating or recorded as shrinking.



Figure 3-5: Identification of seed locations over time. Left: Seed at one time point. Right: FIJJ's placement of each seed for each image in the time sequence is superimposed. The movement of the dot is due to the image at different time points having slight rotation and movement; however, each icon should remain centred around each seed.

Due to this, using a single image to score germination, rather than the change in seed over two images was tested. This was done by thresholding the same image twice [macrocode in Appendix E]; the second threshold was to isolate the seed without any emerging radical. This was used to test the principle established by (Joosen et al., 2010) to count using only one photograph. This was based on thresholding the image for all seed with any radical emergence and thresholding for just seed alone, thus avoiding the problem of seed moving by counting any object that appeared in both thresholds. However, this method did not stop the misidentification of seeds entirely, but it removed the need to know which seed was which. Seeds could be lost in one threshold and appear in another due to fungus or upward radical growth obscuring the seed in the 'seed only' threshold, while it remained in the background subtraction threshold.

Using two thresholds applied to a single image entailed comparing the difference from the background threshold with a combination of the seed and radical

growth to the 'seed only' threshold. This method proved more true to the human reference point than using the method with two images (Figure 3-6). However, it was still not reliably quantifiable because the comparison was still with the same low germination seed dishes; however, a rough comparison of the best estimate, 'area' (see Figure 3-6), shows it always within two seeds of the human scorers' germination estimates.

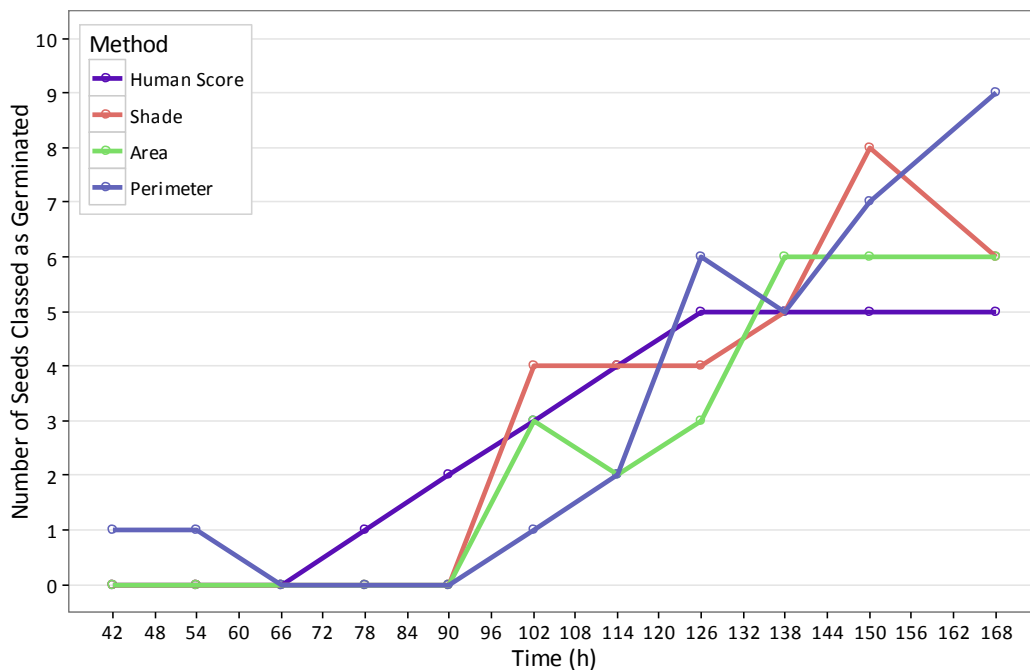


Figure 3-6: Single image germination scores, using the three probable indicators (Shade, area, and perimeter) compared to manual counts.

The Joosen et al., 2010 technique may have been more successful if the seeds were spaced better or on separate dishes. However, the two-image method would have also benefited from the seeds being better spaced; because its reduced trueness, when compared with using single images, was primarily due to thresholding problems leading to incorrect identification of which seed was which.

The two-image method proved to be ~50-80% true to the human reference depending on the sample condition (quality of images, presence of mould on

samples), when tested on individual images of seeds with the seed centred in both images (Figure 3-7).

Due to the low germination, fungal growth, and misidentification of the seed, developing an entire dish protocol for automated germination identification was ineffective. Therefore, selected subsets of seeds from the photographs (unaffected by fungus, germinated and clearly photographed) were used as a set of individual images over a time sequence (Figure 3-7). These had the FIJI data, collected with the '3D object counter' tool (this tool provided most of the same data on the object as the 'particle analysis' tool, but could be used on single objects); this data was linked to the time and human score for germination.



Figure 3-7: One seed photographed over nine time points (24 hours apart) during germination.

This was manually selected seed from the range finding experiments.

When using the individual seed images, measuring the area of the seed object proved a somewhat reproducible way of detecting germination, with an increase in object area often being just after or at the same time as germination (Figure 3-8). However, because the radical can emerge on top (in Figure 3-7, the seed's area would not change until the fifth image) or beneath the seeds, seed area will not always change after radical emergence. Therefore, the secondary method of shade measurement was also used. Figure 3-8 shows an example of the same four seeds analysed using both methods. This could detect a seed's change in shade with radicle emergence, for example, seed 18 in Figure 3-8 shows little change in seed area, but germination is noticeable by an increase in shade. However, only seed movement detection could detect radical emergence from the

bottom of the seed, and this would require a particularly stable set of photographs, such as a time-lapse sequence taken *in situ*.

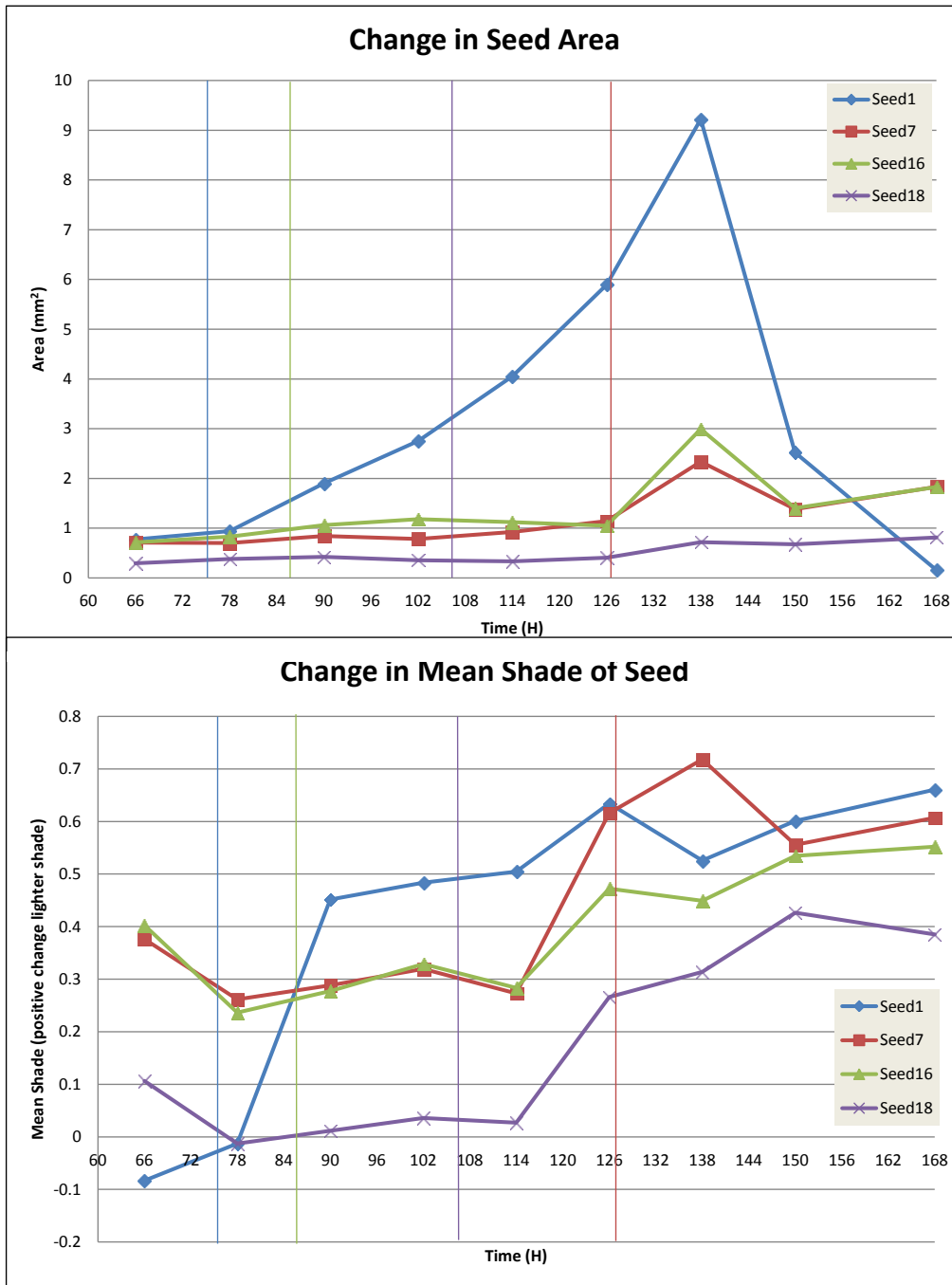


Figure 3-8: Comparison of four exemplar seeds using change in area (top) and change in shade (bottom), all data is the Δ from a 0 hours image. The vertical lines represent the times each individual seed was scored as germinated by the human scorer (colour coded).

To produce a true to human estimate of the total germination, it is clear from Figure 3-8 that neither shade nor area of the seed gave an effective germination score when checking on a seed-by-seed basis. A combination of factors was tested to produce a model that worked for most of the seed-image time sequences. After Δ shade + Δ area and then Δ shade \times Δ area did not yield better results, increasingly complex adding and subtracting of factors was attempted, e.g. standard deviation in object shade \times Δ surface area \times volume at start. This model (shown in Figure 3-9) is one of the better models but this was normally a time point late in having a notable increase at germination and there was no absolute value above which a could be classed as germinated. Often these models could be made to work on a sub set of the seed, then when more were added the model failed to predict their germination. Because complexity was added to account for more seeds the purpose of the rational for the model parameters were lost e.g. equation (2) where S is shade, V is volume, SA is surface area, and sd represents the standard deviation while i denotes the initial value.

$$\begin{aligned} & \left(((\Delta Ssd + \Delta SA) \times (\Delta V + \Delta Smax)) - ((Ssd_i + SA_i) \times \right. & (2) \\ & \left. (V_i + Smax_i)) \right) \times \left(((\Delta Ssd \times \Delta SA) + (\Delta V \times \Delta Smax)) - \right. \\ & \left. ((Ssd_i \times SA_i) + (V_i \times Smax_i)) \right) \end{aligned}$$

A method of checking many factors may work better if they were checked individually; this tactic was developed and tested in Section 3.3a below as the main FIJI germination count.

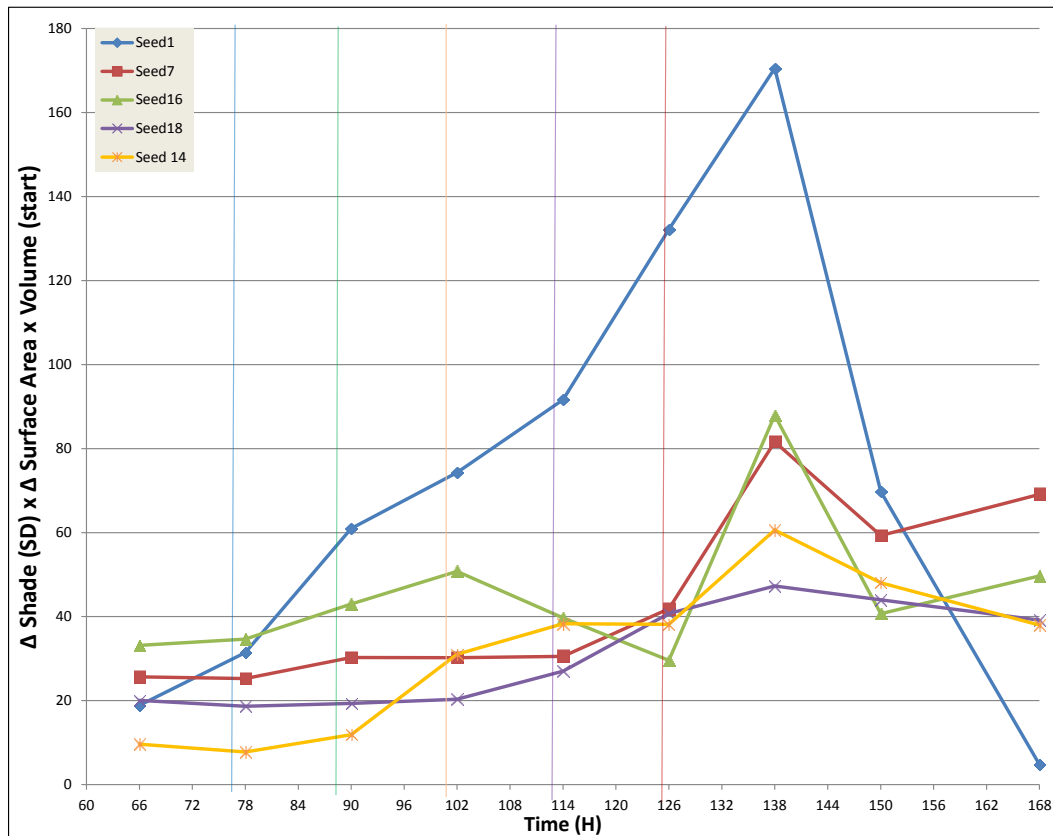


Figure 3-9: An example of a complex model being used to predict germination time for a set exemplar seeds over 168 hours. The human scored germination times are marked as vertical lines.

A new approach was tried whereby the number of pixels at each shade could be multiplied together to give lighter seeds a higher score. The number of pixels would also increase with radical emergence and those pixels would be a lighter shade than the dark brown seed coat of *Miscanthus*. This approach proved to be a simpler way of combining shade and size than using the complex method applied in Figure 3-9 and above. The data from the histogram of a grey scale individual seed image is illustrated in Figure 3-10, where a spike forms in the histogram at 54 hours. The data was used to create a single value, the sum of the product of the pixel shade and frequency of the pixel shade values across the seed image.

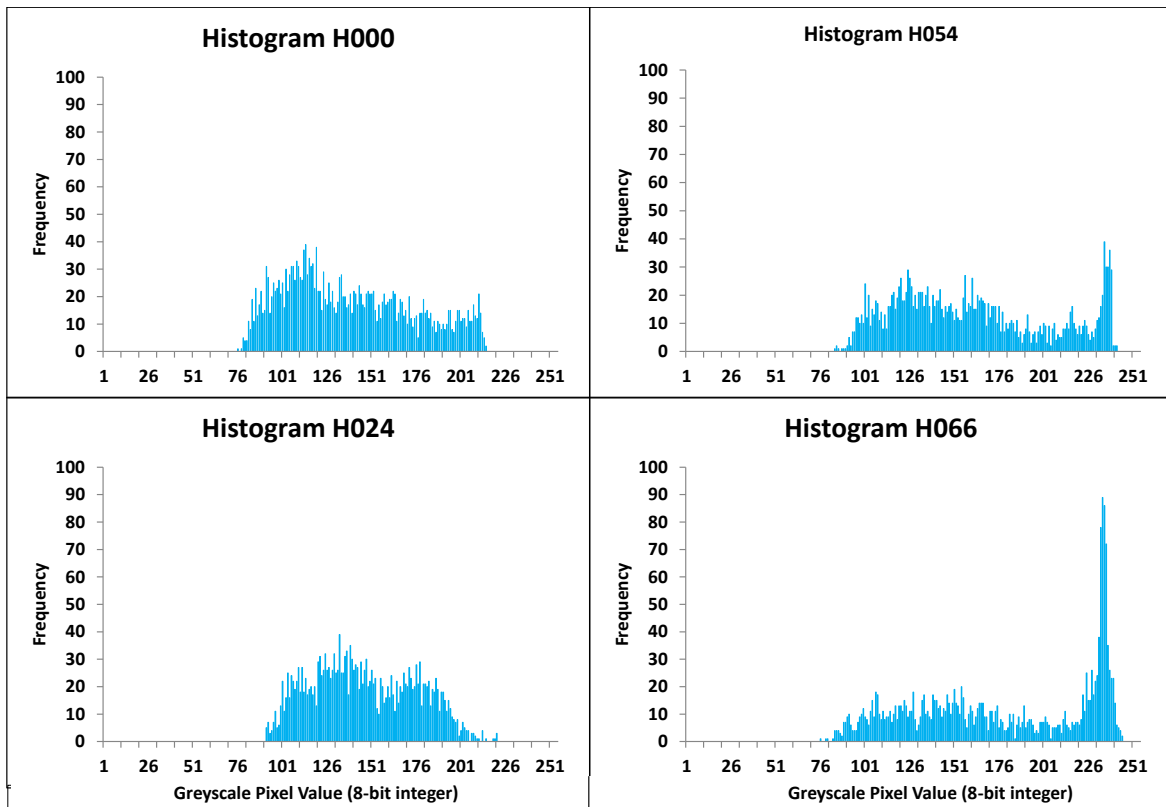


Figure 3-10: Sample of the histogram values for one seed over time (top left 0 h to bottom right 66 h). Germination starts at 54 hours when the distribution of seed pixels shifts to the right.

The combined histogram was tested by setting a value on the combined scale and marking all seeds as germinated if they exceeded this value. From this, it was possible to identify a value at which all of the tested seed images crossed this threshold within 24 hours (example seeds shown in Figure 3-11). This value is set to 40,000 in Figure 3-11, seed 18 was detected 18 hours late for germination, and seed 3 dropped below the 40,000 value after exceeding it at 66 hours, which was the time it was germinated. Seeds that never germinated were added (black lines Figure 3-11) and were easy to exclude. An exact germination time for the seeds that germinated (relative to the human score) was not achieved. Overall, the 40,000 value was accurate to within a day for the seeds in this subset, and allowed the macro to be calibrated and run on all the images.

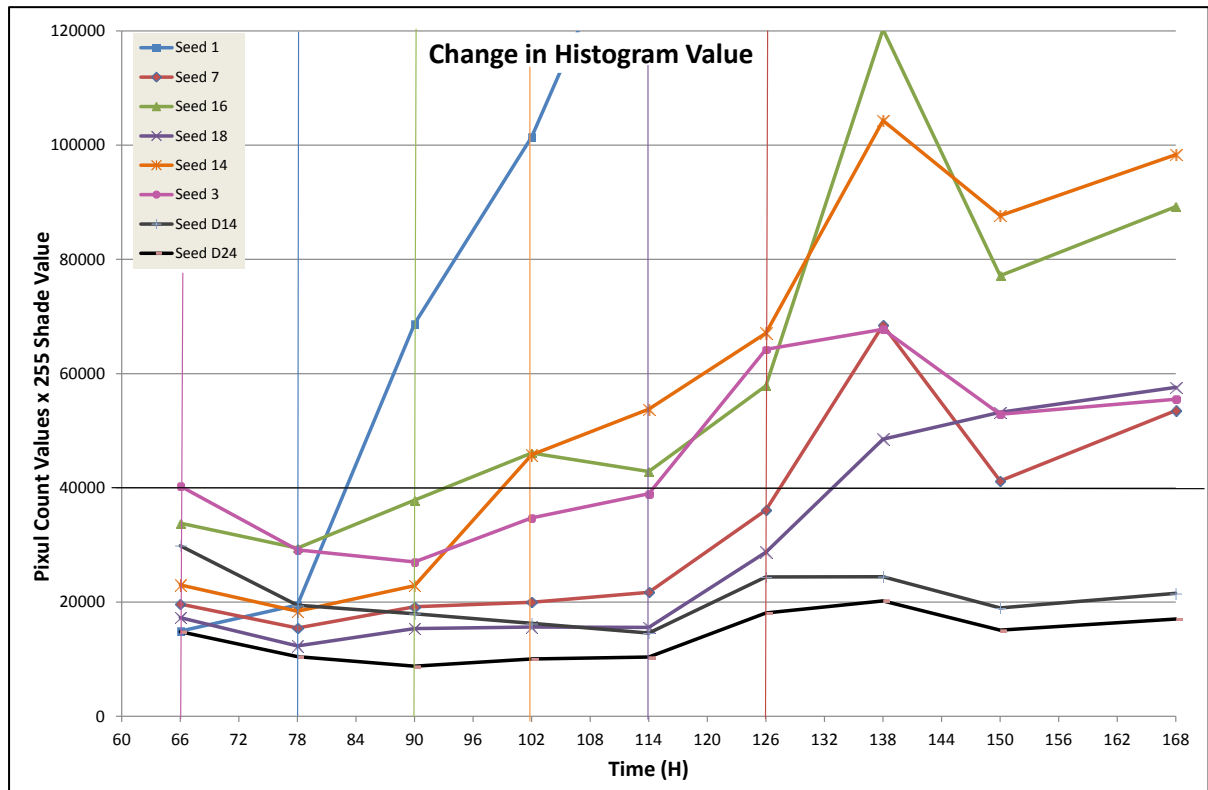


Figure 3-11: Set of seeds tracked with Δ histogram scores, derived by deducting from the histogram scores from a start image (D14 and D24 in black are seeds that did not germinate). Vertical lines represent the human scored germination times coloured for the individual seed.

When run on a wider variety of the sets of individual seed images, the outcome was poor (63.2% correct for if it was germinated or not at each time) this was primarily due to image quality and mould issues as discussed earlier, because the subset of seed images were selected for quality and lack of mould. Mould was a particular problem because it made the seed appear bigger and normally much lighter, causing the seed to clearly cross the germination threshold. This resulted in many more un-germinated seed scoring above the 40,000 threshold set for germination. Some seeds were comparable to seed 3 in Figure 3-11, in that they were germinated yet remained under the threshold.

The experimentation in this section was used to inform two testable methods for germination detection using the information from a FIJI object analysis, this is finalised and is presented in Section 3.3 below.

3.2c TESTING REPRODUCIBILITY OF HUMAN GERMINATION SCORES

It was necessary to test to what level the germination algorithm would have to perform to be as good as human scoring seed germination, and conversely what was the stability of the ground truth that the computer was being measured against. As discussed in 'Repeatability of Manual Scoring' (Section 3.1a above) the computer should be more repeatable, particularly in high throughput imaging, independent of trueness; yet should match the trueness of a human scorer as closely as possible.

3.2c-i IN PERSON GERMINATION SCORING

An experiment was conducted to determine how consistent germination scores were between four people, two with prior experience of scoring *Miscanthus* germination. Seed was scored for germination every 24 hours using a photograph of the seeds taken at the start of the experiment, so that each person could circle the germinated seeds in the photograph. Each person was given the same description of seed germination as stated in 'Germination Scoring' (Section 2.1a): "A 1 mm protrusion of the radical from the seed coat". However, they were not allowed to see which seeds they had indicated as germinating previously, or the total number of germinated seeds they previously indicated. Differences between the scoring by people were analysed using a one-way ANOVA (with seed trays as the error factor) for each time individually, and checked using the human scorer as the error to detect differences between the seed trays.

The differences between the human germination scorers were significant at 54 and 78 hours ($P < 0.05$ & $P < 0.01$ respectively), but not significant at 102 hours despite a wide variation, as shown in Figure 3-12. When the scorers were the error value, the seed trays were significantly different at every time point ($P < 0.05$ at 54 hours, and $P < 0.001$ at 78 and 102 hours).

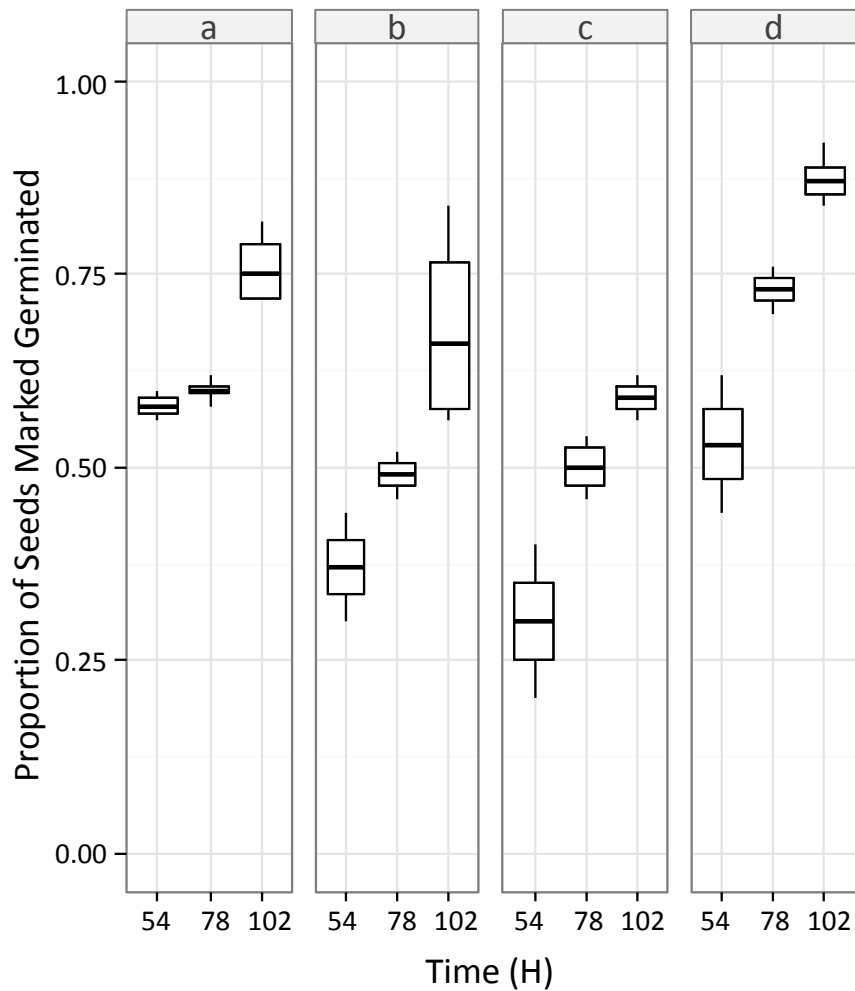


Figure 3-12: A boxplot each box represents the error between people scoring germination over three time points on four trays of seed. The graph has been sub divided to show the 4 trays as panels (a to d).

The average standard deviation of human scores across all times was 3.1 seeds in the tray of 50 (6.2%). This should be a standard for the computer vs. human scorers. This result is good for the total germinated seeds at each time; however, it was observed that people were inconsistent over time about which seeds had germinated so a seed previously marked as germinated may be missed in a second time point. This was identified as a problem when scoring individual seeds in Section 4.2a below.

3.2c-ii IMAGES OF SEEDS

To explore further the error between people scoring seeds, and to improve the image analysis, an experiment was conducted in which people were required to score pictures of seeds. Using a set of seed images (~712) three people scored seed as either 0 (not germinated), 1 (maybe germinated), or 2 (definitely germinated). The scores were combined to give a total likelihood of germination (Table 3–2). Each person scored up to 712 seed images; they all were given the definition of a germinated seed (Section 2.1a). The pictures were not arranged by each individual seed; instead, images of the same seeds at multiple times were given separately.

Table 3–2: Example data for three people scoring individual seeds (referenced in the first column) as three-point likelihood, and as a combined score (in the fifth column). This was later used later as a comparison for the image analysis. This table is a representative example of the analysis table.

Seed & Time	Person 1	Person 2	Person 3	Score
S071_t5	2	2	2	6
S089_t2	1	1	1	3
S159_t0	0	0	0	0
S040_t4	1	2	1	4
S257_t6	2	2	0	4

This was designed to estimate the consistency of human scorers, where a six would indicate that all scorers are certain of germination and a zero would indicate certainty that the seed is not germinated. The scores could have been averaged but summing the scores allowed each total to be a discreet category. Because not everyone scored all of the images, it was difficult to judge the level of reproducibility in the human scorers, but 26.5% of seed images received an odd numbered score implying that was a minimum number of maybes.

These scores were then used in Section 3.3a directly below as an approximation for a germination ground truth to calibrate the FIJI germination detection system.

3.3 METHODS

This section explains the final developed methods that have direct counterparts in the results.

3.3a FIJI GERMINATION COUNTS

An experiment was completed to develop further the results of 3.2a to test the trueness to the human reference of the seed germination detection. This experiment minimised the list of factors that reduced trueness in earlier experiments.

Firstly, mould was an issue in all of the experiments to refine the imaging methodology, stopping the software from recognising the radical by changing the shape of the seed, and sometimes obscuring the seed enough that the thresholding reduced visible seed to a few pixels that were excluded on size from the analysis. These problems may be surmountable with better thresholding or a shape analysis on germinating seed radicals (to remove mould from the side of the seed), or by sterilising the seeds before the experiment. Sterilisation is the better option for removing variables, but may reduce the germination rate. However, this reduction would probably not have as great an effect on reproducibility as mould growth on the samples and therefore sterilisation was used on seeds in future image analysis testing unless otherwise specified. The effect of sterilisation on germination was examined in Section 2.4 above.

Secondly, small rotations in the dishes had caused problems in following the same seed over time (Section 3.2a above Figure 3-5). Improvements in the

method were used to combat this problem and obtain images that were more consistent. Square petri dishes were used with a setsquare [Appendix F] to prevent dish rotation, and the camera was mounted to a fixed copy stand for the duration of the test to provide more stability than a tripod this was similar to the method used by Wang et al. (2009).

Thirdly, sometimes an issue occurred where seeds would obscure each other by growing over or into each other. This was because the seed were placed on the dish randomly, and could end up too close together. Yet being too close to the edges of the dish provides more difficulties for the background thresholding. To reduce this problem the seeds were placed in the dish in a regular grid pattern, so that all seeds were the same distance apart. The grid approach also helped reduce the final problem of seed misidentification. For images from Chapter 4 below, seed could be followed over time by using the position in the grid to identify the seed. A problem of seed misidentification had occurred in earlier experiments, because a seed was tracked through a series of images by recording its order in the dish. The algorithm would label seeds from left to right, top to bottom. This meant that if a seed moved or became undetectable (e.g. due to germination or mould), it could be mislabelled or left out altogether, affecting the order of subsequent seeds. This problem was reduced by instead recording a coordinate location for each seed in the image. Then a seed could be tracked by selecting, in each subsequent image, the seed closest to that coordinate.

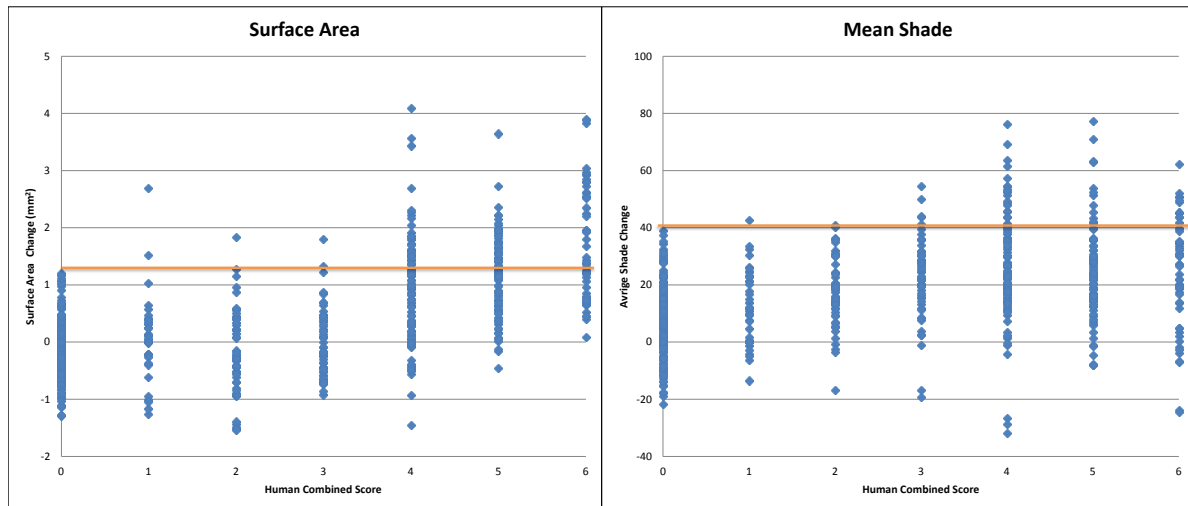


Figure 3-13: An example of thresholding the seeds by combined human score to determine seeds certainly not germinated. Seeds above the line get a point towards a pre-set threshold for scoring germination. The line is always set above the highest seed scored by humans as a zero.

All the seeds scored in images by humans in ‘Counting Germination’ (Section 3.2c above) were combined with twelve traits from each seed image measured using FIJI’s ‘3D object counter’. For each image in the time series, the values for each trait for the starting image were deducted from the values for the new image to quantify the amount of change. For the twelve traits, these changes were then graphed for each seed, arranged in columns on the x-axis by the human observers’ confidence in germination (see totals in Table 3–2). The horizontal lines in Figure 3-13 show the highest levels at which a seed has changed, but has not germinated by the estimation of the human scorers. Next, new scores were given whereby for each trait, all seeds that fell above the line were given a point. The sum of all the points for each seed then provided a confidence value for germination, up to a maximum of twelve. A comparison was made between the number of seeds that fell into each new confidence bracket and the total number of seeds that were classed as definitely germinated (scored a six) by humans; this was 7% of the seeds. The confidence bracket that contained the closest number of seeds to this total was chosen as the threshold for

whether a seed had germinated, i.e. if the closest bracket was ten, any subsequent seeds scoring ten or above could be said to have germinated [details in Appendix G].

Using this algorithm, once a seed was marked by FIJI as having germinated that decision was applied to all future time points even if the seed later dropped back below the threshold. This was because the algorithm was conservative in its approach, because it only ranked the brackets against the total number of seeds humans had scored as a six, when a lower score might not mean that a seed had not germinated.

Testing this method (done in Section 3.4a below) required using a new set of seed images, so individual seed images were extracted from those taken during the experiments in Section 4.2a below. Two methods of change in the seed image were tested: First, the total difference for each of the traits from the first image to the current image was calculated. Second, a percentage difference between the two was used. In each case, the seed image gained a higher score for each trait that had changed adequately (based on the thresholds like the example ones in Figure 3-13). For example if the seed image passed the threshold of plus 1.4 mm in area it would get a point, and passing the threshold of 40 in shade would get it a separate point. Through this process, each image would get between 0 and 12; referred to as a confidence value.

Results were analysed with a ROC (Receiver Operating Characteristics) curves and used the area under these curves as a single score of the classification, this is a popular approach (Fawcett, 2006). However, these areas under ROC curve scores will only calculate false positives and negatives at each seed germination time and not account for the closeness of predicted germination times. For example, if FIJI has estimated the seed has germinated at time 4 and the

human identified the seed as germinating at time 5; time 4 would be just as much a false negative as if FIJI had not estimated germination at all.

3.3b k -NEAREST NEIGHBOUR GERMINATION COUNTS

A machine learning approach was used as an alternative to the basic FIJI method, because it would allow a different classification system to be created based on the data from the previous, manually parameterized method. A k -nearest neighbour (k -NN) method that contrasted the tree style of the FIJI macro method was chosen for the machine learning approach (for more on why this method was chosen see ‘Machine Categorisation’ in Section 3.2a above).

k -nearest neighbour works by finding each point’s nearest neighbours in an n -dimensional Euclidian space, then grouping that point with the k neighbours with which it is most closely associated (Bishop, 2006, p. 125; Shalev-Shwartz & Ben-David, 2014, Chapter 19). The training data is loaded into an n -dimensional matrix, with n being the number of parameters. The uncategorised data is added, and each data entry has its parameters compared to all parameters in the training data. The k closest parameters by Euclidean distance (the nearest neighbours) are used to classify the new entry by majority vote. An odd number can be selected for k so that the vote is unambiguous, otherwise a tie will be broken at random. Larger numbers of k produce more smoothing in the classification boundary (Bishop, 2006, p. 126).

This method was trained on a random set of half the seed (from ‘Physical & Chemical Germination Factors’ (Section 4.3b below)) and tested on the other half. [See Appendix G for script]. This step was repeated multiple times to test and improve trueness by refining the value of k and the amount of classifiers to include in the training set. Traits (area, shade, etc.) from the FIJI object detection were used as well as RGB and HSB histogram values for each thresholded

seed object (e.g. R0 to R255), to give a colour distribution for each image (Dell' Aquila, 2009). Because these traits had different values, values for all traits were normalised to between zero and one. Due to the number of these traits, an alternative to just normalising the data was also tested by putting the trait data through a principle components analysis (PCA) (stats package: R Core Team, 2015) to combine and summarise the main components of variation between images; this restricted the output to twenty-one principle components.

The human scoring of time sequences as described in Section 4.2b below produced the presumed ideal score against which to compare. Pictures of seed from time zero before the test started were excluded from the k -NN method because this added an extra ~5000 un-germinated images, their purpose as a starting point in the FIJI classification wasn't necessary for k -NN.

Due to the scoring of time sequences in Section 4.2b, once a seed was marked as germinated all images after that time in the sequence were marked as germinated. This resulted in a problem; seed images from later time points for seed that germinated and then died, and were originally scored by a human as germinated, would not appear germinated in isolation. To circumvent the problem the index of ground truths was reviewed by running the k -NN classifier and outputting the certainties (between 0.5 - uncertain, 1 - certain). The number of possible values was dependent on the value of k , so if all k of the nearest neighbours were the same the certainty would be 1 and if 4 of say 7 nearest neighbours agreed the certainty would be 0.57. The images that were classified as least certain in each run were manually checked by a human observer, and updated if necessary. Hereafter this set of image-identified germination amended by a human operator will be referred to as the 'amended human assessment'.

ROC curves were calculated and a combined score was determined to assess the final success of the k -NN methods once optimised. The final success of each method test was determined using a single measure from the ROC, the area under the curve, this is statistically equal to the chance the algorithm will rank a random germinated image more highly than a random un-germinated image (Fawcett, 2006).

Fawcett (2006) suggests that averaged ROC curves are a better indication of success, because the random seed could have been picked to give the best possible chance of success. In this study, the time required to run all the k -NN classifiers multiple times prohibited this so a common random seed of 1,234 was chosen. This method was not used to detect germination instead of a human in this study, but it was tested on seed images from 'Physical & Chemical Germination Factors' (Chapter 4), and could be valuable in high throughput work. The results from testing this method are in Section 3.4b below.

3.3c FIELD BIOMASS ESTIMATION

Using the experiment described in Section 6.2a below an experiment was done to use green pixel count from images of first year growth in the field to estimate second year harvested biomass. Plants were photographed while small and manageable (see challenges for field photography 3.1b above) at the end of the first year, just before senescence (October). A white board (1.4m × 0.6m) was held behind the plot to improve contrast between plant and background and the photograph was taken with a Nikon D90 from a distance of approximately 1.5m with a 10-24 mm wide-angle lens (see Figure 3-14a). The resolution of the pictures was approximately 1.44 pixels per mm at the plant, depending on how accurately the 1.5m distance was measured and a ruler was included in the image to standardise the scaling factor. The images were processed with a

simple semi-autonomous ImageJ script [Appendix I] to produce green pixel counts. As discussed in Section 3.2a above, there are many ways to count green pixels in an image, of which hue is often a component. In this case, a simple thresholding of hue and saturation was used because the plants contrasted highly with their background.

Within the ImageJ script, the user defines a line in the image along 10.16 cm of the ruler (in image Figure 3-14) to produce a scale; the number of pixels along the line was divided by 102 to get the pixels per mm. The green pixel count was produced by splitting the image into two grey scale images one of hue and the other of saturation, using two inbuilt FIJI thresholds the hue image was thresholded using an inverted 'IsoData' threshold (Ridler, T.W. Calvard, 1978), while the saturation used a 'Li' threshold (Li & Lee, 1993; Li & Tam, 1998). These were then added together into a binary image (only black or white); from this image, the total number of black pixels was counted [code in Appendix I]. It was necessary to crop the first ~4-5 cm of the bottom of the plants in the image as in Figure 3-14b, since the stems were brown not green at the bottom and they were difficult to threshold against a soil and weed background. In order to achieve this cropping, the photos were cropped manually to the size of the board so that all 108 images had the same area. Cropping was done manually by the user.



Figure 3-14: End of year side on photograph of first year *Miscanthus* plants with the stem data recorded on them (a). The binary threshold as used for pixel counts (b).

The pixel counts were then combined with the second year dry weights from the same plots. This data was tested using three methods: Firstly, with a multi-way ANOVA where second year biomass was the result and the predictors were all the measurements taken in the first year (stem height, number of stems, stem thickness and number of leaves) plus pixel counts, to determine the predictive power of each measurement. Secondly, a Kendall's rank correlation was calculated between and pixel area and second year dry mass, as well as between each

of four alternative first year field measurements (stem height, number of stems, stem thickness, number of leaves) and second year dry mass. Height \times Stem count was tested against second year dry weight as a non-destructive breeding measure of biomass. In addition, second year measurements of height and stem count were tested against the second year dry weight to show the improvement in accuracy in the second year. Thirdly, the R^2 for linear models of each correlation were reported. These results are presented in Section 3.4c below.

3.4 RESULTS

3.4a FIJI CLASSIFICATION

The refined FIJI method based on people's classification of seed images returned an estimate of the place of the first image in the sequence that had germinated (aka the estimated time that seed germinated), it is important to note this is not which images were of germinated seeds. It did not return that the image of seed 10 on day 5 was germinated; just that seed 10 is estimated to have germinated on day 3. This matched up with the data used from Chapter 4 where each seed had been scored for germination time (see Section 4.2b for details).

The area under the ROC curve is used as the measure of success in Figure 3-15; this shows the subtraction change in the traits was more effective than the percentage change. Figure 3-15 also shows the effect of changing the confidence value (number of traits over the set thresholds) required for FIJI mark a seed as germinated. It appears the best confidence values are 4 for a subtraction change and 5 for a percentage change. The percentage change is more dependent on the selected confidence value, with three times the variation between 2 and 7 as the subtraction change (~ 0.03 and ~ 0.01 respectively).

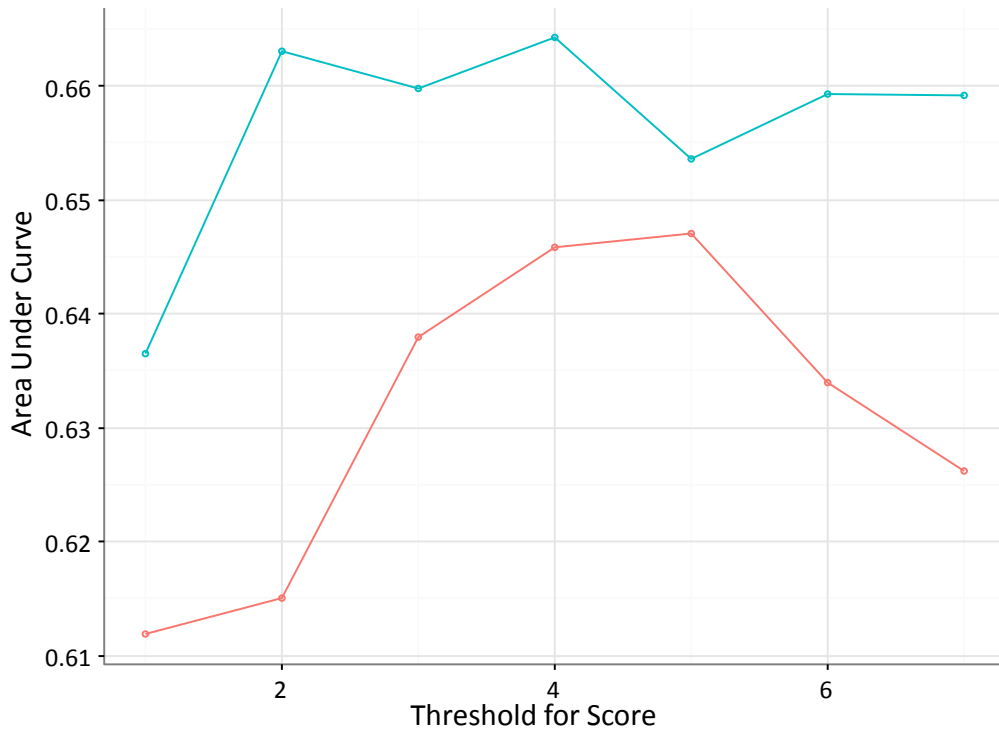


Figure 3-15: The area under the curve for confidence value thresholds of FIJI scoring, the red line is a percentage change and the blue line is a subtraction change. The ROC curves were performed on the estimates of germination time by human scoring and FIJI.

The number of successes was adjusted to the optimum level for each (4 & 5), these optimum ROC curves are shown in Figure 3-16 below for subtraction and percentage change. Figure 3-16 shows the subtraction change ROC curve in black has a higher but very similar area underneath (0.664) to the percentage change ROC curve in grey (0.647); this also shows the results of subtraction and percentage are smooth and not notably biased towards false-positives or false-negatives.

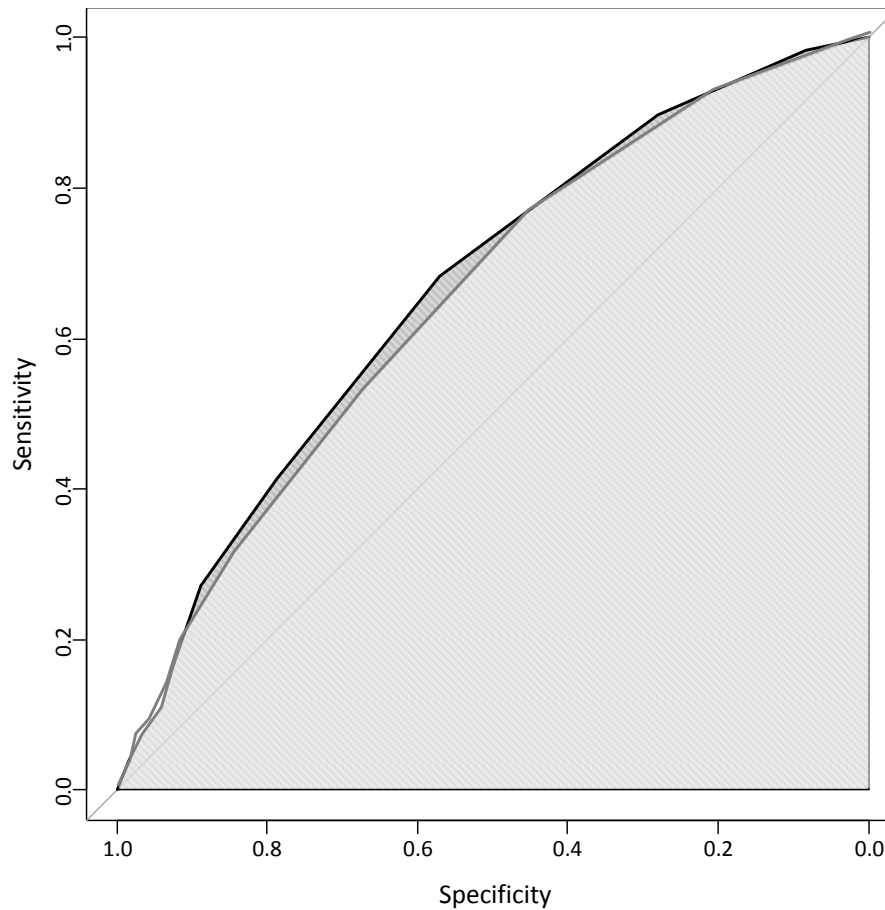


Figure 3-16: ROC curves for the best levels percentage (confidence value 5) and best subtraction (confidence value 4). Higher black ROC (subtraction) has an area under the curve of 0.664; the lower grey ROC (percentage) has 0.647.

However, the ROC curves represented in Figure 3-15 and Figure 3-16 only show the possibility of a true positive and true negative vs. false positive and false negative, this does not account for an incorrect time of germination (i.e. FIJI estimating the time a seed germinates early or late). This distribution of seeds is visible in the Figure 3-17 below.

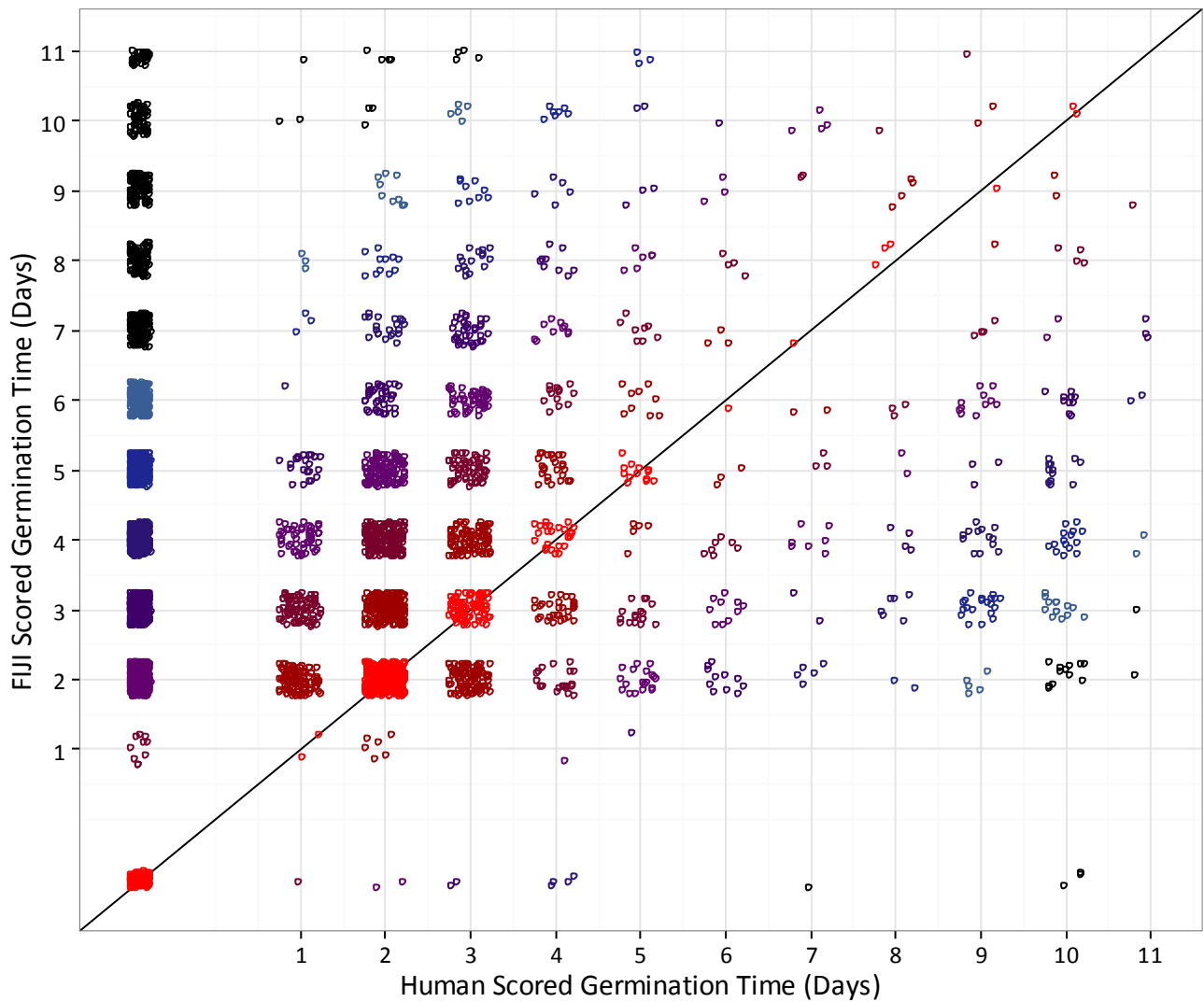


Figure 3-17: Germination Human vs FIJI for the best ROC of subtracted (confidence value used is 4) points that lie along the diagonal line are correct. Points have been spread out into squares to make their density visible, they are also coloured from red through blue to black dependent upon how far away they are from correct. Not germinated seedlings are near the border.

Figure 3-17 shows that FIJI tends to be late in its scoring of germination particularly in the first three days, it is also infrequent for FIJI not to predict any germination while this was frequent for the human. Seeds scored by the human scorer on day 3 were the most likely to be scored early or late in FIJI, and seeds scored by the human scorer on days 9 and 10 were likely to have been scored by FIJI on days 2 to 6 (Figure 3-17).

3.4b k -NEAREST NEIGHBOUR CLASSIFICATION

For the main testing of the k -NN method, the 16,896 seed images (from 4.2a below) were used for which 25 variables given from FIJI object detection (area, shade, etc.) and an additional 1,536 variables were produced from RGB and HSB histograms of the thresholded images. The images were separated into two random groups using a seed of 1,234, one for training the k -NN classifier the other for testing.

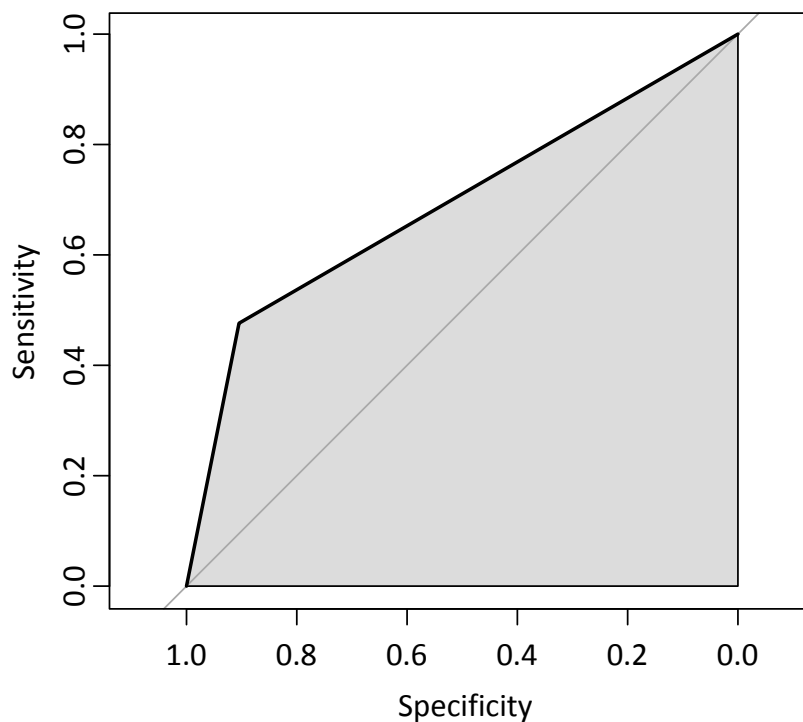


Figure 3-18: A ROC curve of just the FIJI data (traits like area, shade etc. not the histogram values for RGB and HSB). This k -NN output was compared to the amended human assessment of germination (random seed 1,234).

The results with just 25 variables produced by FIJI's object detection using all 16,896 seed images, assessed in comparison to the amended human assessment and a k value of 7, gave an area under the ROC curve of 0.69, with 558/8394 (0.066) false positives and 1345/8394 (0.16) false negatives (Figure 3-18).

To test reducing the total number of variables from 1,561 a PCA was used to limit the number of variables (Figure 3-19). When using the PCA, only the first 21 principle components produced were used (this was based on visual assessment by plotting the principal components); this easily accounts for most of the variation (Figure 3-19).

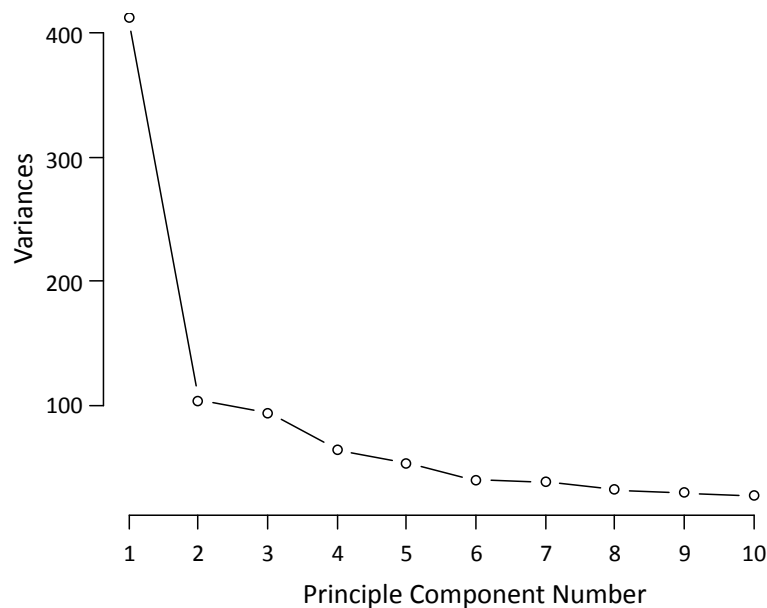


Figure 3-19: A scree plot of the top variances of the top 10 components in the PCA on the seed data of 16,896 seed images, with 1,561 dimensions.

As the PCA had reduced the number of variables for k -NN, the process could be run repeatedly, this was used to amend the human assessment where necessary (as described in Section 3.3a above) until there were no more seeds for which an amendment was necessary. The k -NN was run against the amended human assessment (Figure 3-20) and gave an area under the curve of 0.706 and 561/8,502 (0.066) false positives and 1,298/8,502 (0.153) false negatives.

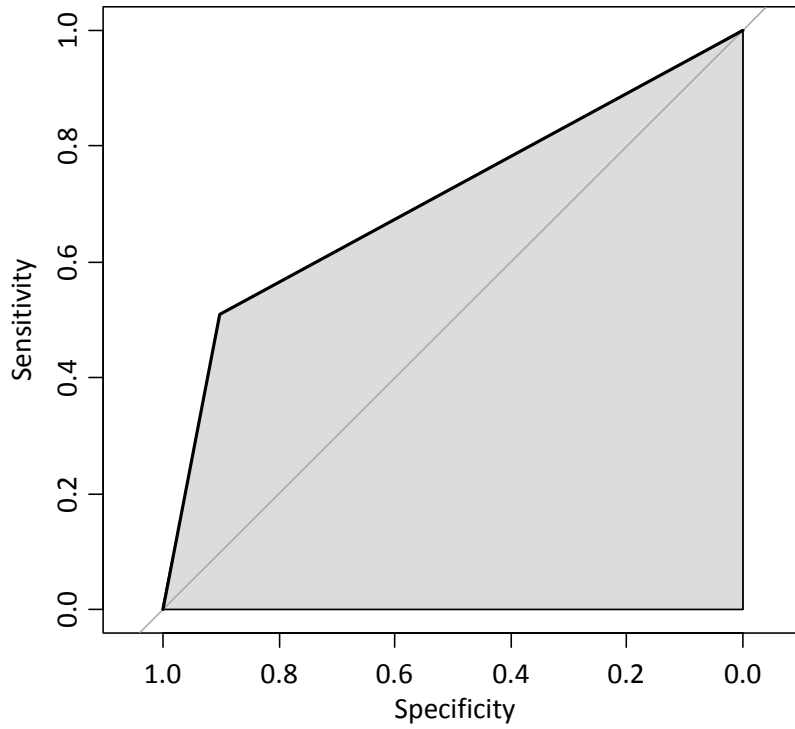


Figure 3-20: A ROC curve of the test set from the 16,896 images' PCA results tested with a k-NN analysis after manual improvements of image labelling.

This was the best overall result on the full set of image data; hence, all of the tests in this section use the amended human assessment, to reflect the improvement in the consistency of the labelling of the human reference point.

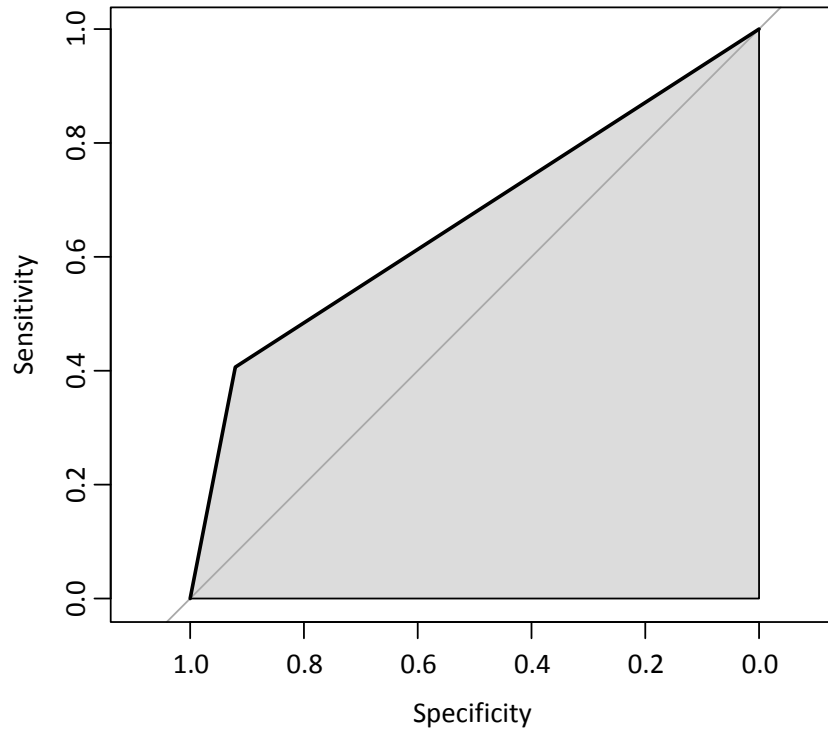


Figure 3-21: A k-NN of all 1,561 variables without a PCA to limit them, tested against the amended human assessment on all 16,896 seed images.

Without a PCA, using all 1,561 variables (and thus producing a 1,561 dimensional space to assess the seed) proved too computationally intensive for much testing to be carried out. One run with a k of seven resulted in an area under the ROC curve of 0.664 and 458/8,394 (0.054) false positives and 1,526/8,394 (0.153) false negatives (Figure 3-21).

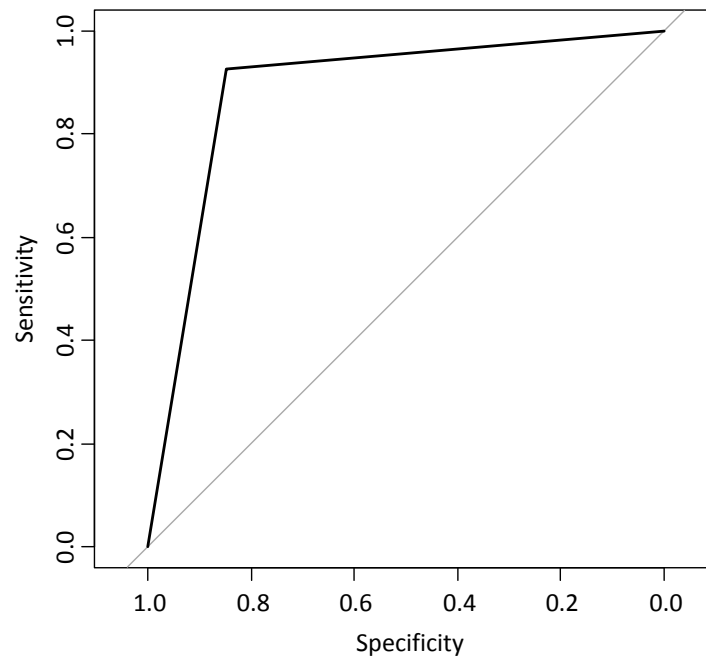


Figure 3-22: A ROC curve for a final idealised k -NN test using a subset of 711 seed images scored in approximately equal quantities as germinated and not germinated. Clear images with no mould present were chosen. The data was divided into two groups using a random seed of 1234.

An idealised subset of 711 seed was tested, split unevenly to provide only 233 test seed. This simplified the inputs to the 25 FIJI variables based on object detection. The k -NN gave a false positive of 8/233 (0.034) and a false negative of 19/233 (0.082). This gave an area under the ROC of 0.887 (Figure 3-22).

The optimum result using the entire image data was with the full set of traits reduced with a PCA; this was plotted against the individual dishes daily germination scores (Figure 3-23). Figure 3-23 shows the k -NN underscores germination early in the experiment yet is close to the correct total germination by day eight. The overall R^2 for the k -NN to the real counts is 0.7; however, this increases to 0.86 for day eight (last time point in Figure 3-23), and 0.89 for the last time point (day eleven).

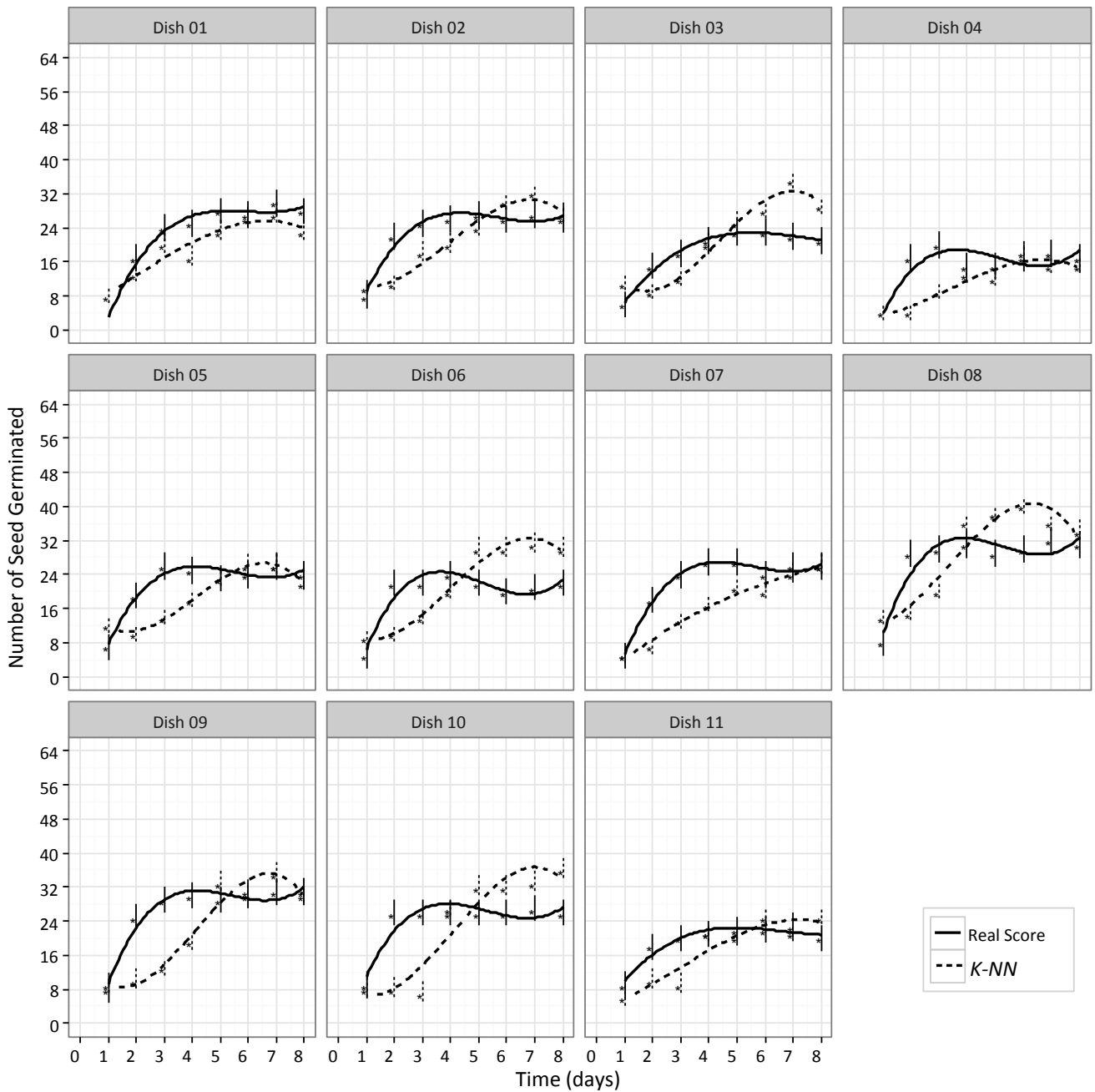


Figure 3-23: The daily total germinated seeds for the first 11 dishes (as a sample), with human counts (using the system in section 4.2b below) and k-NN image analysis for germination. The human standard deviation of 3.1 seeds is represented as vertical error bars.

3.4c FIELD BIOMASS ESTIMATION

First year green pixel area was compared to other empirically determined first year field traits such as plant height, number of stems, number of leaves, and thickness of stems. These were tested to determine which best predicted the second year growth as measured by above ground dry weight. The dry weight was not normally distributed and was therefore transformed to normality using \log^{10} transformation so that an ANOVA could be used to assess the best predictors of second year dry mass. Green pixels were the most significant predictor of dry weight ($P < 0.0001$). This was followed by height ($P < 0.0001$), then number of stems ($P < 0.05$). The other indicators (number of leaves & thickness of stems) were not significant ($P = 0.14$ & $P = 0.61$ respectively) in predicting second year dry mass from the plots' first year measurements.

As the dry weight data and all the first year variables to be correlated did not follow a normal distribution, correlations utilised Kendall's rank correlation. Out of the correlations of the first year measurements, green pixels had the strongest positive correlation (0.71), followed by stem count (0.69) and stem height (0.62), all three of these had high R^2 values (0.75, 0.71, & 0.72 respectively) (Figure 3-24A, C, & D). The other first year variables of first year stem thickness and number of leaves per stem had lower correlations (0.4 & 0.49) with lower R^2 values (0.23 & 0.23). In Figure 3-24B the non-destructive breeder's estimate of height \times stems had a higher correlation (0.7) than any of the first year measurements, apart from the green pixels count; however, it had a lower R^2 value (0.62) than the top first year predictors (pixels, stems, & height).

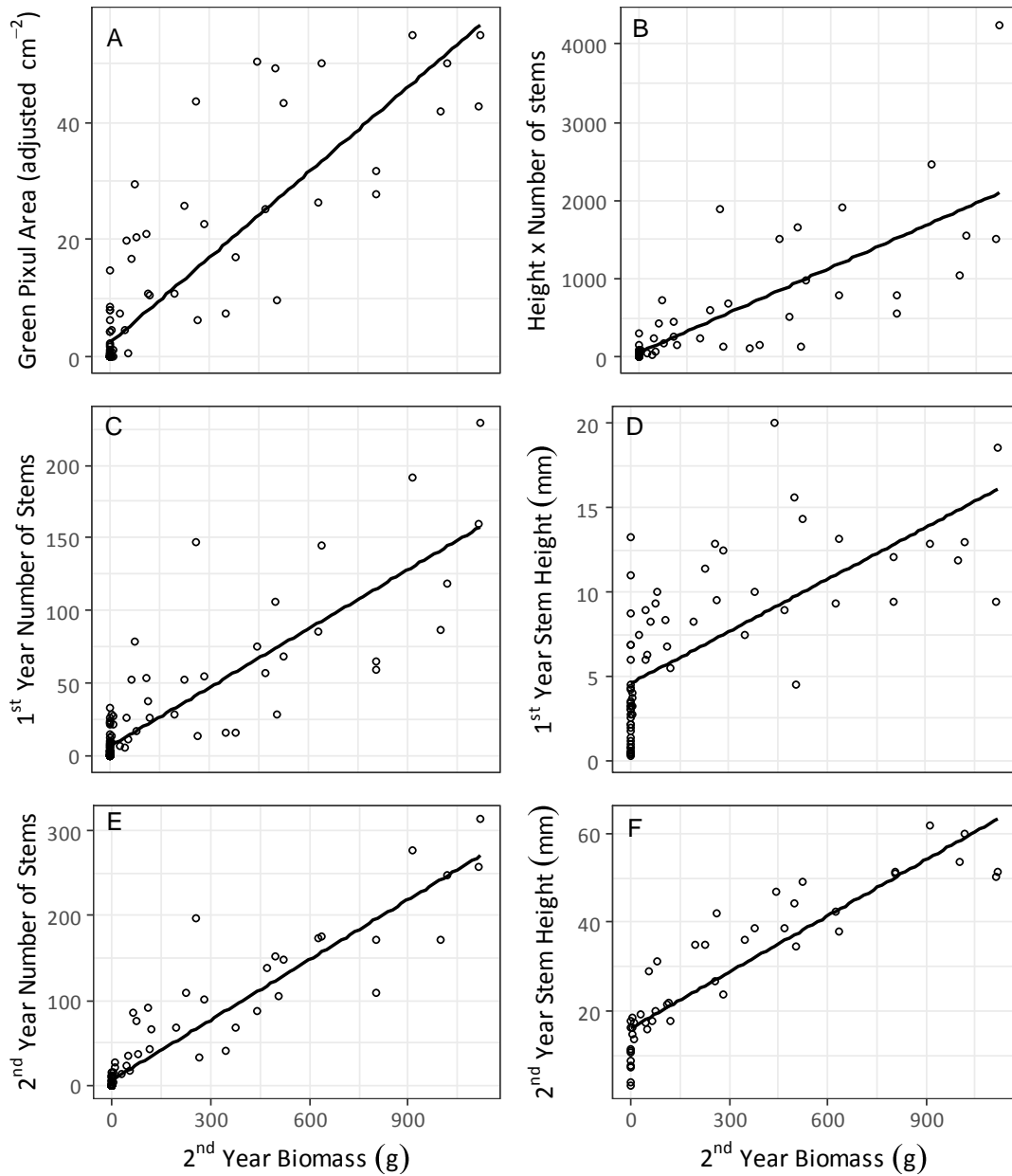


Figure 3-24: Aberystwyth second year biomass correlated against first year pixel count [converted to mm] (A), first year height and number of stems (D & C) and biomass estimate stems \times height (B). Second year height and number of stems correlated in (F & E).

For comparison, the best two empirical measurements (stem count, & stem height) in the second year were correlated against second year dry weight (Figure 3-24E & F). Stem count had the highest correlation and R^2 (0.85 & 0.87 respectively), while stem height was lower ($\tau = 0.81$ & $R^2 = 0.82$); both outperformed all of the first year measurements including green pixel area.

3.5 DISCUSSION

In this chapter, image analysis has been developed and tested in order to answer some basic questions.

Most important to the application of computer vision is its reproducibility and speed over a human. If computer vision offers no advantage, there is no reason to switch from a manual approach. The field and germination methodologies developed here were originally intended to be used to aid the collection of data throughout this study. However, due to the constraints of time and the lack of trueness to human scores, these methods were instead developed throughout this study and tested on the data produced by observations that are more traditional.

3.5a GERMINATION COUNTING

REPEATABILITY OF HUMAN COUNTING

First, the experiments needed to benchmark human counting of seed germination in order to determine how reproducible the automated germination counts need to be.

Human variability was measured at an average standard deviation between scorers of 6.2% in total germination (Section 3.2c-i). It was shown that the differences between the human scores were significant in the earlier scorings; this was probably caused by individual differences in how they interpreted the 1 mm radical emergence instruction for germination. This also did not take account of times scorers were observed not scoring a seed that they scored as germinated the previous time; this problem was encountered again in Section 4.2a below. Although, within person difference should be less than between

people variation, as it was observed individual's total germination scores steadily increased.

Human scorers in Section 3.2c-i were also able to manipulate the seed physically in order to check for germination, this is an advantage over the image based germination scoring. In Section 3.2c-ii, humans scored images of seed, as the computer would use. From the images, in at least 26.5% cases the human scores were unsure about if the seed was germinated or not.

Therefore, it can be estimated from the work in this chapter that the agreement between humans when scoring seed in person is around 93.8% per time point, with less deviation in one person's scores between time points. It can also be approximated that human scores were less repeatable when scoring photographs of individual seed images, being confident of seed germination in less than 73.5% of cases. This gives the image analysis algorithm a target of about 93~94% agreement with a human scorer. However, as it is scoring images the achievable trueness may be 70~80%.

REPRODUCIBILITY OF AUTOMATED COUNTING

The methods tested to determine automated germination scoring developed in this chapter could be true to the human scoring.

Both methods were effective to some extent on a large 16,000 seed set of images/5,760 seed sets. The k -NN method was more effective (0.69 area under ROC), with the FIJI method being less so (0.66 area under ROC).

The problem with the k -nearest neighbour result was that it required adjustment in the human assessment order to be effective, due to how the human assessment was produced. Once a seed had been marked as germinated in the ground truth all subsequent images of the same seed were marked as germinated, but because k -NN scored the images individually, later images could still

reasonably be classed as not germinated (due to mould or death), conflicting with the ground truth. The problem was solved to some extent by manually checking any seeds that the k -NN was both uncertain of and conflicted with the ground truth, and manually adjusting the ground truth where the human scorer could see the seed did not match the ground truth. However, this process was time consuming and so not all possible conflicting seeds could be manually checked, potentially limiting its efficacy. The k -NN method also required a large starting human assessment for training, which would be time consuming, particularly if the images had all been individually assessed (Section 3.3b).

While the FIJI germination scoring is on the surface less effective (66.4%), it was scoring over the time sequence, which could allow it to score seed as germinated at a more consistent time than a human could. It could also be provided with more time points. It should also be remembered that a computer's level of temporal information on the seed could cause it to detect germination at an earlier time point in an image sequence than a human might, as noted by Ducournau et al. (2005). Further investigation would be required in order to unravel the real errors from the ground truth improvement that FIJI may have done (Section 3.3a).

The best outcome achieved with the human scorers was on a sub sample of the seed that would be clear to a human if the seed were either germinated or not. With this subsample of seed images, the k -NN achieved 0.89 (area under the ROC curve).

Overall, the methods developed may be comparable to a human when scoring small images of seeds, but not to a human scoring seeds physically.

OTHER STUDIES

This automated germination scoring producing a seed-by-seed analysis, this was primarily tested on individual seeds using ROC curves, rather than a number of seed germinated over the whole plate. Other studies have fitted the germination over a time series to calculate the fit of the curves (Joosen et al., 2010), or tested against total emergence to determine if the system could arrive at the same conclusions as with human scoring (Wagner et al., 2011). This was used to allow for high throughput imaging where the difference between seed lots was of paramount importance, not the status of each seed at each time. When compared in this manner the k -NN produced a strong correlation of 0.94 across all dishes at the last time, with a R^2 of 0.89. Joosen et al. (2010) shows individual seed scores; however, these include seed that a human may score as germinated before the algorithm.

In Ducournau et al. (2005) the median time for 25 seeds to germinate had a standard deviation of 0.8 hours on average between human scorers over 18 dishes (photographed hourly); while the standard deviation of the computer to the mean human score was 1.32 hours. This is similar to the test of human reproducibility. However, in Ducournau et al. (2005) the human scores lagged behind the automated germination curve, while the k -NN result found in this study did the opposite.

A direct comparison of this chapter's automated germination results is difficult because this technique takes an alternate approach to identifying germination, by identifying the time each seed germinates (FIJI method) or the status of each seed at each time (k -NN method).

3.5b FIELD BIOMASS ESTIMATION

The primary question to ask about the field green pixel counts was whether they can replace empirical first year measurements, used to predict second year biomass.

The field first year pixel counts were as effective as or more effective a predictor of second year harvest yield than common empirical measurements while being quicker to carry out (Section 3.3c). These pixel counts could have provided an even better correlation with biomass in the second year. However, second year photographs at this site, proved impractical when the crop was taller. This was because a bigger more unwieldy board was needed to capture the whole image, and the photographer could not stand far enough away without being in the next plot. These problems may not make this an ideal solution to rhizome or plug based first year crops, because they tend to be bigger plants by the end of the first year, unless plots were specifically placed to allow room for photography.

The predictive ability of both stems and height to the final biomass means that breeders often use some combination of the two to non-destructively assess biomass, for example number of stems \times height; this correlated better than other first year measurements but not as well as the pixel counts. The first year green pixel counts did not correlate as well to biomass as second year stem or height measurements. However, the point of the field photographs was to provide a faster, more effective method of assessing biomass. This could be tested on plots of other types as a quick scoring method for biomass plants when at early development. However, at early plant development stages breeders often score plants by eye, which would be an even faster method.

Time-consuming stem and height data collection could be substituted for good photographs in the first year, unless the data was needed for phenotypic analysis anyway. Simple two person photographs would give a strong correlation with second year yield as accurate as any standard first year measurement.

3.5C FURTHER WORK

Both germination approaches could be taken further to yield results truer to a human reference. The FIJI method is somewhat effective at detecting germination and has an intentionally low false-positive rate by only marking germination for seeds that have passed above the minimum-recorded sample level for any un-germinated seeds on multiple traits. Therefore, most of its error comes from false negative recording of germination this is skewed towards recently germinated seeds due to incorrect estimates of the time to germinate. However, by missing the first day the seed is germinated, the algorithm overestimates the germination time of the seed.

The machine learning k -NN approach could take the time of the picture into account, which may make it more effective; however, this was not done, because it would be difficult to weight the times correctly, so that times do not over bias the result. For example if a seed lot had an 80% germination by day 6, the k -NN would have a eighty percent chance of saying any seed over day 5 was germinated and being correct. Essentially this could lead to a worrying distribution of false positives and false negatives, as early germinating seed could be more likely to produce a false negative, and un-germinated seed could be more likely to produce a false positive at later time points. This would undermine the point of using machine learning on germination testing.

If a higher temporal resolution was required, or the number of seed involved in a trial made a human counter impractical and the errors less important, an in

situ time-lapse sequence could be carried out. In this case, a variation of one to two time points from when a human would have called germination would be less relevant. However, this was tested briefly as mentioned in 'Alternate method' (Section 3.1a above), and would not be suitable for the experiments in this study.

Therefore, for the testing of germination done in the rest of this study, a 70% trueness to a human reference of germination through any of the tested methods was not enough, even if there is a benefit in consistency and impartiality. This was decided because the throughput of seeds was not high enough to account for even a 10% error rate, because the effects tested on the seeds in Section 4.3 below may have small effects on germination.

The technique tested in this chapter could be used for high throughput imaging particularly where the identification of individual germinated seeds is of importance. It could also be expanded; Dell' Aquila (2004) used the analysed properties of the seed/seedling image after germination to measure early seedling elongation.

4 PHYSICAL & CHEMICAL GERMINATION FACTORS

4.1 INTRODUCTION

Experiments were undertaken to assess a range of physical and chemical factors' effects on *Miscanthus* germination. These consisted of laboratory germination tests culminating in an orthogonally designed multi factorial experiment of Taguchi design (Section 4.1c below).

4.1a UNDERSTANDING GERMINATION

Germination of *Miscanthus* seed was the focus of these tests, in order to select variables to alter germination.

TEMPERATURE

A major factor in germination is temperature. Monteith & Squire (1982) investigated this on a thermal gradient bar in pearl millet; the rate of germination increased linearly with temperature from the base to the optimal and then decreased linearly to the maximum temperature. Temperature will not only effect the rate of germination but also the fraction of seeds germinated (Garcia-Huidobro et al., 1982). Soil temperature has an impact on long term survival of seedlings, although genotypic variation affects the plants' response to soil temperature (Clifton-Brown & Lewandowski, 2000). Because germination rate increases linearly with temperature between base and optimum (Garcia-Huidobro et al., 1982; Trudgill, Squire, & Tompson, 2000), the temperature over

time can be regarded as thermal time. Aso (1976) found that the optimum temperature for *M. sinensis* germination was just over 25°C; however, this would not be viable in most real world situations.

The base temperature is genotype-dependent and unaffected by seed quality; however, high quality seed may require less thermal time (Ellis & Butcher, 1988). Base temperature also varies with plant origin; this can vary within a species, although different species have different levels of variation (Trudgill et al., 2000). Often the less cultivated a plant the more variation in germination will occur within its temperature range, because it will not have been bred for uniformity (Trudgill et al., 2000). This is important because *Miscanthus* originates in a range of climates allowing it to be bred for a range of temperatures.

PRIMING

Priming seed before sowing is an increasingly common commercial seed treatment (Sathish et al., 2011). Priming aims to synchronise and enhance the rapidity of seed germination by starting germination under controlled conditions, but not allowing the seeds to chit (emergence of the radicle or cotyledon) (Sparks, 2011). The seeds are moistened until at a specific water content, specific for the batch of seeds e.g. from 8.8% to 38% in onion seeds (Ellis & Butcher, 1988). The seeds are kept at that water content until about to chit, before being dried back to their initial moisture content (Sathish et al., 2011). Priming seed suspends seed germination in phase 2 (lag phase), in doing so the vigour of the seed is improved (Hussian et al., 2014). Once non-dormant there can be a continuum of reaction with some seeds more likely to enter secondary dormancy whilst others are less likely (Baskin & Baskin, 2004; Shen et al., 2001).

Priming is becoming common commercially and may improve *Miscanthus* seed reliability in the field. Because primed seeds emerge quickly growers are more able to pick the most effective sowing window based upon more reliable weather (Farmers Guardian, 2008a). However, priming is claimed to also improve seed vigour and yield (Elsoms Seeds, 2013), and lower thermal time to germinate (Ellis & Butcher, 1988). Primed seeds that are dried and stored will continue to use seed resources and therefore priming results in reduced longevity (Ellis & Butcher, 1988). As Sathish et al. (2011) suggests, the reason that priming can have positive effects on the strength, yield and longevity of the population is that it boosts the strongest and weakens the weakest, therefore this will mean that priming can lower overall germination percentage.

Research by Ellis & Butcher (1988) into onions concluded that priming had a positive effect on seed at sub and supra optimal temperatures. However, it had no consistent effect on base temperature and little effect on ceiling temperature of germination (Ellis & Butcher, 1988). At lower temperatures fewer primed seeds germinated compared to unprimed, yet at higher temperatures more primed seed germinated (Ellis & Butcher, 1988). This may be because seeds with a low germination rate were less affected by temperature than seeds with a higher germination rate; this was more pronounced in primed seed (Ellis & Butcher, 1988). Also because the viability of the seed is limited once primed there may be differences in longevity and vigour of the seeds depending on storage methods (Ellis & Butcher, 1988). Primed seed has been used widely and is increasingly common in industrial agriculture (Sathish et al., 2011; Sharma et al., 2014). Priming seed with water should reduce the thermal time to germinate and improve the consistency of germination for some time (Ellis & Butcher, 1988). However, priming can age seed faster (Hacisalihoglu et al., 1999) and improvements in vigour seen in the final crop may be a side effect of weakening

the weakest seeds (Sathish et al., 2011). However, the advantage bestowed by the uniformity of the crop outweighs these disadvantages.

There has also been research into priming with solutions other than water e.g. low salt concentrations (Sathish et al., 2011). The salts raise the osmotic potential in the seed, and if the salt itself is not lethal the treatment improves water uptake upon sowing (Sathish et al., 2011). It may be that germination depends on the temperature and conditions under which the seed are primed, because this could be inducing secondary dormancy (Ellis et al., 1985a). Priming effectiveness is very variable between seed particularly because they are easily affected by previous exposure to different environments (Ellis & Butcher, 1988). It may be prudent to test a range of priming conditions when priming *Miscanthus* seed. This has the potential to be a very broad area that merits further research.

LIGHT

Light is a simple and often vital trigger to end dormancy or to initiate germination, which can be altered in the field using different sowing techniques. Different seed species can be positively or negatively affected by light depending on their natural germination strategy (Ellis et al., 1985a). Light should not be necessary for germination in non-dormant seeds, but lack of light can cause some species to re-enter dormancy (Ellis et al., 1985a). Light dependency on germination in *M. sinensis* seed has previously not shown a significant effect (Christian, Goggi, & Moore, 2014). However, the effect of light on dormancy in other species varies between seed batches and light may be necessary even if other conditions, such as water availability, are met (Kucera, Cohn, & Leubner-Metzger, 2005). Sensitivity to light varies with genotype and temperature (Ellis, Hong, & Roberts, 1989) and only short (5 minutes far-red) periods of light may

be required to aid germination (Hsiao & Vidaver, 1989). The energy given by each quantum of light is dependent on the light's wavelength; however, short wavelength high-energy waves beyond the visible spectrum can be damaging to the seeds through ionization. The main wavelengths of light that affect germination in most species are 600-680nm & 700-760nm, with occasional effects at 400-500nm (Ellis et al., 1985a). Both density of photons and the photoperiod can also be altered to break dormancy, and when temperature is reduced the effect of the light decreases (Ellis et al., 1989). By using the most influential wavelengths of light, *Miscanthus* seed can be tested for other factors under optimal conditions. Light levels, periods, and wavelengths can also be tested to inform a commercially relevant seed treatment that could be applied to *Miscanthus* seeds in bulk before sowing.

HORMONES

Hormones are mainly used to trigger dormancy or release from dormancy (Kucera et al., 2005) and could therefore be useful in controlling *Miscanthus* germination. While different species vary in their reaction to hormones, "Knowledge gained from individual species can assist in developing optimized conditions for enhancing germination" (Sarath et al., 2006). Shallow dormancy, where seeds are only dormant for a short period of time, is better understood than deep dormancy, because most modelling species (e.g. *Arabidopsis*) only experience shallow dormancy (Koornneef, Bentsink, & Hilhorst, 2002). In addition, there is primary and secondary dormancy (Baskin & Baskin, 2004). Primary dormancy is the dormancy seeds acquire from the parent plant (Baskin & Baskin, 2004). Secondary dormancy is when seeds re-enter dormancy after being non-dormant (Baskin & Baskin, 2004; Sarath & Mitchell, 2008; Shen et al., 2001). This can be due to seeds being exposed to poor germination conditions (Ellis et al., 1985a). Seeds within an accession can be placed on a

continuum for how easily they can germinate and re-enter dormancy (Shen et al., 2001). In addition Ellis et al. (1985a) discusses environmental dormancy as being when current conditions are temporarily preventing the seed from germinating. Baskin & Baskin (2004) suggest that such seed are non-dormant, but have an environmental requirement for germination that is not being met.

In most seed varieties, dormancy may be overcome through a sustained period above base temperature or by varying the temperature, causing the release of hormones within the seed (Baskin & Baskin, 2004; Trudgill et al., 2000). Finding the main hormone regulators of dormancy has proved difficult due to variations in dormancy type, a variety of physiological mechanisms and seed environment (Koornneef et al., 2002).

Abscisic acid (ABA) is widely indicated to have an effect on inducing and maintaining seed dormancy (Baskin & Baskin, 2004; Finch-Savage & Leubner-Metzger, 2006; Grappin et al., 2000; Shu et al., 2016). Studies have been carried out into effects on germination when artificially added to seed, as well as effects due to natural variation (Finch-Savage & Leubner-Metzger, 2006). Therefore, ABA may be of primary interest for study with *Miscanthus*, which has unreliable germination rates, to understand better the mechanisms and quantities of ABA promoting seed dormancy. Tseng et al. (2003) reported 20 mg L⁻¹ may stop germination and 120 mg L⁻¹ reduces growth in *Miscanthus* if applied to a plant. This growth effect may be because ABA has been shown in Maize to prevent the radicle extending out of the seed coat as normal (Bewley, 1997b); ABA may also reduce root growth by regulating auxin (Wang et al., 2011b).

Gibberellic acid (GA) is the best known hormone for promoting germination (Yaldagard, Mortazavi, & Tabatabaie, 2008). Concentrations as low as 0.1 mg L⁻¹ have a positive effect on *Miscanthus* germination (Aso, 1976). This implies

Miscanthus can be sensitive to the correct hormonal signals. However, despite most (as low as 0.1 mg L⁻¹) concentrations of GA having a positive impact on the proportion of seeds germinated, the actual concentration was irrelevant (Aso, 1976). The presence or absence of GA is critical for breaking dormancy; however, the seeds' ability to produce GA in *Arabidopsis* was found to be sensitive to the other environmental responses of the seed (Barua et al., 2012). Finch-Savage & Leubner-Metzger (2006) suggest that after warmth, cold stratification can cause an increase in GA within seed. GA has also been found to substitute a red light trigger in release from dormancy (Kucera et al., 2005), allowing seeds to germinate before light levels are optimum. This suggests that GA is interacting with other hormones produced by the seed under specific environmental conditions, yet it is certainly promoting germination. When studying the effect of GA with *Miscanthus* germination Aso (1976) found that the effect of the hormone GA was only clear at 20 - 30°C. However, it should be noted that stimulating growth may overstretch the seeds' capacity and may reduce long term survival (Ellis et al., 1985a). Pushing the seed to germinate and grow may be necessary in *Miscanthus*, but this will require the seed to grow correctly, in a way that is suitable for long duration survival. GA addition may also disproportionately lead to growth of a particular part of the seed at the detriment of other parts (Aso, 1976). GA could be powerful at counteracting the effects of ABA (Steber & McCourt, 2001), as it may also regulate the creation of ABA as shown by Grappin et al. (2000) in *Nicotiana plumbaginifolia*.

Brassinosteroid (BR) seems to work with other hormones to promote germination; however, the germination boost does not come from BR alone, and may result more from an interaction with GA and possibly light (Kucera et al., 2005; Steber & McCourt, 2001). It is not known whether BR stimulates production of GA or just enhances its signalling (Shu et al., 2016). Steber & McCourt (2001)

suggested BR might be required to mitigate the dormancy effects of ABA on the seed allowing germination. However, BR itself may not be required for germination because seeds that do not produce BR will germinate, albeit at a lower rate (Koornneef et al., 2002; Steber & McCourt, 2001). This may be due to BR promoting GA production as shown by Tong et al. (2014) in rice.

BR signalling may be complex, for instance it has been associated with germination reduction when applied to seeds under salt stress (Wang et al., 2011a). The effects of BR on seedling growth are not clear; Steber & McCourt (2001) showed in *Arabidopsis* that BR may inhibit root elongation yet Müssig, Shin, & Altmann (2003) showed a positive effect on root growth in *Arabidopsis*. This may be part of a more complex process with stimulated GA production being used to regulate elongation (Tong et al., 2014). Unterholzner et al. (2015) showed in *Arabidopsis* that BR is necessary for GA generation.

Auxin is primarily associated with its effects stimulating root growth (Müssig et al., 2003) and regulating root shape (Rosquete et al., 2013). Auxin as well as ethylene up regulate root hair growth and auxin is required for proper root hair growth in *Arabidopsis* (Pitts, Cernac, & Estelle, 1998). Auxin also regulates seed dormancy against GA (Shu et al., 2016), and may interact with ABA at the end of dormancy (Wang et al., 2011b). It has long been known that auxin has a faster effect on elongation than BR, 15 minutes against over 45 minutes at the cellular level (Clouse et al., 1998; Evans & Ray, 1969). This may be due to the mechanisms used to regulate each hormone, for instance there is an interaction between BR and auxin in root growth which could be auxin responding to BR levels (Bao et al., 2004). With high concentrations of auxin inhibiting root growth by promoting ethylene production (Müssig et al., 2003), increasing the uptake of the auxin signal in the roots may inhibit growth (Wang et al., 2011b).

Other hormones to consider include ethylene, which promotes germination in non-dormant seeds, but is rarely enough alone to release dormancy (Kucera et al., 2005). Unlike GA, photo-dormancy (where there is a light requirement to break dormancy) is not released by ethylene and seeds would still require light to germinate (Kucera et al., 2005). However, the ethylene signalling pathway may regulate the amount of light and other germination signals (Zhu & Benková, 2016). Hydrogen peroxide, nitric oxide and sodium nitroprusside have all been shown to aid germination and break dormancy in grass seed by Sarath et al. (2006), because they are all reactive oxygen donors, increasing oxygen uptake in early germination. Other hormones such as kinetin and thiourea have also showed some potential positive effects on germination (Hsiao & Vidaver, 1989). Kinetin is similar to GA; thiourea in some cases can be more effective than GA, but normally only effective on pre-stratified seed (Ellis, Hong, & Roberts, 1985b). The use of hormones was confined by the method used to test them in Section 4.2b.

pH

Partially due to the annual burning of *M. sinensis* in Japan, the soils it is found in can be acidic, between pH 3.5 – 7.5. However, *Miscanthus* has been found growing in soils with a pH as low as 2.7 (Stewart et al., 2009). The natural pH variation may have an impact on the germination and dormancy of *Miscanthus* seed. In *M. sinensis* Aso (1976) found that at low and high pH (4 – 8.5) there was some slowing of germination; later the stems of the low pH group extended disproportionately in comparison to the root. Abnormal growth patterns may make a difference to long term survivability (Ellis et al., 1985a). Resistance to extremes of pH is interesting because the pathways plants develop to cope with adverse conditions will likely be less efficient than standard pathways. Use of these alternative pathways will therefore be negative to yield (Abadikhahdeh Ali

& Gholam, 2011). If the optimum pH for *Miscanthus* is low, the establishment could be aided by altering pH, which is a widely used agricultural technique.

WATER STRESS

Determining the amount of available water *Miscanthus* needs to germinate is important to modelling the commercial geographical range of *Miscanthus*. Dry conditions can prevent germination by inducing secondary dormancy (Ellis et al., 1985a). In addition, 2-3 week old *Miscanthus* seedlings can be killed by a drought of as little as two weeks (Christian et al., 2005). Having perennials that can cope in a changeable climate is important for a biofuel crop, and for energy security. This is most important in adult plants, but for effective establishment the effects of water stress on germination will also be important.

Salt produces an ion toxicity effect in plants (Bajji, Kinet, & Lutts, 2002); however, plants can be broadly classified into two groups: halophytes that are effected mostly by the lower water potentials found in salt, and glycophytes that are also effected by the ion toxicity salt generates within the cell (Zhang et al., 2010). PEG (Polyethylene glycol) is widely used to water stress plants without toxicity effects. Plants can be placed in PEG containing media with different water potentials, allowing measurement of the effect of water stress (Knight et al., 2004; Rao, Roberts, & Ellis, 1987) without influence from physical factors such as root effectiveness. Seeds can also be primed using PEG to end dormancy and prepare the seed for germination without absorbing the water required to germinate (Burgass & Powell, 1984; Sathish et al., 2011), so that when sown the seed imbibes water easily.

The natural influence that leads to water stress in a wet environment is salt stress; this is more of an issue for *Miscanthus* because it will be grown on marginal land. Much useful land is salt contaminated (6% of all land is affected

by salt (Zhang et al., 2010)). A bio energy crop that could thrive on land with salt contamination would be highly valuable. Salinity has an effect on germination due to osmotic and ion toxicity, but terrestrial plants have ways of regulating salt flux (Zhang et al., 2010). However, intracellular salts can inhibit metabolism, retarding germination or leading to cell death (Zhang et al., 2010). Different salts have been found to affect germination differently; Sathish et al. (2011) tested the effect of NaCl, KCl, CaCl and KH₂PO₄ salts on germination, finding that KH₂PO₄ was most effective but only as a pre-treatment (a form of priming). Seeds that germinated at lower osmotic potentials germinated faster (Zhang et al., 2010), especially when incubated at higher osmotic potentials. However, pre-treatments would not solve the problem of salt-contaminated land unless a pre-treatment of high salt could allow a tolerant seed to still obtain water in a slightly saline environment (Zhang et al., 2010). Pre-treating seed commercially requires the seed to be re-dried before sowing; however, this causes germination to take longer (Sathish et al., 2011). Some seeds may be better at coping with water stress, and whilst an evolutionary advantage, this could lead to a decrease in yield. This was as shown by Abadikhahdeh Ali & Gholam (2011) when investigating cyanide resistance in sorghum and wheat. Therefore, a treatment that quickens germination in a proportion of seeds, may not be promoting the highest yielding seeds. It will be necessary to look at a range of natural markers of water stress as well as looking at the underlying biology using PEG to assess the reaction of *Miscanthus* to water availability.

STRATIFICATION

Temperature during germination and during storage will also have an effect on viability (Ellis & Butcher, 1988). A form of pre-germination seed treatment involves exposing the seeds to low but not deadly temperatures (stratification) (Shen et al., 2001). This triggers release from dormancy when the temperature

is raised, simulating overwintering. All treatments depend on the climate the seeds originate from, because they are to some extent simulating natural triggers. An example of stratification in *Panicum virgatum* (Switchgrass), seeds were stored wet for 14 days at 5°C before being germinated in warmer conditions or dried and germinated (Shen et al., 2001). There has been little research into stratification in *Miscanthus*; however, Christian et al. (2014) found wet pre-chilling of *M. sinensis* seed without drying boosted germination percentage.

The problem with stratification as a commercial improvement of germination is that dry seed are easiest to sow; yet when the seeds were dried they could enter secondary dormancy (Shen et al., 2001). The main factor was the length of time the seeds were stratified for at the colder temperature, where insufficiently stratified seed entered secondary dormancy easily (Shen et al., 2001). Stratification for 3 – 4 weeks is now more commonly used for Switchgrass to ensure seed does not revert to dormancy (Walker, 2009).

Additionally, diurnal cycles can also give seeds an environmental trigger to end dormancy (Finch-Savage & Leubner-Metzger, 2006). Whether this is relevant to *Miscanthus* germination requires further investigation and it is believed that dormancy is less significant in *Miscanthus*.

SEED SIZE

Miscanthus seed vary considerably in size even within a genotype; this difference may be up to two or three times. Seed size may be a selectable trait and large seed may have more germination success or may survive the priming process better than smaller seed. In a study of *M. sinensis* Aso (1976) found that larger seeds germinated faster but with no impact on final total germination. Parisi et al. (1991) expected seed weight would be an important component of seed vigour, yet there was no significant change of germination speed. A related

factor to seed size is density; large seeds may be less dense than small seeds, and density may affect survivability. As flotation can be used to separate seeds based on density, or sieving seed for size, this could be an easy method to separate seed in commercial systems.

SEED COATINGS

If understanding the biology of germination in *Miscanthus* reveals a potential chemical treatment to promote germination and/or successful establishment of *Miscanthus* in the field, it will be important to find the best way of applying treatments to *Miscanthus* seed in the field. Spraying a product on a field is expensive, so many farmers opt instead to spray lines parallel to the sown seed; but to be more precise additives can be applied directly onto seeds (Scott, 1989). Seed coatings are applied in equipment similar to a concrete mixer (Scott, 1989) to keep seeds moving and apply treatments evenly. However, whilst improving germination the effect of seed coats can be inconsistent (Scott, 1975).

The key mechanism of the coating is the attraction and repulsion of water to the seed, this alone can have a wide ranging effect on germination (Scott, 1989). Coating can be of particular benefit to grass establishment, with its small seed (Scott, 1975). Scott (1989) explains that coating seeds can provide some predation protection from both micro and macro organisms, a supply of oxygen to the seed, protection from weathering and freezing effects, and an ability to bind the seed to the soil, as well as more specific ingredients to quicken germination. It has been found that the best adhesives for binding seeds and earth are Methyl Cellulose and gum Arabic (Scott, 1989). Gum Arabic is most effective but in reality methyl cellulose is used because it is easy and cheap to use (Scott, 1989). The problem when using binding agents is there is a tendency to cause the seeds to agglomerate which is wasteful (Scott, 1989). Useful substrates for

binding include vermiculite, dextrin, and aeretion (Scott, 1989). Examples of non-synthetic coatings can be wide ranging and include: activated charcoal, bone meal, guano and mucilage (Scott, 1989). Beyond this, there are coatings that encapsulate pesticides, fungicides, herbicides, or hormones.

However, coating with herbicides or pesticides for better establishment may not provide any notable benefit over a plain hard coating (Scott, 1975). Experimenting with coating *M. sinensis* Christian et al. (2005) found that seeds coated into a pellet were less successful, probably due to worse transfer of water. Despite the potential benefits all coatings can lower germination a little (Scott, 1975), this may simply be due to the extra mechanical force required to germinate or the lack of light and water to the seed. Despite the findings of Christian et al. (2005), coatings offer a wide range of possibilities and it may be too soon to rule out the potential benefits.

4.1b SELECTION OF VARIABLES

It was necessary to select which germination variables to study and which to exclude from those outlined above. This was mainly based on achieving a consistent method so the most interesting variables could be used in the Taguchi experiment. The tests not selected and reasons for that are outlined below.

The effect of temperature was tested in 'Agronomic Modelling' (Sections 6.2e & 6.2f); however, it would have complicated the methods in this section to apply temperature as a germination variable. The effect of physical factors was also intrinsically linked to agronomic treatments such as mulch film.

Diurnal cycles also had the problem of having many potential combinations. They were not studied primarily because they would have required successive

experiments in a controlled environment to be treated as one experiment, or more controlled environments to be used simultaneously than were available.

Hormones were selected based on, whether they could be provided to the seeds in a liquid treatment (e.g. excluding Ethylene gas) to maintain the same method, and if they had previously been shown to have a direct effect on seed germination or development. Therefore, the hormones selected were ABA, GA, BR, and auxin.

PH was not tested due to difficulty with keeping the chemicals used the same over a wide range of PH values, and because high acidity treatments would interact with the blue germination paper.

Salt and PEG water stress were tested but seed only priming with water was done to lower complexity and give the best chance of success.

Pelleting seed was not covered during these experiments for three reasons. Firstly, the equipment needed large (more than 500 g) quantities of seed to test coating seed, this would have used a large percentage, or all, of any of the *Miscanthus* seed lots that were available. Secondly, there are many ways of pelleting seeds outlined in 'Seed Size' above that any testing could not be thorough. Thirdly, the only study done before this project on pelleting *Miscanthus*, was not successful in improving germination (Christian et al., 2005).

4.1C SELECTION OF TAGUCHI METHOD

As discussed in Section 2.1a above, there are wide varieties of factors that can affect *Miscanthus* germination. Due to this, analysing the effect of each factor individually would require an impractical quantity of experiments. Therefore, the application of novel statistical methods is required.

The Taguchi method, first used in manufacturing (Taguchi, 1986), is “one of the most well-known and widely adopted robust design methods” (Yaldagard et al., 2008). It is designed to test multiple factors together statistically by first defining the range of the variables and then defining the noise (Tong, Su, & Wang, 1997). Orthogonal arrays can then account for the noise using multi-variant statistical techniques (Tong et al., 1997). Taguchi’s orthogonal tabulated arrays allow a maximum number effects to be compared orthogonally in an unbiased manner, with a minimum of experiments (Rao et al., 2008).

The method uses ANOVA to identify which variables in a group are contributing to the variation (Pourjavadi et al., 2006). This makes it useful for processes such as germination with many variables, although Tong et al. (1997) suggests that it is less suited to studies where the variables react interdependently with each other. However, Yaldagard (2008) employed the Taguchi Method to identify the most important variables in germination in barley, which suggests that it would be suitable for studying germination in *Miscanthus*. The Taguchi method has not been widely adopted in the biological sciences (Rao et al., 2008).

4.2 METHOD DEVELOPMENT

4.2a PRELIMINARY TESTING

An experiment was carried out to investigate the effect on germination of NaCl and ABA. This experiment used eleven concentrations of NaCl and twelve concentrations of ABA, plus one control. In-plate replication was used, with each seed as a replicate, because replication of dishes for so many concentrations would be impractical. Using seeds in a dish as experimental units is not ideal, as the environment is not replicated; therefore, no statistics using indi-

vidual seed can be taken as definitive proof. Since the seed is the experimental unit, this method relies upon being able to monitor each unique seed over time. Therefore, the seed were laid out on the blue germination paper in a grid pattern (7x7), with one extra seed in the middle, to give 50 seeds per treatment. Building on 'FIJI Germination Counts' (Section 3.3a above), a system of germination scoring was used where the human scorer marked which seed locations contained germinated seed at each time point.

The seed genotype used was SYN55. To prevent problems from mould, application of a fungicide in addition to surface sterilisation was considered, but this was believed to be an excessive complication because it could interact with the seed and the hormone treatments. Therefore, surface sterilisation alone was used to reduce mould.

The cabinet was set to 25°C and 75% RH. A dish was set up with one 10 cm × 10 cm piece of blue germination paper atop two pieces of round filter paper to act as a reservoir. 20 mL of solution was prepared from a stock solution and added to each dish. Because it had been observed in previous experiments (Section 3.2a above) that the seed could move around due to water sloshing on the plate or dripping from the lid, only enough water to wet the paper fully (20 mL) was used on the dishes. Images were captured daily alongside the human germination count, using a Nikon D90 DSLR camera attached to a stable copy stand.

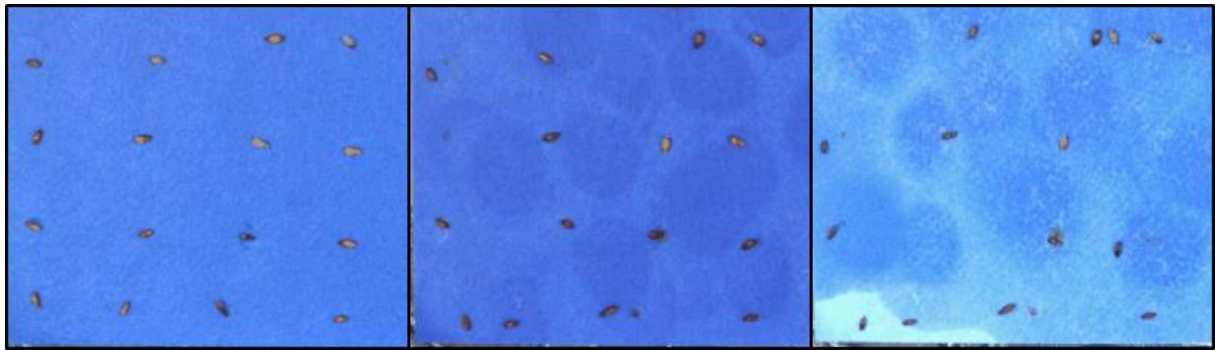


Figure 4-1: Problems with basic method for physical and chemical germination factors. Days 0, 3, and 5 (left to right) are shown. The problems visible are seed movement and the blue germination paper drying over time.

Three problems occurred with this method (Figure 4-1): Firstly, despite the low water input (which led to poor imaging, due to colour variation) the water still condensed on the lid and dripped onto the seeds, moving un-germinated seeds freely across the blue germination paper. This interfered with the grid pattern to the extent that some seeds' starting locations could not be identified, making them unable to be used as replicates.

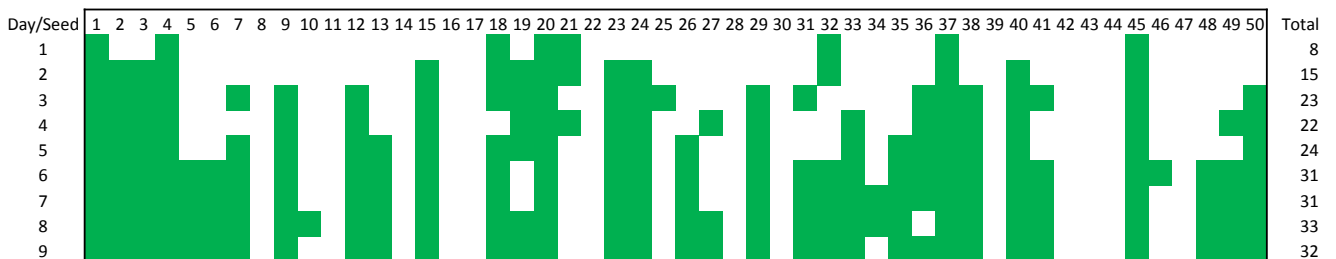


Figure 4-2: The seeds scored by the human on just the control plate. The chart shows fifty seed over nine days; only when each seed was compared at each time can the problem of individual seed being inconsistently marked as germinated; the total germination loosely follows a normal germination curve.

Secondly, when reviewed, which individual seeds were marked as germinated at which times by the human scorer varied from time to time (see Figure 4-2), showing a lack of repeatability by the human scorer. This compounded the lack

of over-time replicated seed monitoring. Thirdly, the plates became dry over the course of the experiment, resulting in poor consistency of photographs. Despite the high RH in the cabinet, the plate was showing signs of dryness by day five of eleven (Figure 4-1). Adding water during the experiment would have been difficult, as the correct concentrations of chemicals needed to be maintained.

4.2b DEVELOPING A TIME SERIES CRUCIAL METHOD WITHOUT IMAGE ANALYSIS

Section 4.2a shows people are poor at tracking seed germination over time. This could be mitigated by providing access to the entire time series, which would allow the human scorer to make comparisons to the previous and future states of the seed. As germination counts using image analysis had also not proved reliable enough for using seed as replicates in Section 3.5a above, and locations of each seed were important for replication (Section 4.2a above), the method required development to allow individual seed to be captured in a time series. The developments made in the method to improve consistency included adding dents in an 8×8 grid to the blue germination paper; this allowed for 64 seeds per dish. The blue germination paper had a folded sheet of blue roll placed below it to allow for more of a reservoir. This could then be imaged over time (Dell' Aquila, 2005), and the time series could then be extracted by FIJI for each individual seed and scored by a human as a time series [see Appendix J]. Human scorers were therefore presented with images of individual seeds instead of looking at the whole dish. This would allow the most repeatable human scoring at the expense of not being able to manipulate the seed physically when counting. However, manipulating the seed during the time sequence may lead to damage of the emerging seedling anyway. In addition, reducing the time the lid was removed from the dishes should reduce water loss by evaporation, and reduce the chance of mould affecting the seeds.

4.2c FLUORESCENCE IMAGING

At the end of the 'Preliminary Testing' the seed were scored for fitness on a 0 to 5 scale (dead, inactive, barely alive, struggling, alive, thriving). This scoring system did not represent the health of the seeds at the end of the experiment, because they could die but still look healthy (Figure 4-3). To combat this problem, at the end of future experiments, before elongation was measured, the seeds' chlorophyll fluorescence was imaged using a CF Imager, from Technologica Ltd (Colchester, UK). The Fv/Fm false colour image was extracted [Appendix J] and used to gain a more accurate idea of the end state of the seed (see Figure 4-3).

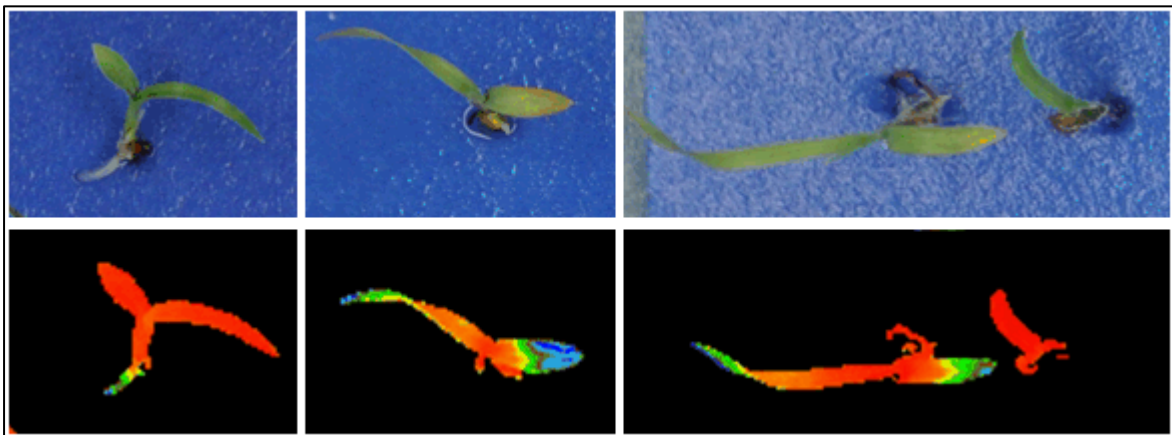


Figure 4-3: Fluorescence images reveal extra information about the state of the seed. The left image is small but shows up well on the fluorescence, the middle image is larger but is patchy on the fluorescence; however, does not look as green. The right image shows a pair of seeds, the right one small and mouldy the other much larger two leaves; however, the fluorescence reveals the right seedling is not as weak as it looks.

To assess difficult seeds a decision tree was used [see Appendix K] based on both the light photograph of the seedling and the fluorescence image. The decision tree allowed more consistency in scoring.

4.3 METHODS

To find the appropriate range of each factor for the Taguchi method, experiments were carried out with the above factors each at a broad range of levels, to create a range over which the effect of each factor on the seeds' germination and early growth was known. Unless otherwise stated in the individual methods the seeds used in this chapter are SYN55. The methods used for each factor are detailed below, split into categories by the type of treatment used on the seed. All germination testing in this chapter, unless otherwise stated, used a Fitotron 120 Plant Growth Chamber. The 'Method Development' Section 4.2 above provided a basis for the methods used in the experiments below. Where this applied, seed were placed in a grid in square Petri dishes and were imaged daily with a DSLR camera. These were scored by humans using time sequences of individual seeds. After eleven days, end measurements were taken and the seeds were fluorescence imaged.

4.3a HORMONES EFFECTS EXPERIMENTS

All the hormone tests were carried out on blue germination paper with each seed being a replicate. An 8 × 8 grid of seeds was used in each plate and the seeds were germinated while being photographed every 24 hours as well as at the start. After 8 days, extra SDW was added (amount varied). The experiment continued until day 11 at which point the seeds were fluorescence imaged and each seed had the maximum elongation of its root and stem/leaf measured for the success of above and below ground growth (details in Section 4.2b above).

Each hormone test was analysed for germination time, mould time, root elongation, and stem elongation from the data for each seed. This allowed average values to be calculated for each dish. These were each analysed using a one-way

ANOVA against the concentration of the hormone, or if a normal distribution was not present and could not be achieved with a simple transformation a Kruskal-Wallis rank sum was used as a nonparametric alternative. A Pearson's product-moment correlation (PPMC) was calculated for the normally distributed data, and it was stated if a strong correlation was present. For nonparametric tests, a Kendall's rank correlation was used. Summary statistics for each dish were analysed using a PPMC or Kendall's rank correlation if an un-replicated one-way ANOVA showed a significant difference. These statistics were germination percentage, mould percentage, and Germination index (GI). Correlations were used because the trend in the data across the dishes was the important result.

ABSCISIC ACID

The abscisic acid (ABA) experiment was repeated using the method in Section 4.2b above. The 64 seeds were placed in thirteen dishes, with concentrations of ABA at 0, 0.05, 0.1, 0.5, 1, 2, 5, 10, 20, 30, 40, 50, and 60 mg L⁻¹. This was done by incremental dilutions of a 60 mg L⁻¹ stock solution. The failed ABA test in 4.2a above had used 2 to 300 mg L⁻¹ in 12 dishes plus a control; this appeared to limit germination in all dishes so the range was altered to be lower. The concentration range was based on Belin et al. (2009) who used 0.2 to 1 µM of ABA on *Arabidopsis*. Similarly, Steber & McCourt (2001) used 0.5 to 3 µM. Therefore, a complete range would be from 0.2 to 3 µM (0.05 to 0.8 mg L⁻¹). This was extended upwards to include any secondary effects.

GIBBERELIC ACID

Gibberellic acid (GA) concentrations of 0, 0.15, 0.75, 1.5, 7.5, 15, 75, 150, 300, 500, and 750 mg L⁻¹ were used, with one concentration applied to each dish using the method above. Aso (1976) had tested 10 to 100 mg L⁻¹ of GA on

Miscanthus and found all concentrations had the same effect. Dong et al. (2006) had also used 100 mg L⁻¹ on rice, while testing on *M. sinensis* seed has been as high as 500 mg L⁻¹ (Christian et al., 2014). The concentration range was extended in both directions to capture a wider range of effects. A stock solution of 1.5 g L⁻¹ was prepared and diluted to get each concentration.

AUXIN (NAA)

The type of auxin used was 1-naphthaleneacetic acid (NAA), due to it being frequently used for germination and seedling root growth studies (Belin et al., 2009; Müssig et al., 2003; Rahman, 2001; Rosquete et al., 2013; Wang et al., 2011b). Therefore, this choice should give this the best chance of an effect from auxin.

The concentration of the stock solution was kept below 0.38 g L⁻¹, the maximum dissolvable in water (TCI America, 2005); however, because it was 0.3 g L⁻¹, 1 mL of ethanol was added first to the stock solution to aid dissolving. The upper range was based on Rosquete et al. (2013) who used 100 to 500 nM of NAA which equates approximately to 0.02 to 0.1 mg L⁻¹. This provided the experiment with a lower range. Müssig et al. (2003) used lower concentrations of 0.1 to 10 nM but observed the same inhibition of root growth. Belin et al. (2009) used up to 1000 nM (\approx 0.2 mg L⁻¹) and (Jeong et al., 2009) used an upper concentration of 27 μ M (\approx 5 mg L⁻¹) when working with ginseng roots. This was extended to observe a very wide range of concentrations or secondary effects; therefore, concentrations of [0, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100, 200] mg L⁻¹ were used over 11 dishes.

BRASSINOSTEROID

Epibrassinolide was used as the brassinosteroid (BR) because it has previously been used in germination (Steber & McCourt, 2001; Wang et al., 2011a), root growth (Müssig et al., 2003), and in hormone regulation (Unterholzner et al., 2015) experiments. Thus, epibrassinolide should provide a reasonable BR for testing of *Miscanthus*. The experiment was conducted using the method above. A stock solution of 2 mg L⁻¹ was made and diluted to get concentrations of 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 1.5, and 2 mg L⁻¹. This range was based on Müssig et al. (2003) who used a range from 0.1 to 10 nM of BR on *Arabidopsis* roots, Wang et al. (2011) who used 0.1 to 10 µM of BR in NaCl which affected cucumber germination, and Steber & McCourt (2001) who used 0.5 to 2.5 µM of BR in *Arabidopsis* germination. This range could be restricted because Wang et al. (2011) only saw a noticeable negative effect on the wild type starting at 1 nM; giving a range of 1 nM to 10 µM of BR which would become a range of 0.005 to 4.8 mg L⁻¹. However, due to the cost of epibrassinolide the upper limit was reduced to 2 mg L⁻¹ (4.2 µM).

4.3b PHYSICAL STRESSES AND PRE-TREATMENTS

The below experiments test the seed under levels of physical stress to assess the effect on germination and early growth.

SALT (NaCl)

An experiment using NaCl to reduce water availability but also add salt toxicity was conducted using the refined method laid out in Section 4.2b above. The levels of salt were based a wide range of possible desired water potentials (Ψ), which were calculated using a derivation of the Hoff equation (Lewis, 1908) (3);

where Π is water potential, i is the number of ions formed, M is the molarity, R is the pressure constant, and T is the temperature in °K.

$$\Pi = iMRT \quad (3)$$

Seed were put into 12 Petri dishes with Ψ of 0, -0.05, -0.1, -0.2, -0.3, -0.5, -0.8, -1, -1.2, -1.6, -2.2 & -4.1 MPa, each created by diluting a -4.95 MPa (1 M) stock solution of NaCl with SDW. This range was used because Zhang et al. (2010) used -0.5 to -2.5 MPa range of NaCl in barley and germination did not cease within this range. Other germination studies used similar or lower ranges of NaCl (Dodd & Donovan, 1999; Gummerson, 1986; Koger, Reddy, & Poston, 2004).

The NaCl experiment was analysed using individual seed information for germination time, mould time, root elongation, and stem elongation. Each of these were analysed using one-way ANOVAs, or if a normal distribution could not be achieved with a simple transformation, a Kruskal-Wallis rank sum of these factors vs. Ψ was used. If the result of this was significant, the PPMC was calculated, or if a nonparametric test was needed a Kendall's rank correlation was used. The single values for germination percentage, mould percentage, and GI (Ranal & de Santana, 2006; Walker-Simmons, 1987) in each dish were also correlated against Ψ , using the appropriate correlation statistic.

POLYETHYLENE GLYCOL

An experiment was carried out to investigate the effects of water availability using PEG (polyethylene glycol) instead of NaCl to avoid the effects of ion toxicity. This experiment had three hurdles to overcome. Firstly, the desired osmotic pressures for PEG should align with those used in the Salt (NaCl) test above. Secondly, a large PEG molecule with a molecular mass of 8,000 or above could hypothetically be large enough to become immobilised within the blue germina-

tion paper or the tissue reservoirs, and therefore not provide a full water limitation on the seeds. Thirdly, there is some evidence that PEG molecules of low molecular masses e.g. 4,000 could penetrate a plant and cause a toxicity effect (Lagerwerff, Ogata, & Eagle, 1961; Lawlor, 1970). The second and third problems were mitigated by running the PEG experiment at both a high (8,000) and a low (4,000) molecular mass. However, this increased the complexity of the first problem, because NaCl obeys the Hoff Equation and each molecule of salt binds a predictable number of molecules of water (two in this case). This is not the case for PEG which is a large coiled molecule that does not follow the Hoff Equation (3) as an 'ideal solution' (Mcclendon, 1981; Steuter, Mozafar, & Goodin, 1981); it follows a curve with more PEG disproportionately tying up water molecules (Figure 4-4). This is because PEG is not completely a solution because it is not dissolved in the water but binds to the water to render it inaccessible, known as the Tyndall effect (Steuter et al., 1981).

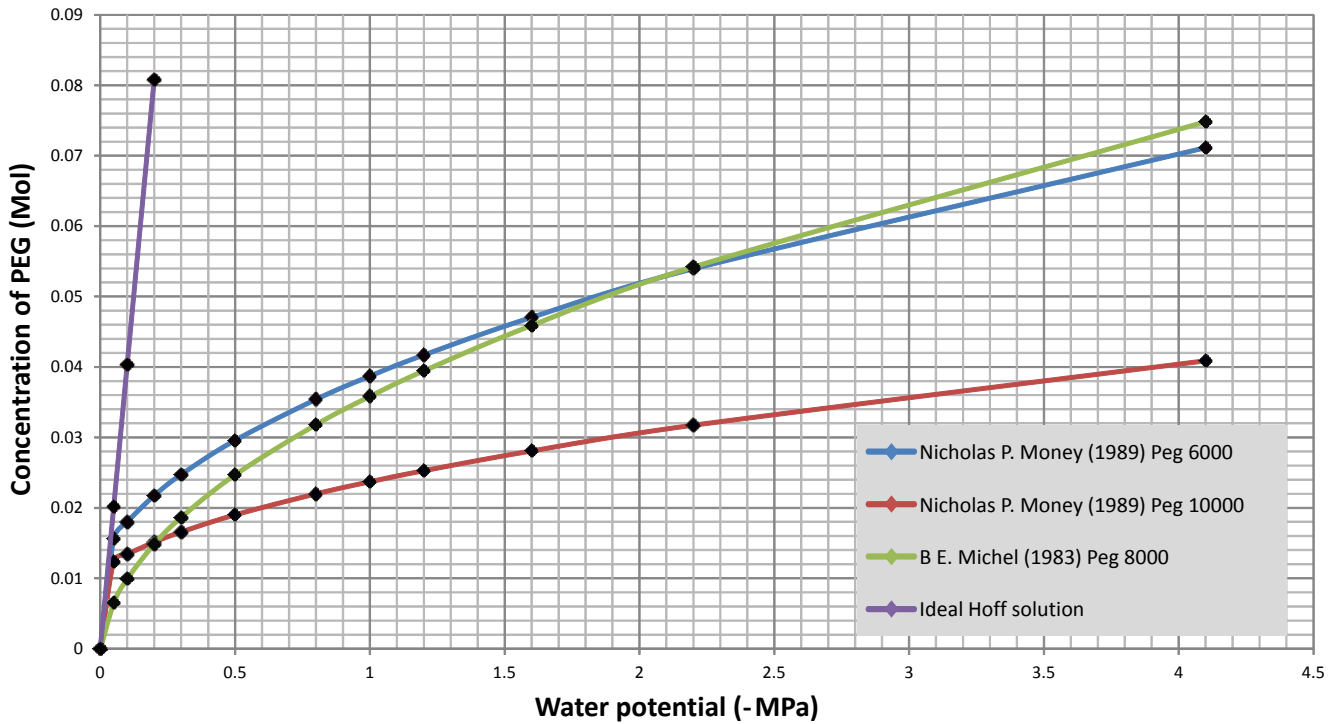


Figure 4-4: Water potentials for polyethylene glycol sizes in the literature. Data from experiments done on PEG 6,000 and 10,000 by Money (1989) and PEG 8,000 by Michel (1983) is shown. Lastly, an ideal linear solution has been plotted using the Hoff equation.

Money (1989) used an equation (4) to model the osmotic potentials of different none ideal solutions. α and β are listed for a variety of PEG molecular masses, from these a variety of curves can be calculated for water potential of PEG (Figure 4-5).

$$\Pi = \alpha \cdot C + \beta \cdot C^2 \quad (4)$$

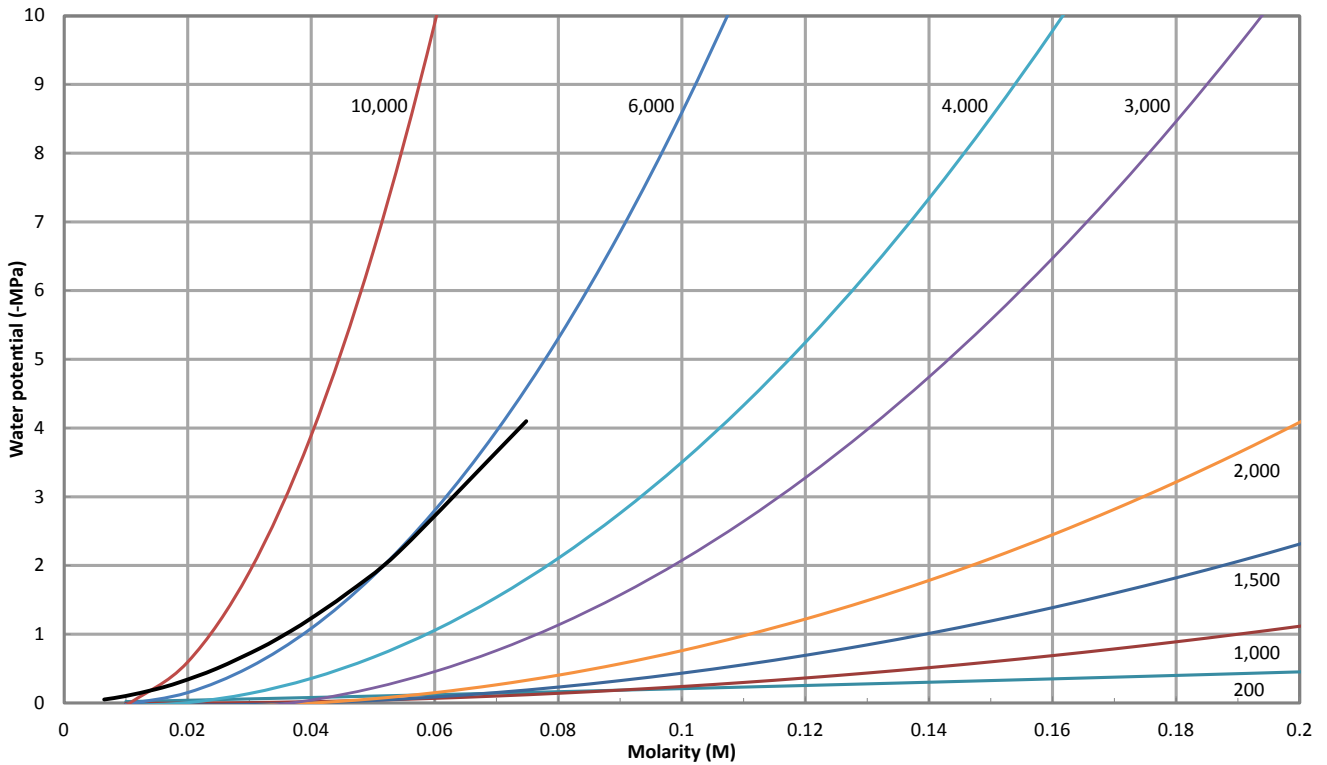


Figure 4-5: Polyethylene glycol solutions as calculated using the regression curve equation from Money (1989), the PEG values have been recorded at the end of each line. The black line represents PEG 8,000 as measured by Michel (1983).

Due to the mismatch of the lines in Figure 4-5 it was decided to use the general PEG 8,000 equation (5) from Michel (1983) to determine the quantities of PEG 8,000 to use; where T is temperature in degrees Celsius and Ψ is target water potential in bar.

$$[\text{PEG}] = \frac{4 - (5.16\Psi T - 560\Psi + 16)^{0.5}}{2.58T - 280} \quad (5)$$

Thus, in the literature PEG 8,000 and 4,000 both calculated Ψ using the equations best suited. The Michel (1983) equation (5) would be best for calculating a wide range of PEG solutions; however, it was safer to go with a PEG 8,000 specific calculation (4) (Money, 1989) because there is a wide gap between PEG 6,000 and PEG 10,000 in Michel (1983). It is difficult to accurately test the Ψ of a PEG solution (Steuter et al., 1981); however, Money (1989) concludes that

complex interactions mean that it is more accurate to measure Ψ than calculate it particularly in a complex medium. This study was not using a complex medium so Ψ was calculated; especially because Steuter et al. (1981) was using vapour pressure deficit.

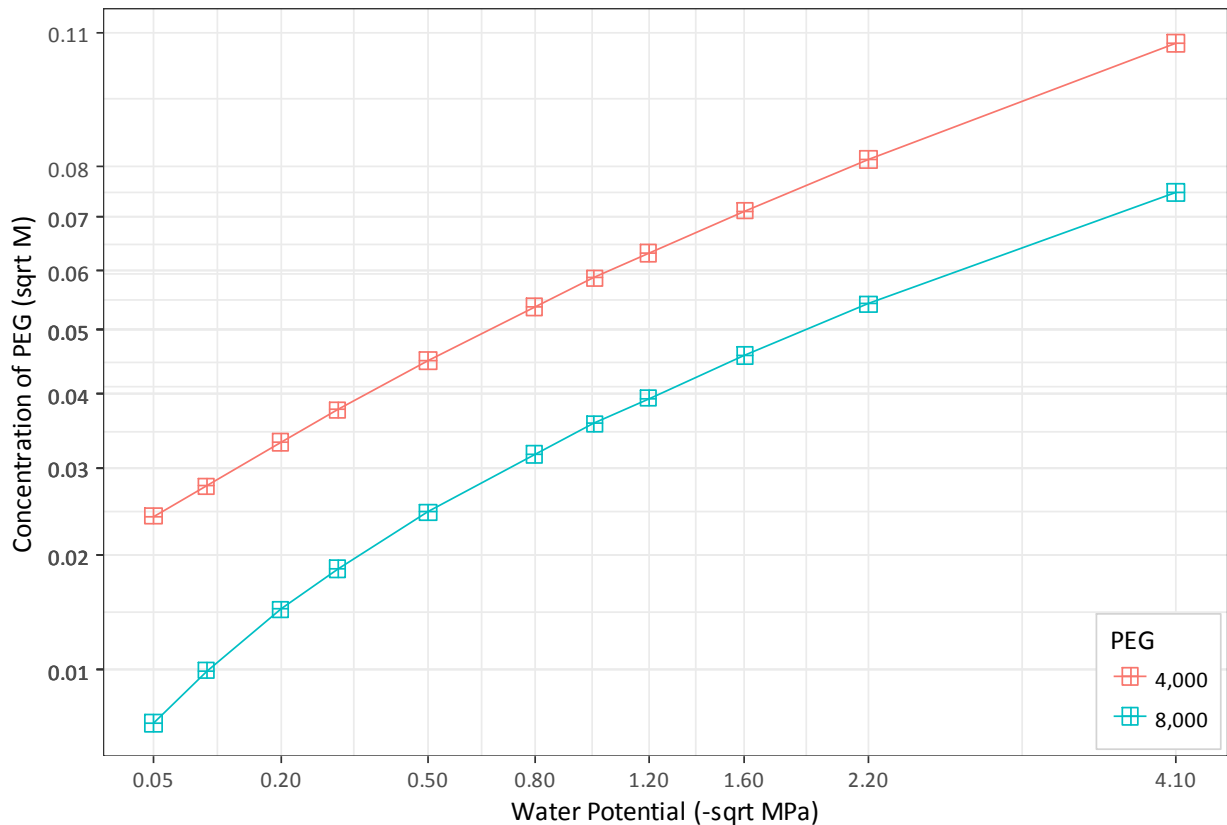


Figure 4-6: The water potentials and concentrations used in this study for PEG 4,000 and 8,000, these are the same water potentials as used in Salt (NaCl) above.

Both PEG sizes were matched to the water potentials of the NaCl experiment (Ψ of 0, -0.05, -0.1, -0.2, -0.3, -0.5, -0.8, -1, -1.2, -1.6, -2.2 & -4.1 MPa) (see Figure 4-6 above). To make the concentrations, 12 PEG stock solutions were diluted in SDW. The stock solutions had a Ψ of -4.1 MPa (107.3 mM) for PEG 4000 and a Ψ of -4.1 MPa (74.8 mM) for PEG 8000.

The experiment was carried out as outlined in Section 4.2b above, and time to germinate, time to become mouldy, root elongation, and stem elongation were

analysed with two-way ANOVAs or Kruskal-Wallis rank sums. If Kruskal-Wallis was used, they were analysed in three parts, as Kruskal-Wallis is limited to one factor: levels of PEG 4000 only, levels of PEG 8000 only and a comparison of the PEG 4000 and PEG 8000. The summary statistics (germination percentage, mould percentage, and GI) were all analysed with two-way ANOVAs to test the basic trends in the data in both Ψ and PEG dimensions.

STRATIFICATION

An experiment investigating the cooling of seeds in water before germination (known as stratification) was carried out. The seeds were wetted and then stored wet at 4-5°C for 1, 3, 7 and 14 days. After storing, half of each set of seeds were removed and dried for 3 days at 25°C and then germinated. The other half of each set was germinated without being dried. Germination was done in the conditions described in Section 4.2b above. None of the seeds were sterilised because the sterilising solution would have to be made separately for each batch and therefore the bleach concentration could differ. In addition, residue from the bleach could remain on the seeds for longer at low temperatures. Sterilising after stratification was dismissed because seeds could have begun the chitting process slowly at low temperatures and the sterilising solution could then infiltrate the seed. The seeds were then placed in nine Petri dishes each representing one treatment, plus one control. The setup of each treatment combination was staggered so that all of the dishes were moved to the germination cabinet at the same time regardless of whether or not they were dried. This ensured that all dishes experienced the same germination conditions in the cabinet.

Germination time, mould time, root elongation, and stem elongation were analysed with two-way ANOVAs if there was a normal distribution, otherwise

three separate Kruskal-Wallis rank sum tests were used; level for wet only stratification, level for dried stratification and a comparison of the wet and dried. All the summary statistics were analysed with two-way ANOVAs. To account for both stratification time and if the seeds were dried, without replicates this can only show if a basic trend exists.

COLD STORAGE

This experiment was used to test seed stored in cold conditions after threshing to identify if this improved germination. This process is similar to stratification but without wetting the seed. Fresh threshed GNT14 seed was kept at room temperature or cooled to 5°C for 2 weeks. Then a non-sterilised germination test was run. This was done with three replicates of 64 seed on blue roll with 50 mL of SDW added. This used a standard 25°C cabinet germination test as outlined in Section 4.2b above; however, scoring was only done at 5, 7, and 15 days.

The data was analysed with a t-test for mould at 15 days and germination percentage at 7 days. These times were chosen to test the germination where it had levelled off and test the mould when it had had the most time to grow.

PRIMING

Two experiments were carried out to investigate the effects of priming on germination and early growth on seed grown on blue germination paper. The priming was done by Elsons Seeds (see Section 2.1g above).

Primed and unprimed SYN55 seed were germinated using the technique described in Section 4.2b above. This was done using two plates, one with 64 primed seed, and one with 64 unprimed seed. The plates were monitored and measurements taken daily, these were tested with a Kruskal-Wallis rank sum or a t-test depending on the normality of the data.

A second replicated experiment into priming was done with three dishes separated into two sections: 50 primed and 50 unprimed SYN55 seed were randomly assigned to left or right sides to give 100 seeds per dish. These seeds were grown for 37 days to better measure the stem and root growth. Photographs and fluorescence images for each plate were gathered throughout the experiment and at the end of the experiment. Water was added as necessary, to keep the blue germination paper fully wet. The mean fluorescence F_v/F_m values and mean total fluorescence areas for each side of the dish were analysed with a paired t-test or a Wilcoxon signed rank when lacking a normal distribution. The stem and root elongations were analysed with t-tests or Kruskal-Wallis rank sums depending on normality. A Kruskal-Wallis was also used to determine if there was a significant difference in the total germination counts at the end of the experiment.

4.3c LIGHT AND DARK

SEED MASS MODEL

With experiments such as the 'Dark Burnout Seed Testing' below, it would have been useful to take the mass of seed individually as this would have allowed a thorough testing of whether seedlings grown from seeds with more mass survived for longer in the dark. However, the time required to measure each seed made this impractical. Instead, the seeds' weights were estimated by taking a subsample and measuring the areas. Two methods were tested to estimate the mass of individual seeds.

First, the MARVIN (MARVIN-fine, GTA Sensorik GmbH) system was used; MARVIN automatically weighs and images seeds, dividing the total weight of a sample of seeds by the number of seeds in the sample. The number of seeds is counted using computer vision. MARVIN was used with five seed lots of 200

seeds each. Seeds were placed by eye into categories: larger seeds were placed in lots 1 and 2 while smaller seed were placed in lots 4 and 5 with medium seed in lot 3. It was hoped that by doing this a relationship between the mass and the size of the seeds could be established. The measurements on each lot of seed were repeated three times to provide reliability to the result.

The second manual method was done by weighing each seed on a Sartorius M-power AZ214 scale (Repeatability ≤ 0.2 mg (Sartorius Mechatronics, 2010, p. 24)) and waiting until a stable reading was achieved (1-4 minutes), then recording this reading. The seeds were affixed to white masking tape to preserve the order, then imaged (using a Nikon D90) and thresholded using FIJI. The size of each seed object was found using the 'particle analysis' tool. A size calibration was done using an in-image ruler. These were plotted against the recorded weights to find the best model to describe the change in seed mass for size.

DARK BURNOUT SEED TESTING

An experiment was done to investigate various effects of time in darkness: The survivability of seedlings, whether there is any benefit from some time in darkness, whether seed size affects the outcome, and how far the seedlings can grow. First, a range finding pre-test was carried out to determine a maximum time range, i.e. at what point seedlings started to die. Collections of 20+ MX300 seeds were tested on wet tissue paper for six (144 h) and twelve days (288 h) time in the dark (TiD). The latter changed the growth pattern of the seedlings but neither period was long enough to kill the seedlings. Therefore, 576 hours dark was chosen for the longest time to keep the seeds in the dark. At 288 hours, the seedlings appeared weak so doubling the length of TiD was thought to be sufficient.

At the beginning of the experiment, seed areas were determined using a method similar to the one in 'Seed Mass Model' above but without the need for masking tape, because the moist seed did not roll around. The seed areas were calculated and then compared to the seed mass model (created using the manual technique above) in order to provide an estimate of the resources each seed had.

Miscanthus SYN55 seeds were pre-sterilised (see Section 2.4 above) and kept in the dark for 13 multiples of 48 hours (0, 48, 72...576 h). The dishes were randomised within the cabinet to control for cabinet temperature differences. The seeds were germinated and grown at 25°C in white light (fluorescent, 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$), at a relative humidity of 77%. This was the highest the cabinet was capable of and was variable by 10%. It was kept this high to prevent the Petri dishes drying out easily.

Each Petri dish was set up with one piece of blue roll folded 4 times as a water reservoir, with one 9.8 cm \times 10.5 cm piece of blue germination paper on top. Because the 8 \times 8-grid system (Section 4.2b) had not been developed at this point, twenty-five seeds per dish were laid out in a five by five grid; this also ensured that seed locations in the dish could be maintained. Each dish was numbered and wrapped in two layers of tin foil. This was done for 39 dishes to allow three replicates to be unwrapped at each of the 13 time points.

Once removed from the dark and unwrapped, the seeds were photographed every 48 hours for 12 days (this was used to record germination and mould). On removal from the dark seedlings also had hypocotyl and root elongations measured. After the 12 days had elapsed, the hypocotyls and roots were re-measured and fluorescence imaged. The control seed lot (0 h TiD) was fluorescence imaged at 12 days using the CF Imager (Technologica Ltd, Colchester UK) and further monitored as a comparison for the remainder of the test. The end status of the

seeds was assessed at each seeds' last observation time using the technique and decision tree from [Appendix K], this along with a final dry weight of the seedlings, done at the end of all testing (dried for four days at ~60°C), was used to assess the success of the seedlings.

The results of this experiment were used to illuminate four questions: Firstly, whether seeds benefit from time in the dark. The proportion of germination was correlated with the amount of TiD (Kendall's rank), the end state proportions of the seeds were tested against TiD (one-way ANOVA and Tukey's HSD), and the final mass of the seedlings was correlated with TiD (one-way ANOVA and Tukey's HSD). Secondly, the maximum elongation the seeds were capable of in the dark was calculated from the point where the average elongation of germinated seeds stopped increasing with time. Thirdly, how long the seedlings survive in the dark; this was based on whether they could recover during the 12-day monitoring period. One indication of this was whether the seed lot stopped growing during the 12 days recovery, the other was at what TiD the number of seeds classed as living when fluorescence imaged dropped to zero. The final question was whether seed size affects the final state of the seed. This was firstly determined by comparing seed size with end state using a one-way ANOVA with a Tukey's HSD. The elongation of the seedlings against seed size was also analysed using a Kendall's rank correlation, as well as end seedling dry weight against seed size, which was analysed with a Kruskal-Wallis rank sum.

DARK VS. RED LIGHT GERMINATION EXPERIMENT

To test for absolute light dependency of germination in *Miscanthus* seed, seeds needed to be tested with absolutely minimal light exposure once other germination conditions were met (e.g. water), as any small amount of light can contribute to triggering germination (Acosta et al., 2013; Hsiao & Vidaver, 1989).

The dark burnout seeds were exposed to some light while being sterilised and at the start of the experiment while being photographed for size. Therefore, an experiment was done using four archetypal seed lots: An open crossed *M. sinensis* (MX300), a *M. sinensis* synthetic cross (SYN55), an interspecific hybrid of *M. sinensis* and *M. sacchariflorus* (GNT14), and a synthetic *M. sacchariflorus* cross (SYN70). The experiment was conducted separately on each set of seeds, due to the size of the controlled environments. Two small identical germination cabinets (Tritech Research DT2-MP-47L with in house modifications) were used; these had lighting and temperature control. Both were put on identical settings, one using a red lighting array (outputting a PAR of $\sim 80 \mu\text{mol m}^{-2} \text{s}^{-1}$ measured using a Skye SKP 215 sensor) and one without. They were monitored for 24 hours before the test to ensure consistency of temperature.

The seed was left to germinate for six days. Six days was chosen to give the seeds time to reach the levelling off point of the germination curve, but was shorter than the standard seven days. This was to limit the effect of mould on the unsterilized seed. On the sixth day, the cabinets were opened and the germination was counted.

One variety of seed used was MX300 because they have high germination, low susceptibility to mould and are plentiful. The seed were placed onto folded blue roll with 64 evenly spaced dents in it; this was placed in a Petri dish. Eight Petri dishes were prepared in this way. These were then divided randomly into the two germination cabinets (four in each). The dishes had 50 mL of SDW added just before going into the cabinets; this was done under a safe light. The seed was not sterilised in line with other germination experiments, because this would have risked light exposure while the seed was wet. Both cabinets were kept constant at 25°C for six days. 25°C was chosen as an optimal temperature

for *Miscanthus* and is high enough for the full effect of any light requirement (Ellis et al., 1989). Then the dishes were removed and the germination of the seeds was scored as explained in Section 2.1a.

This test was repeated in individual experiments on SYN55, GNT14, and SYN70 to get a broader understanding of how common light independent germination is in *Miscanthus*. The result of the six-day germination score for each of the four accessions was analysed with a t-test of the difference between red light and no light after checking for a normal distribution.

4.3d TAGUCHI ASSESSED BY ORTHOGONAL ARRAY

The Taguchi experiment was designed in collaboration with Dr Sreenivas Rao Ravella of Aberystwyth University, and the Taguchi analysis was performed by Dr Sreenivas. Taguchi experimental design focuses on allowing many factors to be tested simultaneously, each at multiple levels (e.g. multiple concentrations) (Taguchi, 1986). From the responses (e.g. germination) recorded in the experiment, the Taguchi can utilise an orthogonal design to determine the effect of each factor.

A mixed design Taguchi method was used based on the 16_L table (see Table 4–1), but with four factors at four levels and three factors at two levels. The factors used were chosen to go into this design based on their effect on seed outcomes (germination, elongation and end state) as well as how easily investigating the multiple factors would fit into one combined method (defined in Section 4.2b above). Each hormone had four levels, with PEG 8000 for water stress at two levels, light at two levels and primed seed used as a last two level factor. The minimum levels selected for each hormone were very low concentrations (that should have no or near to no effect), a low but effective concentration, a main effective concentration, and lastly a very high concentration that may have a

secondary of counterproductive effect. These limits were based on the above range finding techniques.

PEG 8000 was used because in Section 4.4b below PEG 8000 and 4000 performed similarly, implying that PEG 8000 was not becoming trapped in the blue germination paper. Therefore, because the PEG 8000, due to its size, is less likely to enter the seed than PEG 4000, it was the better choice without analysing the seeds to find out if PEG 4000 was imbibed. NaCl was not used for water limitation because the salt toxicity only affected the seeds at high levels, at which the effect of salt toxicity would be difficult to untangle from the effect of the water limitation.

For the light factor, low light was used instead of dark, because the dark test would not allow the germination rate to be monitored without carrying out the whole experiment using aliquoting techniques. Light reduction to the seed was achieved using six layers of bleached white muslin placed atop the dishes, as a neutral density filter. This was recorded as a reduction of 73 percent photosynthetically active radiation, from 300 to 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$, measured using a Skye SKP 215 sensor. It is expected that seedlings would perform better under brighter light, possibly with shorter stems this could be countered by the effect of GA (Lockhart, 1956).

Priming was added because the results for primed seed (Section 6.3e) implied they might have remained in dormancy for too long after being primed; a germination boost only occurred at 800 hours, which was too late for use in the field. Combining priming with hormones could aid in releasing the seeds from dormancy earlier.

The hormone levels chosen for each hormone were based on the range finding results (Section 4.4a below). For ABA values of 0.02, 0.2, 2, and 20 mg L^{-1} were

chosen to represent the full range in which effects were seen in the range finding. GA did not have a noticeable secondary effect in the range finding experiment; therefore, 0.015, 0.15, 1.5, and 15 mg L⁻¹ were chosen as representative for selection of under to over effective values. Auxin's levels were 0.005, 0.05, 0.5, and 5 mg L⁻¹; this cut the long diminishing tail from the range finding experiment short to 5 mg L⁻¹, and selected the other levels to stay to a well-distributed exponential scale. Four BR levels of 0.015, 0.75, 1.5, and 7.5 mg L⁻¹ were chosen; 0.75 mg L⁻¹ as the main section of deviation from the control, 1.5 mg L⁻¹ to represent the highest effects, and 7.5 mg L⁻¹ to extend what was seen in the range finding, which had a small increase in seed performance metrics after 1.5 mg L⁻¹. Because the hormones interact and crosstalk the concentrations that previously had certain effects may no longer give the same results.

The experiment was conducted using the levels shown in Table 4-1 and at the limits explained above. All dishes were prepared and tested at the same time to limit external variation.

Table 4–1: A table of the levels (very low to very high) of each factor (hormone, treatment, or growth condition) that were used in the Taguchi experimental design. Each individual Taguchi experiment is listed down the left side.

Experiment number	Treatment levels for each treatment						
	ABA (mg L ⁻¹)	GA (mg L ⁻¹)	Water stress (Mpa)	NAA (mg L ⁻¹)	BR (mg L ⁻¹)	Light level	Prime Seed
1	V Low	V Low	Low	V Low	V Low	Low	Yes
2	V Low	Low	Low	Low	Low	High	No
3	V Low	High	High	High	High	Low	No
4	V Low	V High	High	V High	V High	High	Yes
5	Low	V Low	Low	V High	High	High	No
6	Low	Low	Low	High	V High	Low	Yes
7	Low	High	High	Low	V Low	High	Yes
8	Low	V High	High	V Low	Low	Low	No
9	High	V Low	High	Low	V High	Low	No
10	High	Low	High	V Low	High	High	Yes
11	High	High	V Low	V High	Low	Low	Yes
12	High	V High	Low	High	V Low	High	No
13	V High	V Low	High	High	Low	High	Yes
14	V High	Low	High	V High	V Low	Low	No
15	V High	High	Low	V Low	V High	High	No
16	V High	V High	Low	Low	High	Low	Yes

The Taguchi method allows for multiple responses (Rao et al., 2008), e.g. germination and stem length can both be output metrics. This was utilised in this study to input a set of important germination and early growth metrics. A set of metrics were chosen to go into the Taguchi analysis, each analysed separately to work out what the main effects of each treatment were and if there were any significant interactions between them. The germination percentage of each dish was used because changes to germination percentage may indicate important effects for direct sowing. Rate of germination both as mean time to germinate and $1/T_{50}$ (1/the time until 50% of viable seed germinated) were both analysed, to give the reactivity of the seed. Stem and root elongation were analysed as well as the stem:root ratio, because quick growing seedlings are inherently

important for direct sowing; the seedling focusing on stem growth is important to compete with weeds. Data from the fluorescence imaging of the seeds was used to determine their activity, both the total area per dish that was photosynthetically active, and the median level of the Fv/Fm reading. GI (Ranal & de Santana, 2006; Walker-Simmons, 1987) was also used to give a broader comparison of germination.

Interactions between the variables were also reported by the Taguchi analysis. These were given a sensitivity index to characterise the interaction signal against the noise. The interactions with a sensitivity index over 70% were used to identify possible effects of factors on each other. Particularly of interest were the hormone interactions and the effect of hormones on the response to physical factors.

$$\text{OEC} = \left(\frac{y_1}{y_{1\max}} \right) \times w_1 + \left(\frac{y_2}{y_{2\max}} \right) \times w_2 + \dots \quad (6)$$

The Taguchi can also apply a summary statistic as an Overall Evaluation Criteria (OEC) if the individual responses measured are not in consensus about the best treatment (Roy, 2001, p. 429). The OEC can then determine which set of treatments were the best for seedlings based on all of the experimental responses. To do this, the outputs are normalised then multiplied by subjective weightings given to each of the experimental outputs, based on their importance to the objective of the experiment (Roy, 2001, p. 54). The OEC calculation is shown in the equation above (6) (Roy, 2010, p. 214; Subba Rao et al., 2008); where y_i is the response measurement, $y_{i\max}$ is the maximum value for the response, and w_i is the weighing of the response. In this study, weighting was done using the principle that the weighting should be higher the broader the output was. Therefore, the GI was weighted highest at 0.25, because it is an index of speed and quantity of germination, which gives a broad picture of

germination success. The stem:root ratio was second, weighted at 0.2 because it used information from stem and root elongation, giving an indication of the overall health of the seedling. Next, the total area and median level of Fv/Fm fluorescence were both weighted at 0.15, because they give an overall impression of the size and health of the seedlings. Then the amount of germination and $1/T_{50}$ of the germination were both weighted at 0.075 because these results represented individual measurements. Lastly root and stem elongation were both weighted at 0.05 because they both represented individual factors, and were thought less important to an overall score than the previous two.

4.4 RESULTS

The results for each of the chemical physical experiments and relevant pre-tests are presented below.

4.4a HORMONES EFFECTS EXPERIMENTS

Below are the results for the four reported hormone tests.

ABSCISIC ACID

The effect of Abscisic acid (ABA) on root and stem elongation appears negative, particularly at higher (more than 20 mg L⁻¹) concentrations (Figure 4-7). There are some positive effects on stem elongation at lower concentrations visible in Figure 4-7. However, this is only a difference between a median of 5 mm for the control and 7 mm for less than or equal to 5 mg L⁻¹, and the mean differences for both round to 7.19 mm. When tested with a Kruskal-Wallis rank sum the stem elongations were significantly affected by ABA ($P < 0.0001$), with a negative Kendall's rank correlation of -0.32. Root elongations also negatively correlate

with concentration at -0.46 (Kendall's rank), this is also significant when tested with a Kruskal-Wallis rank sum ($P < 0.0001$).

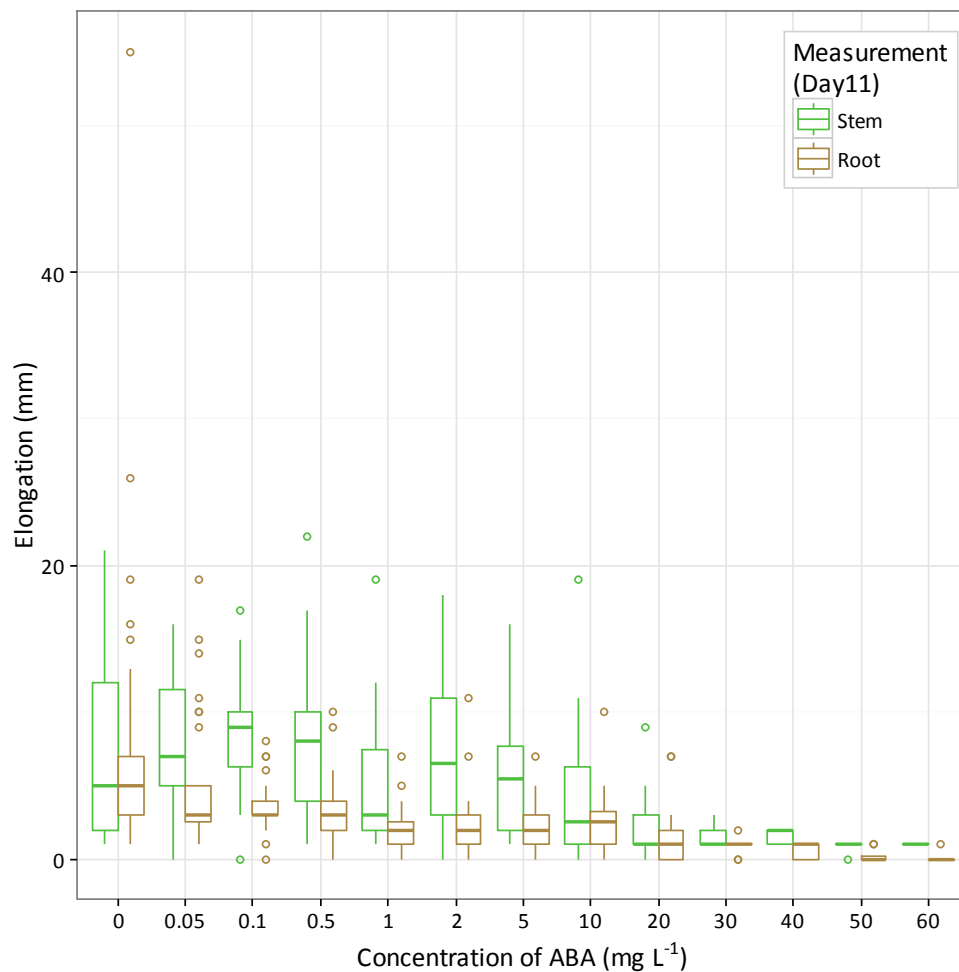


Figure 4-7: Root and stem elongations for seedlings grown under a range of ABA concentrations, as measured on the last day of the experiment, excluding un-germinated seed. Each dish up to 64 seedlings is represented by two boxplots, one for root elongations, and one for stem elongations. The range of ABA concentrations has been drawn on a categorical scale.

To assess the end states of the seedlings, they were placed into six categories. The number of seedlings categorised as 'Thriving' drops rapidly with increasing ABA concentrations (Figure 4-8). The number of seedlings in any living state at the end of the test lowers at 0.5 mg L⁻¹ and again at 20 mg L⁻¹ (Figure 4-8). With the exception of 1 mg L⁻¹, the number of seeds that are 'Inactive' at the end of the experiment remains stable across the concentrations.

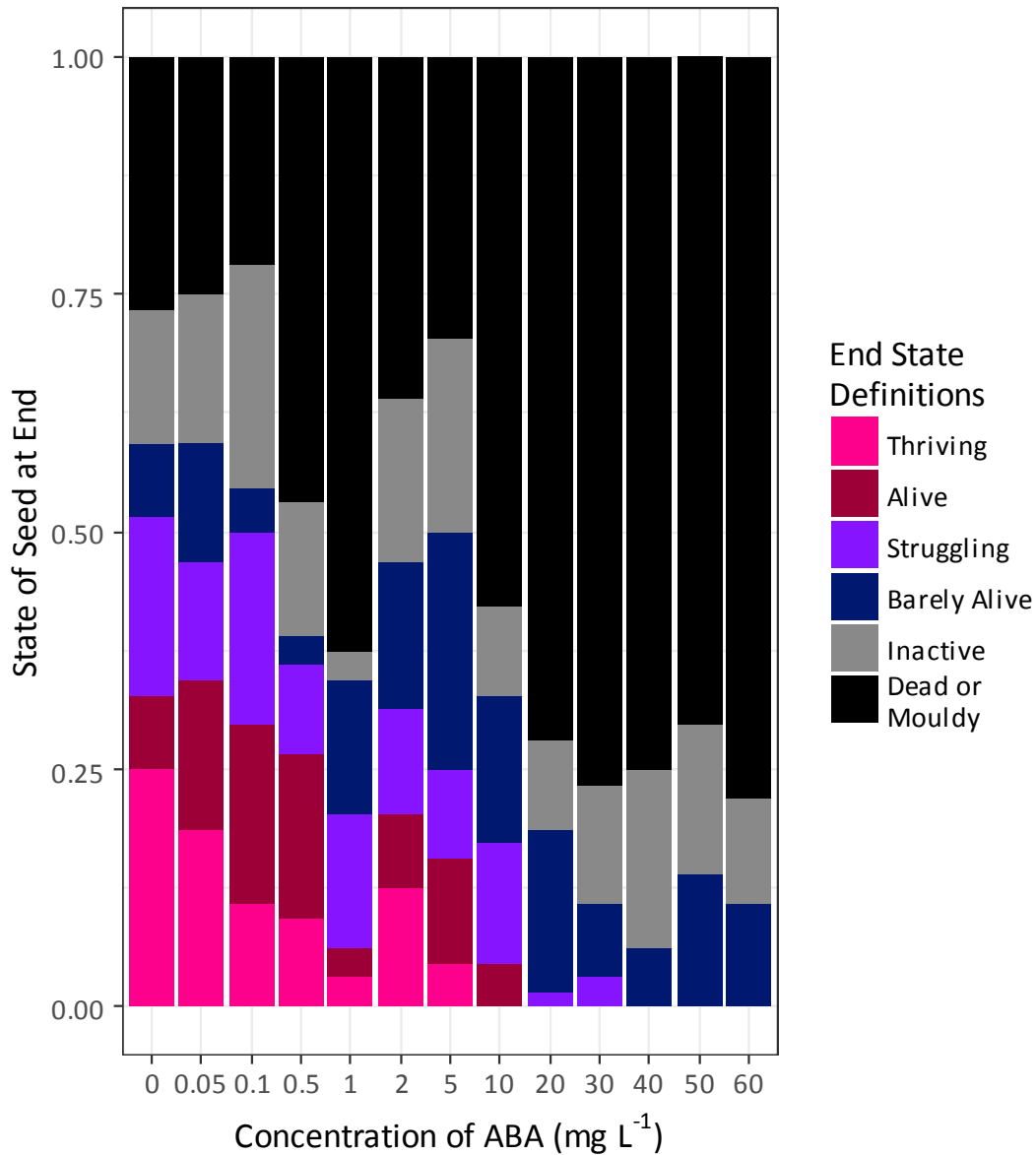


Figure 4-8: Proportions of the seedlings at each end state as assessed visually and using chlorophyll fluorescence, at the end of the test (day 11). The x-axis is categorical, representing the concentrations of ABA tested.

Time to 50% germination was 4 days for the control and for 0.05 mg L⁻¹, before increasing to 8 days at 0.1 mg L⁻¹ after which 50% of seeds do not germinate. This is reflected in Figure 4-9 below, where the time to germinate becomes broader after the first two concentrations.

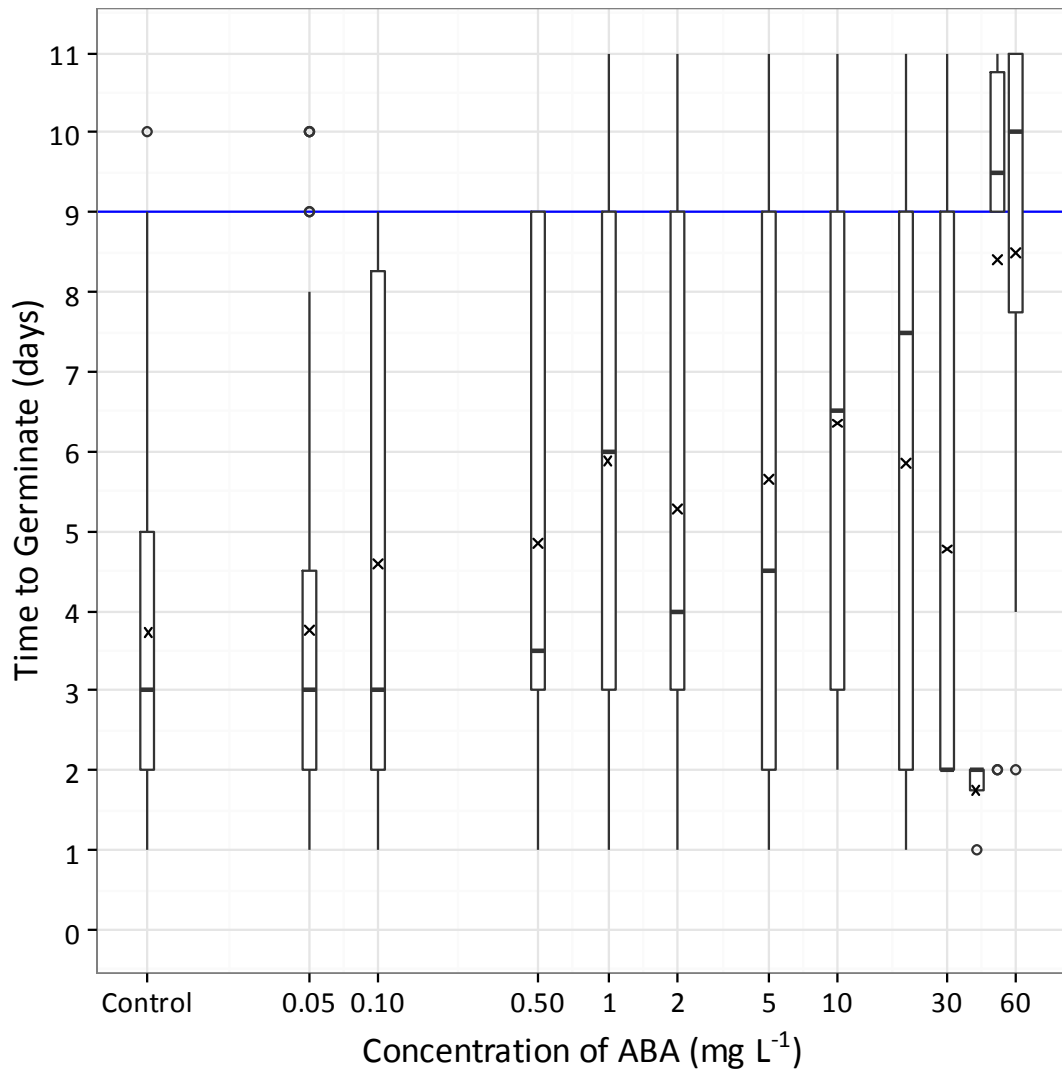


Figure 4-9: Number of days for each seed to germinate while in a solution of ABA (\log^{10} x-axis), the horizontal line represents the day extra SDW was added. Each box represents a dish (up to 64 germinated seeds). The control has been added on the left.

The requisite time for each seed to germinate significantly increases with the increased concentration of ABA (Figure 4-9), when analysed with a Kruskal-Wallis rank sum ($P < 0.001$). In the two highest concentrations, the median time for seeds to germinate was not until after the solution was diluted on day nine (Figure 4-9). The time required for mould to become visible on affected seed was significant when tested with a Kruskal-Wallis rank sum ($P < 0.0001$), a Kendall's rank showed a weak negative correlation of -0.18.

GI attempts to summarise germination in a single score, as described in Section 2.1a. GI dropped from over 4 in the first two concentrations to 3.6 at 0.1 mg L^{-1} then 2.5 at 0.5 mg L^{-1} . It then stabilised around 2 mg L^{-1} until more than 30 mg L^{-1} after which it remained less than 1. This effect on GI significantly affected when tested using an ANOVA ($P = 0.001$), with a strong negatively PPMC correlation at -0.84 .

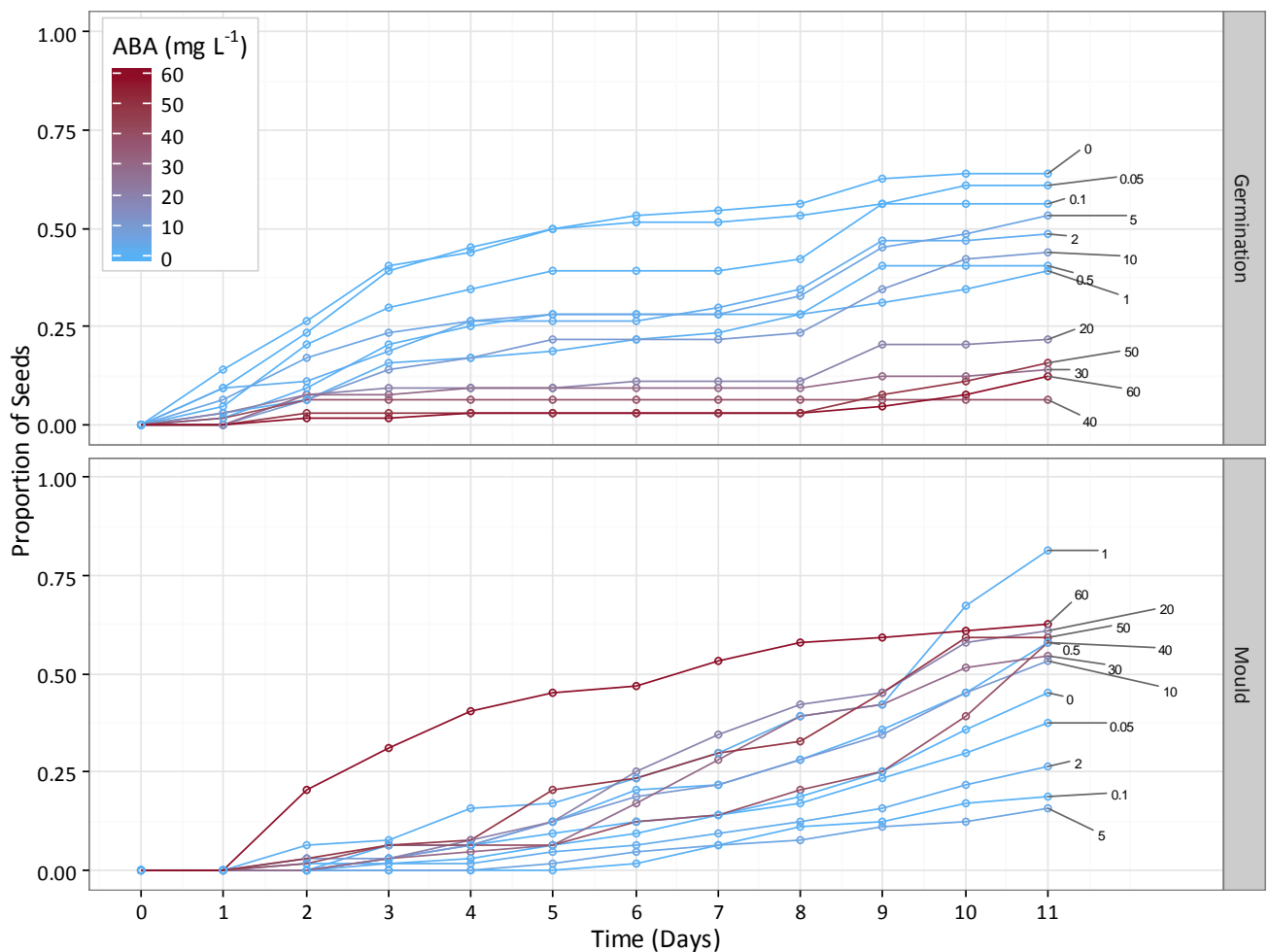


Figure 4-10: Germination and mould of each concentration traced over time. Water concentrations (mg L^{-1}) are annotated at the end of each trace. The points represent values not means and show an overall trend.

For each concentration of ABA, the final germination was reduced, and the cumulative germination curve flattens (Figure 4-10 top). The proportion of seeds germinated at the end of the experiment was significantly affected by ABA

(ANOVA, $P < 0.0001$), with a strong negative PPMC of -0.9. Mould (as shown in Figure 4-10 and Figure 4-8) increases with higher concentrations of ABA, this effect is significant at $P < 0.05$ and shows a positive correlation with a PPMC of 0.66. At 60 mg L^{-1} mould grows much faster, this may be an outlier or because it is the highest concentration it could be triggering a more deadly effect in the seeds.

GIBBERELIC ACID

The final mean elongation of the stems exposed to concentrations of Gibberellic acid (GA) that were more than or equal to 0.75 mg L^{-1} was 22.1 mm, which is longer than the control at 7.7 mm (see Figure 4-11). When tested with a Kruskal-Wallis rank sum there was a significant ($P < 0.0001$) change in stem elongation with GA concentration. A Kendall's rank produces a low positive correlation of 0.21, which is visible in Figure 4-11 as a change over the first two concentrations of GA.

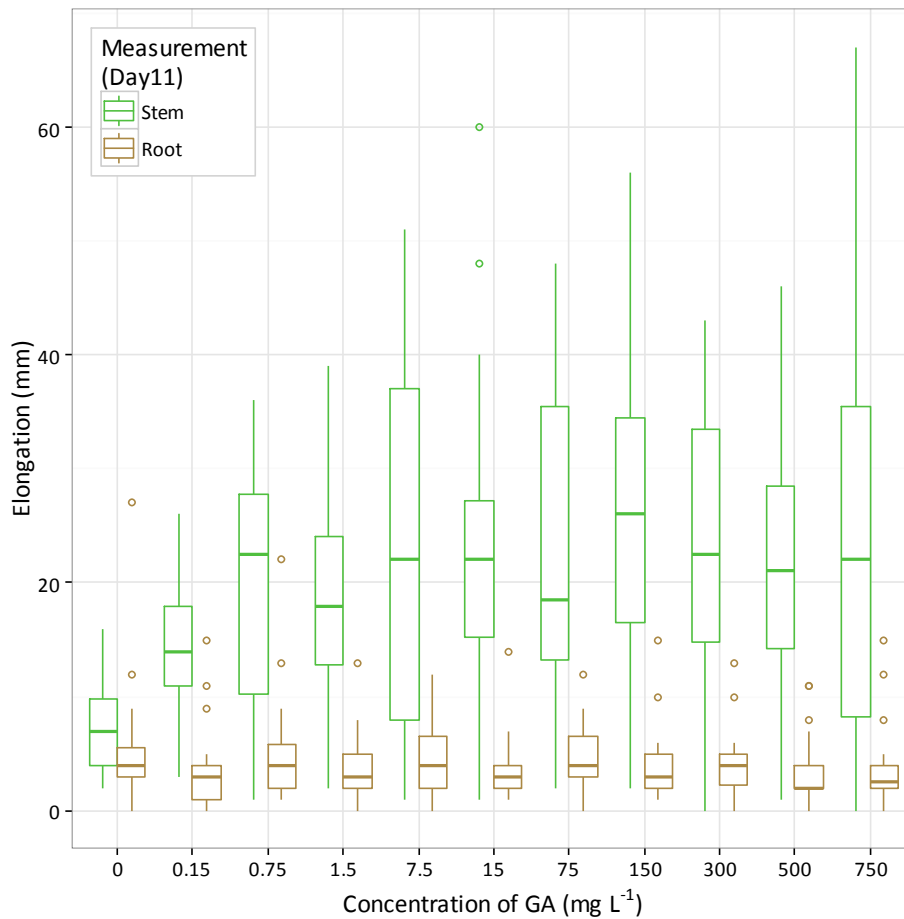


Figure 4-11: Root and stem elongations for seedlings grown under a range of GA concentrations, as measured on the last day of the experiment, excluding un-germinated seed. Each dish up to 64 seedlings is represented by two boxplots, one for root elongations, and one for stem elongations. The range of GA concentrations has been drawn on a categorical scale.

Root elongations were not significantly affected by the GA concentration when compared with a one-way ANOVA ($P = 0.52$). This lack of response is also visible in Figure 4-11.

There is no clear trend for classified seedling end state under the influence of increasing concentrations of GA (Figure 4-12).

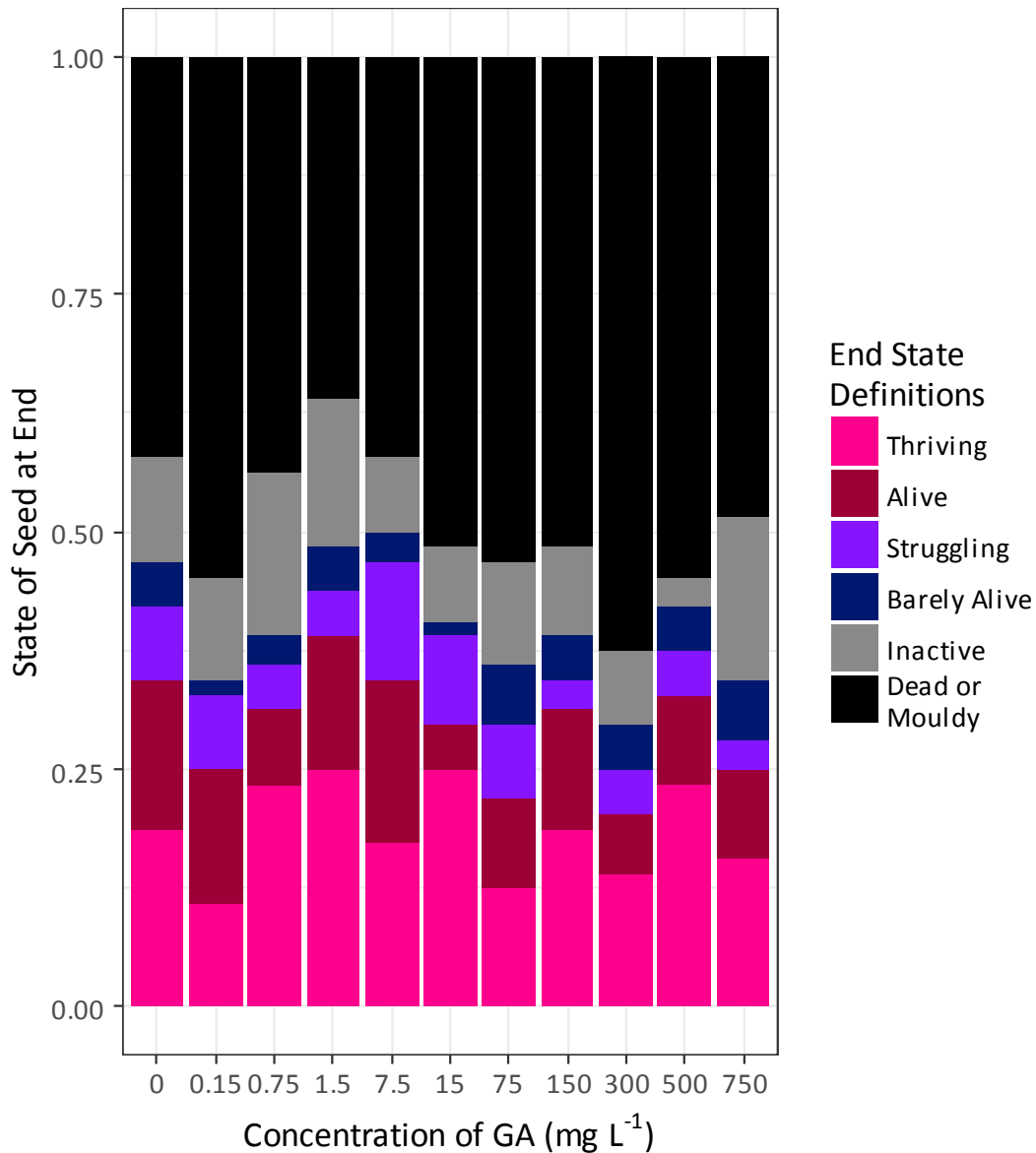


Figure 4-12: Proportions of the seedlings at each end state as assessed visually and using chlorophyll fluorescence, at the end of the test (day 11). The x-axis is categorical, representing the concentrations of GA tested.

More elongated seedlings should be classified as a healthier end state using the system in Appendix K, as they have grown longer stems; however, seeds are classified by their fluorescence and visual health, which affected the number classified as 'Thriving'. The seedlings may have looked paler and/or fluoresced less, and so were scored as being less healthy.

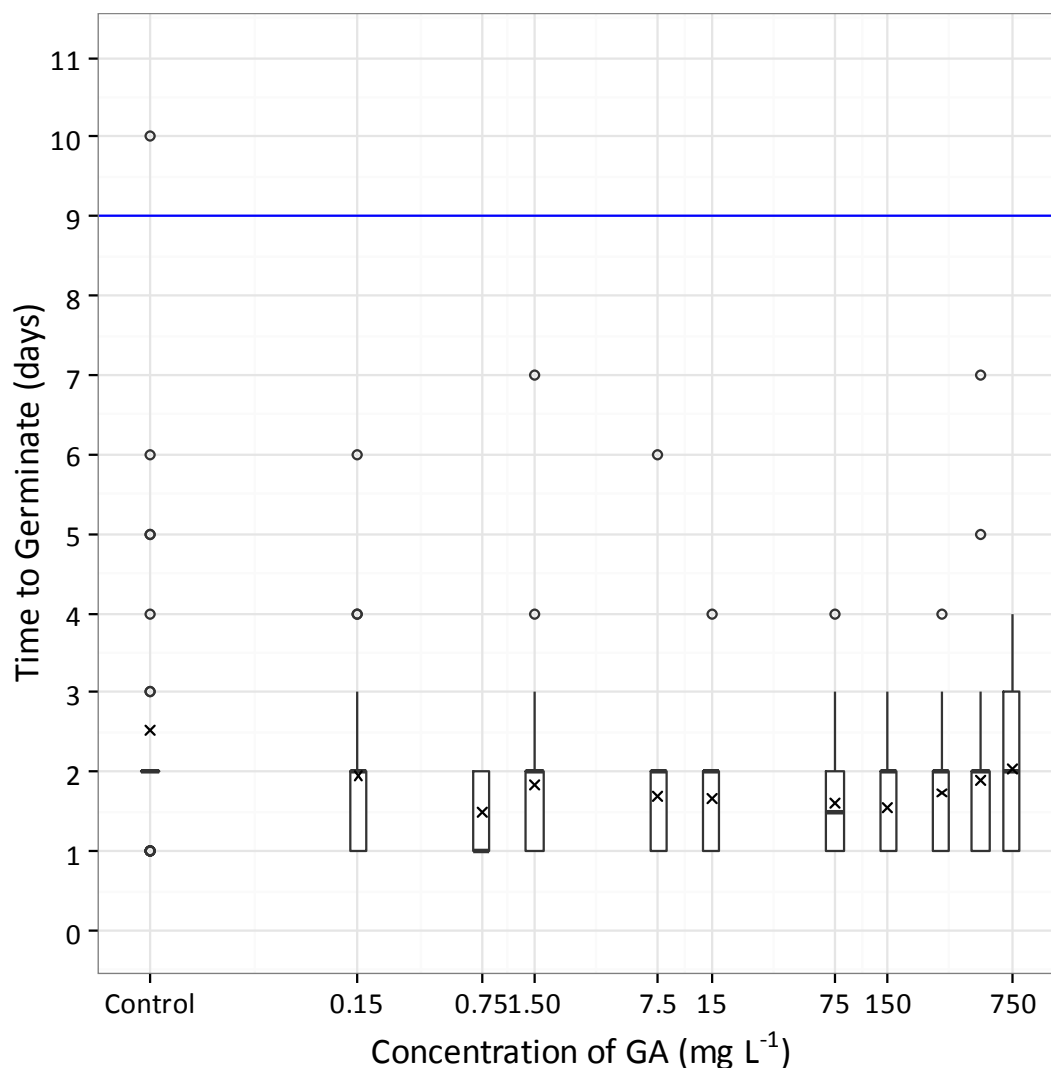


Figure 4-13: Number of days for each seed to germinate while in a solution of GA (\log^{10} x-axis), the horizontal line represents the day extra SDW was added. Each box represents a dish (up to 64 germinated seeds). The control has been added on the left.

The time taken for the individual seeds to germinate under the influence of GA showed no significant effect when analysed with a Kruskal-Wallis rank sum ($P = 0.08$). Figure 4-13 shows the lack of a clear pattern.

The time in which mouldy seed went mouldy, had a significant relationship with GA when analysed with a Kruskal-Wallis ($P < 0.0001$); however, a Kendall's rank showed no correlation (-0.005).

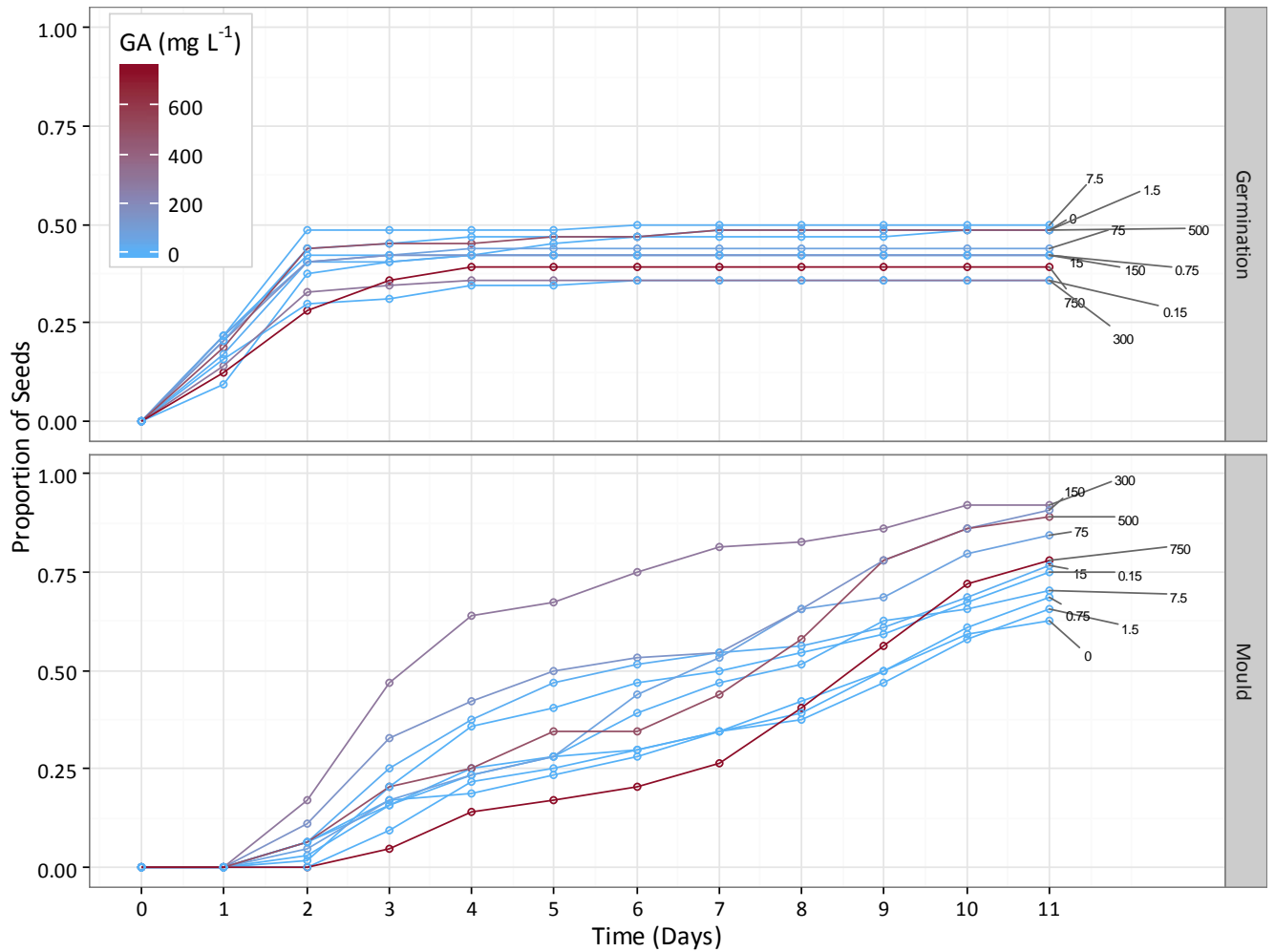


Figure 4-14: Proportion of seeds germinated and mouldy over the course of the experiment.

Each trace is a dish with a different concentration of GA (as labelled).

The proportion of seeds that became mouldy by the end of the experiment was not significant when tested for an effect of GA concentration with an ANOVA ($P = 0.29$). There is also no clear trend visible in Figure 4-14 (bottom) of the prevalence of mould based on proportion of GA.

However, GA concentration produced peak germination between 1 and 10 mg L⁻¹ (Figure 4-14 & Figure 4-12). This is most noticeable for GI, which was 4.09 in the control, rising to 4.65 at 7.5 mg L⁻¹ before staying between 3.3 and 4.4 for the rest of the experiment. When GI and raw germination were tested against

GA concentration with an ANOVA, neither produced a significant effect, with results of $P = 0.41$ and $P = 0.52$ respectively.

AUXIN (NAA)

Auxin (1-naphthaleneacetic acid [NAA]) appeared in Figure 4-15 to have a positive effect on stem and root elongation at 0.01 to 0.05 mg L⁻¹, after which the root elongations dropped below the control. The stem elongations stayed the same or improved until over 50 mg L⁻¹ auxin (Figure 4-15).

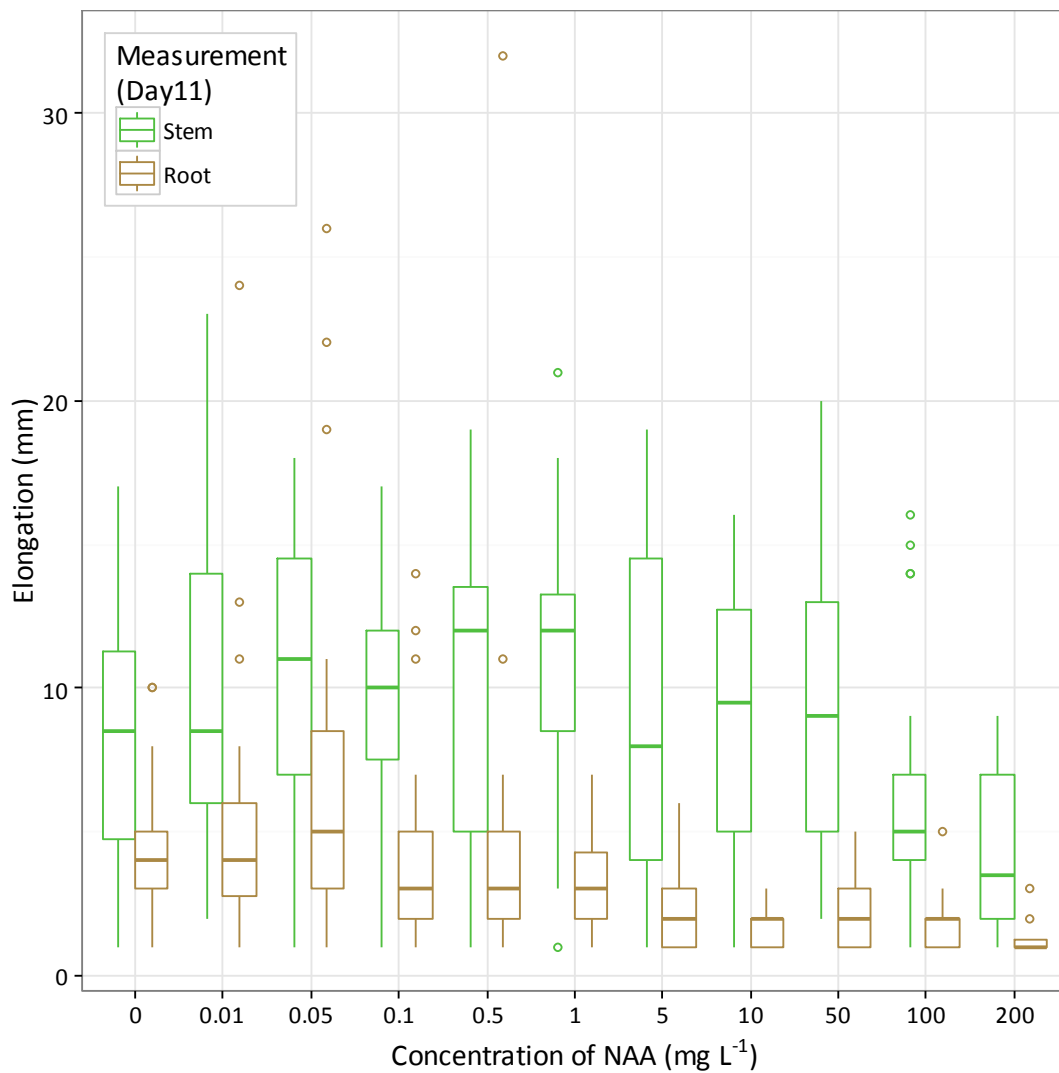


Figure 4-15: Root and stem elongations for seedlings grown under a range of auxin (NAA) concentrations, as measured on the last day of the experiment, excluding un-germinated seed. Each dish up to 64 seedlings is represented by two boxplots, one for root elongations, and one for stem elongations. The range of NAA concentrations has been drawn on a categorical scale.

It was observed during the test that the roots appeared fluffier, probably due to more root hairs being visible in the auxin dishes (Figure 4-16). However, numerical measurements were not taken of this.



Figure 4-16: Seedlings photographed at 5 days, the top row are examples of seedlings without auxin exposure and the bottom row have 1 mg L^{-1} auxin (NAA).

When root elongations were tested with a Kruskal-Wallis rank sum there was a significant decrease ($P < 0.0001$), this had a negative Kendall's rank correlation of -0.39 . Stem elongation was also significantly changed by concentration of auxin when analysed with a Kruskal-Wallis rank sum ($P < 0.0001$). Seedlings with low amounts of auxin (0.1 to 0.5 mg L^{-1}) recorded an end state of 'Thriving' at a higher proportion than the control (Figure 4-17). The number of seeds not classed 'Inactive' or 'Dead' at the end of the experiment appears to stay consistent across all dishes (Figure 4-17), and averages to 43%.

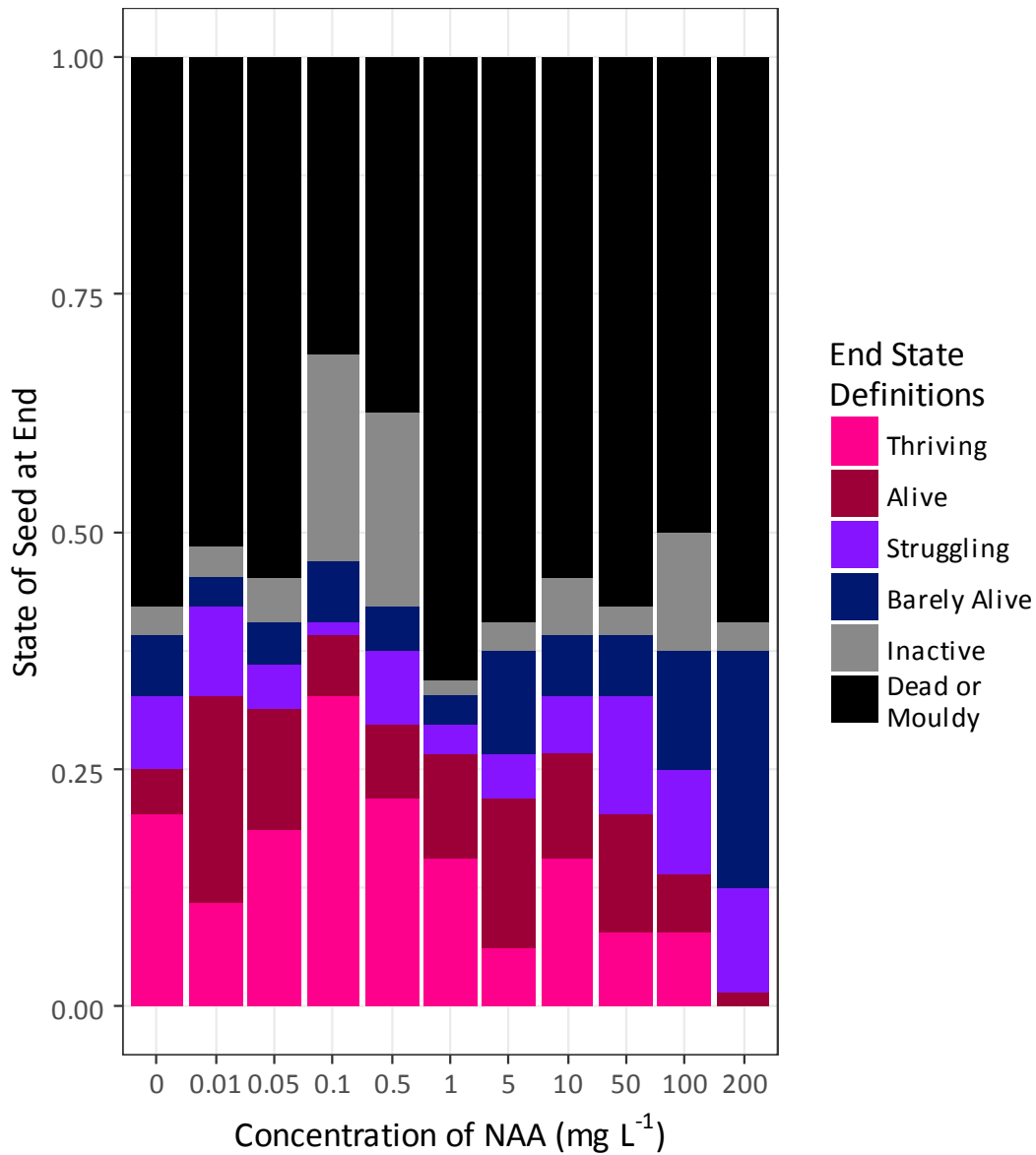


Figure 4-17: Proportions of the seedlings at each end state as assessed visually and using chlorophyll fluorescence, at the end of the test (day 11). The x-axis is categorical, representing the concentrations of auxin (NAA) tested.

Auxin concentration did not significantly affect GI, which ranged from 3.4 to 4.1 ($P = 0.22$). The percentage of mouldy seeds, shown in Figure 4-17, was also not significantly affected by auxin concentration when tested using an ANOVA ($P = 0.09$).

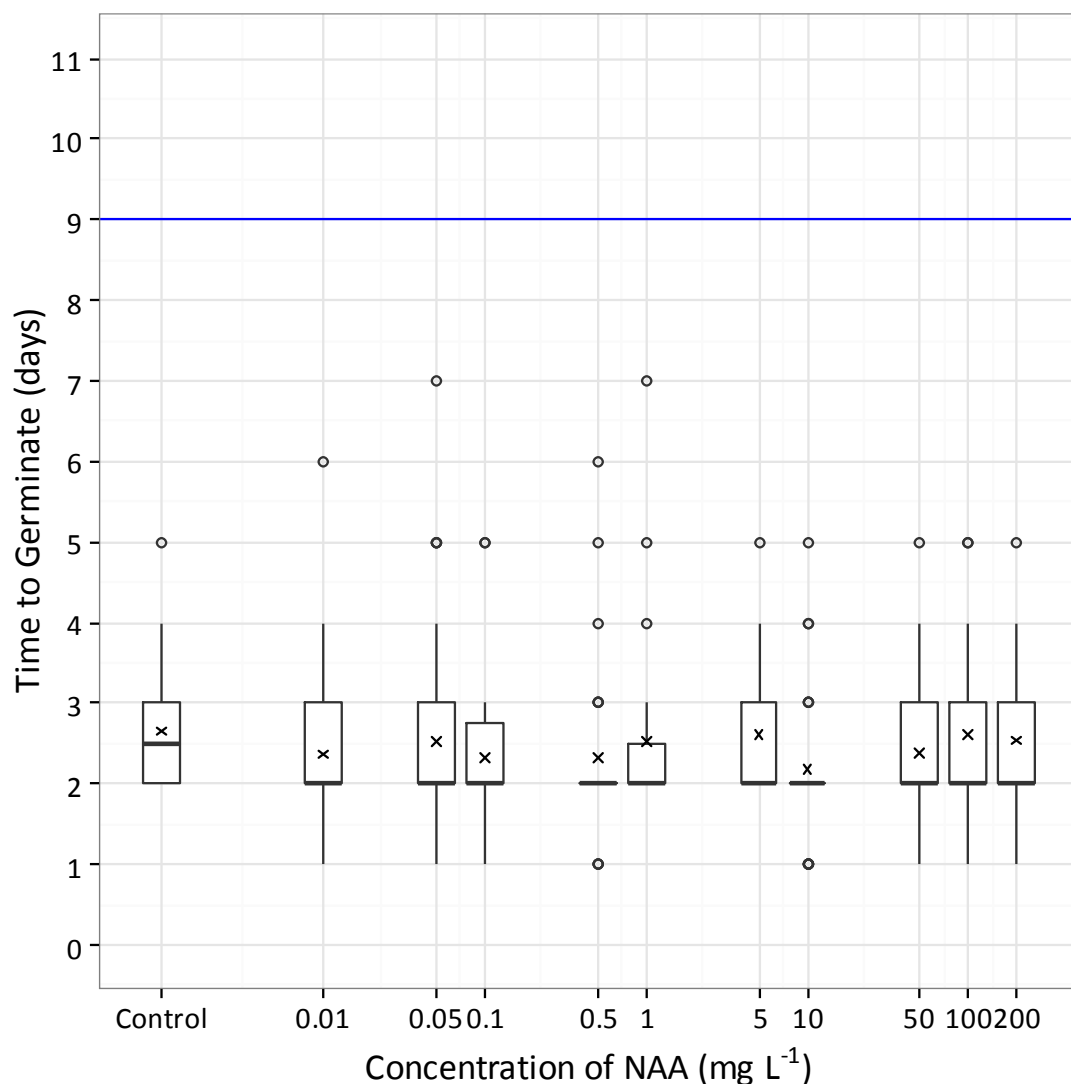


Figure 4-18: Number of days for each seed to germinate while in a solution of NAA (\log^{10} x-axis), the horizontal line represents the day extra SDW was added. Each box represents a dish (up to 64 germinated seeds). The control has been added on the left.

Auxin had little noticeable effect on germination speed. The mean number of days for a seed to germinate stayed at around 2.5 days regardless of concentration (Figure 4-18). However, Figure 4-18 shows a small increase in median germination speed from 2.5 to 2 days, from the control to all the concentrations. A Kruskal-Wallis rank sum test for the time for each seed to germinate against concentration was not significant ($P = 0.249$).

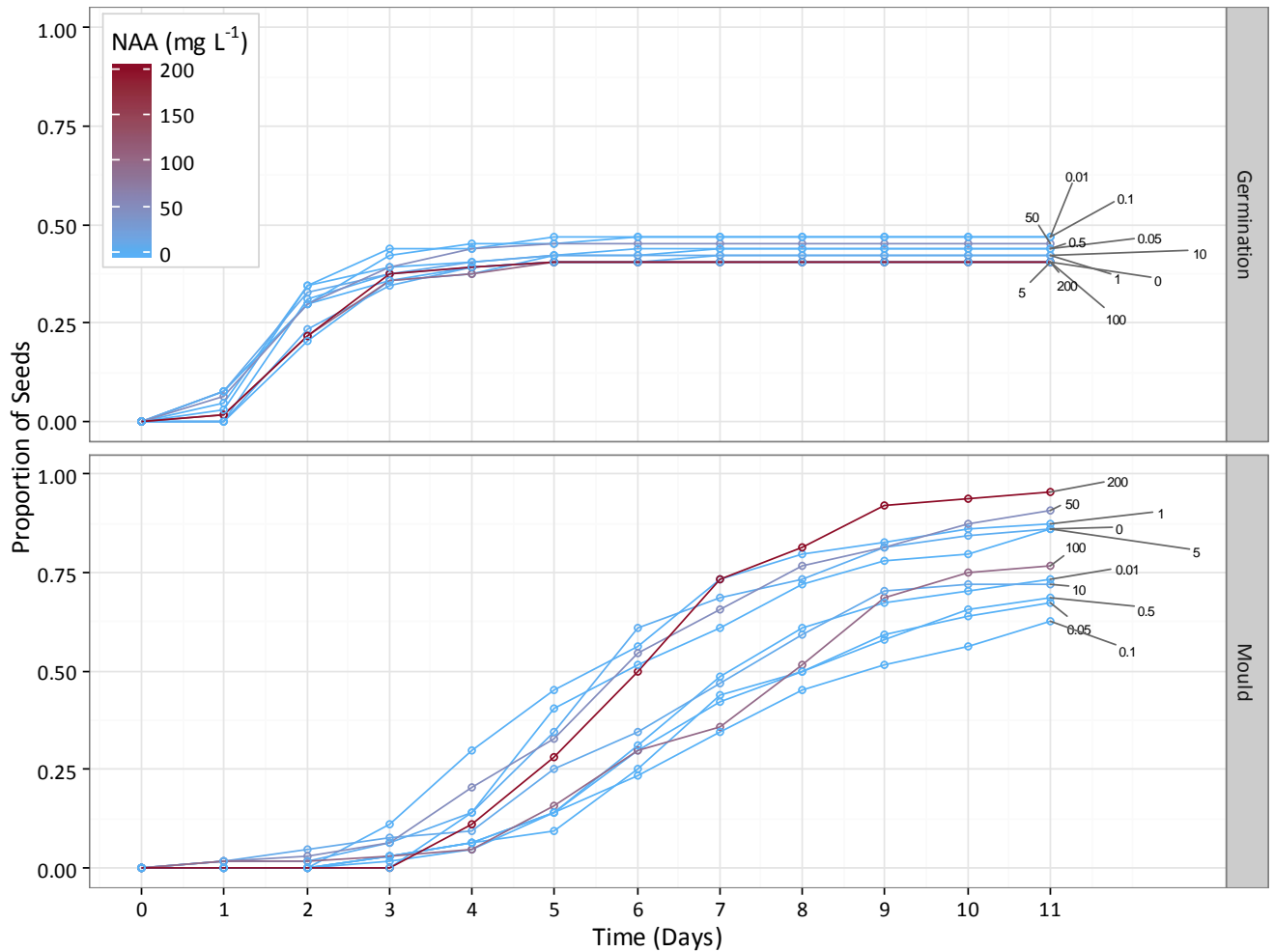


Figure 4-19: Seed germination and mould proportions over time. Traces labelled for each concentration of auxin.

There is no consistent impact on mould or germination proportion by the auxin; Figure 4-19 (top) shows all concentrations responding together regardless of auxin concentration. Figure 4-19 (bottom) shows that mould proportion is more variable than germination proportion, but there does not appear to be a trend based on concentration. The proportion of seeds germinated at the end of the experiment was not significantly affected by auxin, when tested with an ANOVA ($P = 0.22$). As with the time required to germinate and the proportion of seeds germinated, the time required for seed to go mouldy was not significantly affected by concentration, when tested with a Kendall's rank ($P = 0.63$).

BRASSINOSTEROID

Brassinosteroid (BR) appeared to have no effect on stem elongation and only a minor effect on root elongation, which decreased after 0.5 mg L⁻¹, and both stem and root elongation fluctuated more after 0.1 mg L⁻¹ (Figure 4-20).

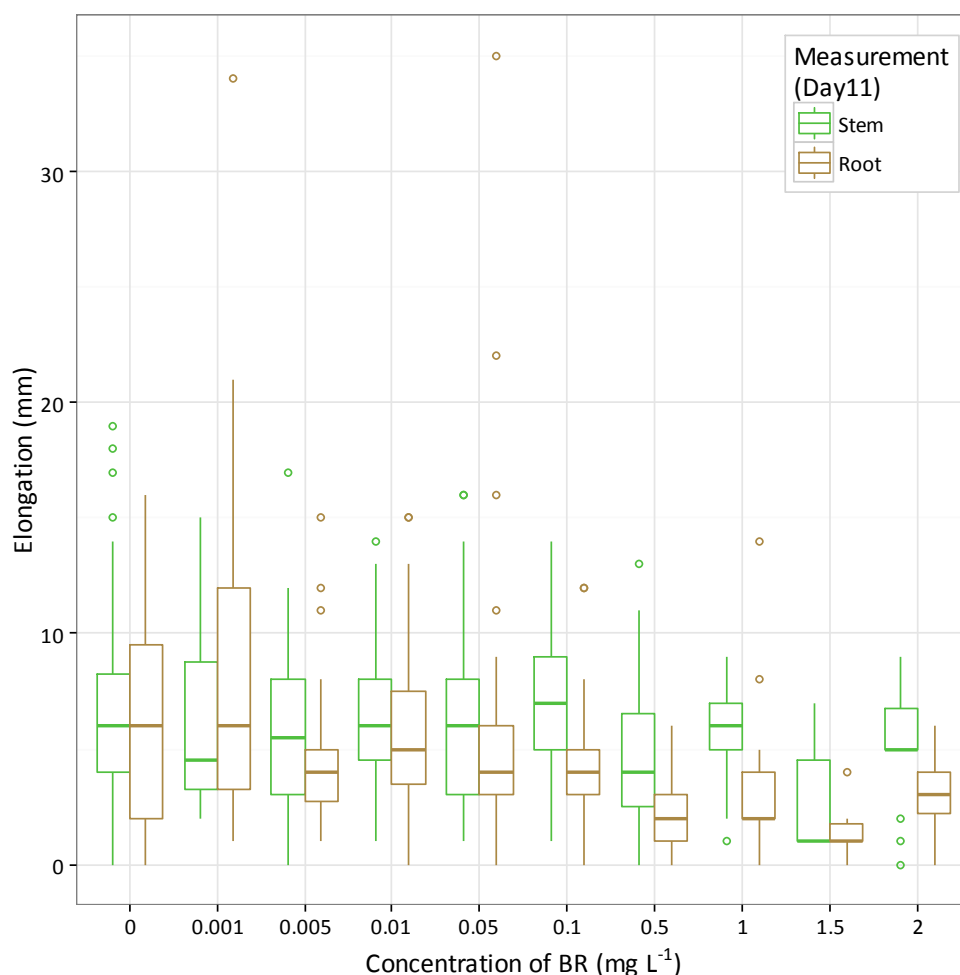


Figure 4-20: Root and stem elongations for seedlings grown under a range of BR concentrations, as measured on the last day of the experiment, excluding un-germinated seed. Each dish up to 64 seedlings is represented by two boxplots, one for root elongations, and one for stem elongations. The range of BR concentrations has been drawn on a categorical scale.

Stem measurements were not normally distributed, but were easily transformed to normality by square rooting, while root measurements could not be transformed so a nonparametric test was used. Applying a one-way ANOVA of the square-rooted stem measurements showed a statistically significant decrease

with concentration of BR ($P < 0.01$), as did the root measurements when tested with a Kruskal-Wallis rank sum ($P < 0.0001$). The change in mean root elongation occurred from a mean of 6.6 mm at 0 mg L⁻¹ to a mean of 2.5 mm for an average of the highest three concentrations (1, 1.5 & 2 mg L⁻¹). Stem elongation went from 7.4 mm to 4.5 mm over the same ranges. This is confirmed by a stronger correlation for root elongation (Kendall's rank, -0.3) than for stem elongation (PPMC, -0.18).

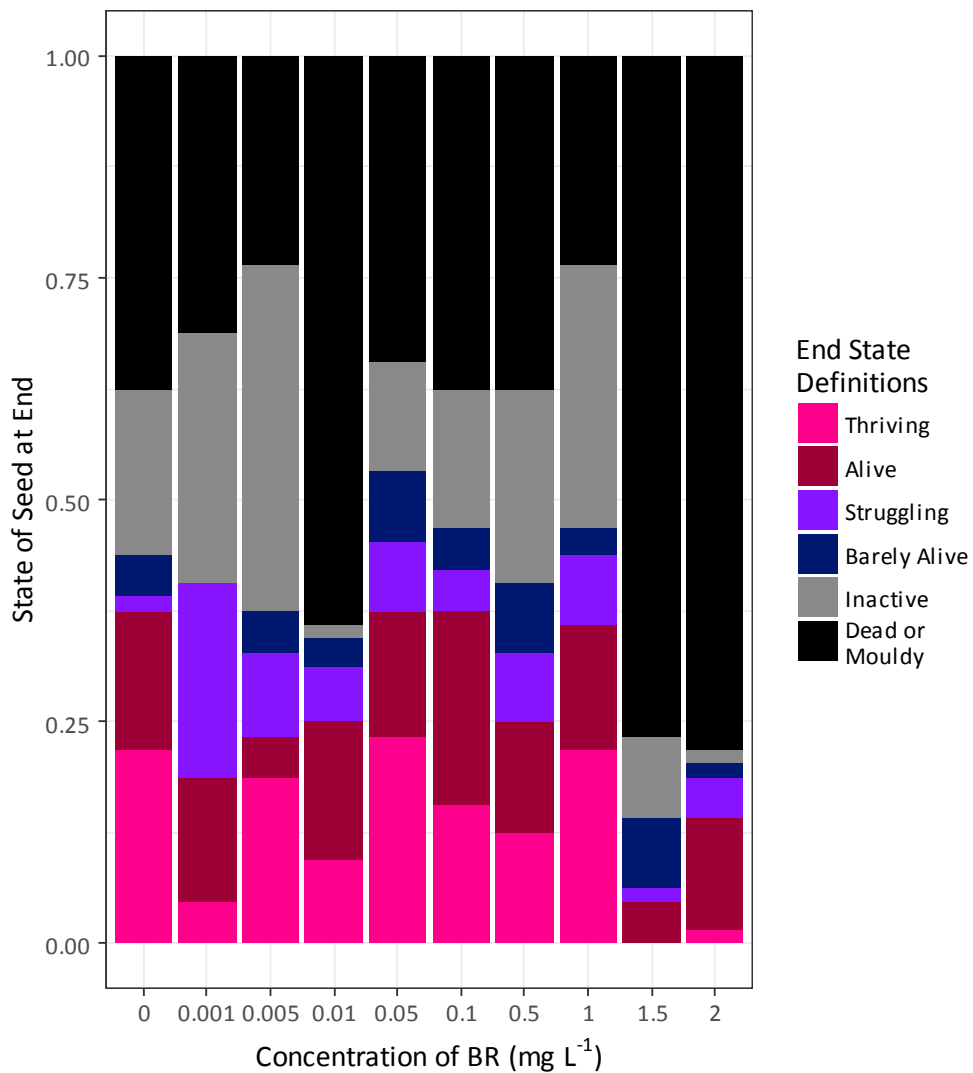


Figure 4-21: Proportions of the seedlings at each end state as assessed visually and using chlorophyll fluorescence, at the end of the test (day 11). The x-axis is categorical, representing the concentrations of BR tested.

End seed state was visually determined and analysed against BR concentration. The proportion of the seeds classed as 'Dead or Mouldy' increases, from an average of 23/64 seeds dead at 1 mg L⁻¹ or before, to an average of 50/64 dead for 1.5 and 2 mg L⁻¹. The proportion of inactive seed decreases in the highest two concentrations of BR. The other seed states, shown in Figure 4-21, do not appear to follow a trend.

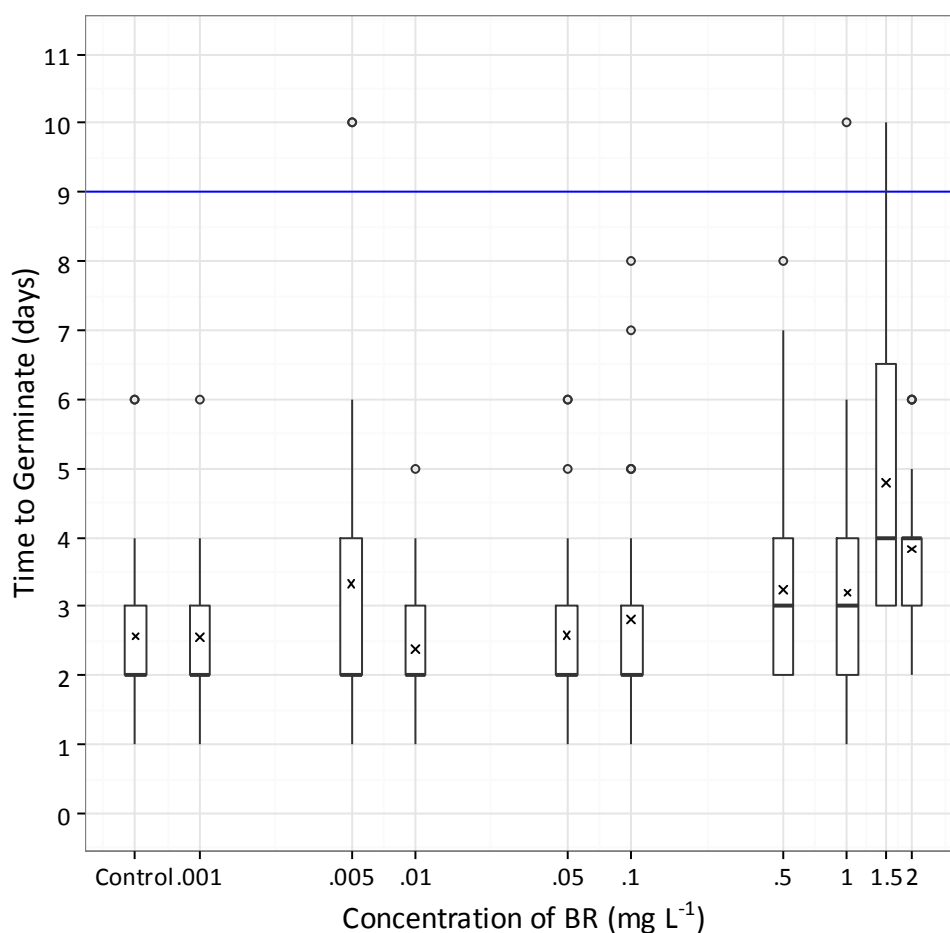


Figure 4-22: Number of days for each seed to germinate while in a solution of BR (\log^{10} x-axis), the horizontal line represents the day extra SDW was added. Each box represents a dish (up to 64 germinated seeds). The control has been added on the left.

The median time taken for seeds to germinate remains at two days at concentrations below 0.5 mg L⁻¹. With concentrations of 0.5 or 1 mg L⁻¹, time taken is three days and when higher it is four days (Figure 4-22). When tested with a

Kruskal-Wallis rank sum this change gives a significant increase ($P < 0.0001$) in germination time with concentration. This trend is the result of the uptick in time to germinate at concentrations over 1 mg L^{-1} .

A Kruskal-Wallis rank sum shows time for seed that became mouldy to be visibly mouldy significantly decreases with concentration of BR ($P < 0.0001$); however, when correlated with Kendall's rank there was not a strong correlation (-0.12).

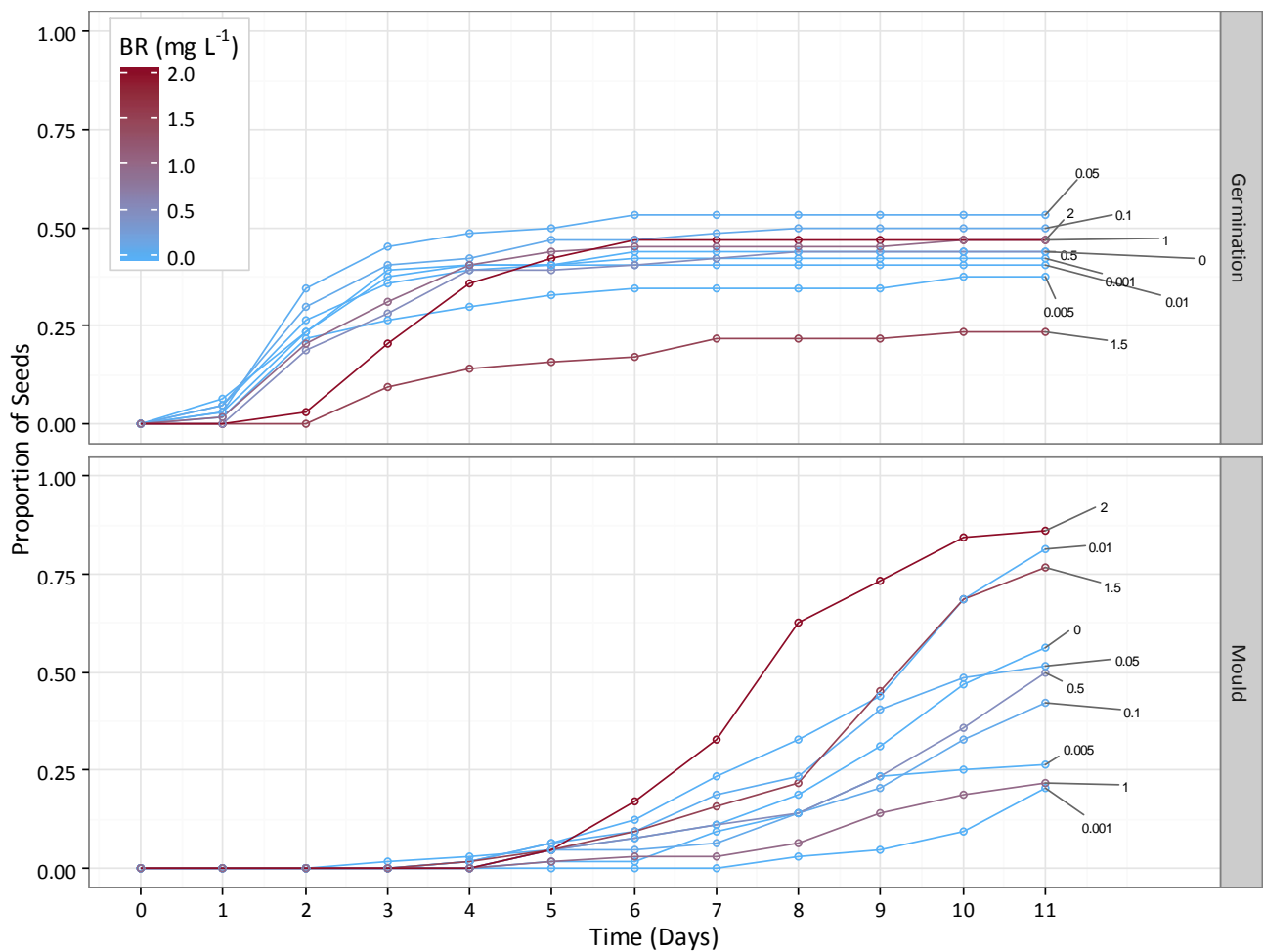


Figure 4-23: The proportion of mould and germination for each concentration of BR over 11 days. The lines are coloured by the concentration of BR.

The mould appears to have no clear trend with 2 mg L^{-1} producing 91.7% mould and 1 mg L^{-1} producing 22.3% mould (Figure 4-23). This is supported by an

ANOVA where the percentage of mould did not change significantly with BR concentration ($P = 0.58$).

In Figure 4-23 only 22% of seed germinate at 1.5 mg L^{-1} ; however, this result appears to be an outlier, because all other dishes' germination is between 41% and 53% regardless of BR concentration. An ANOVA confirms that there was no significant relationship between the proportion of seeds germinated and BR ($P = 0.44$). GI when calculated behaves the same, all results are between 2.8 (0.005 mg L^{-1}) and 4.5 (0.05 mg L^{-1}) except 1.5 mg L^{-1} at 1.4. BR also has no significant effect on GI when tested with an ANOVA ($P = 0.15$).

4.4b EXPERIMENTS ON PHYSICAL STRESSES AND PRE-TREATMENTS

Below are the results for the effects of water stressing, priming, and pre-chilling of seed prior to and during the germination test.

SALT (NaCl)

During the range finding test using NaCl, it was found that the effect of salt on germination percentage is significant when tested with and ANOVA ($P < 0.001$), and has a strong negative correlation (PPMC, -0.89). Germination percentage only visibly lowers at -1.2 MPa (242 mM) (Figure 4-24).

Germination time appears to increase below -0.1 MPa (20 mM) (Figure 4-25). This is a small effect and larger changes in germination time are not seen in either Figure 4-24 or Figure 4-25 until less than -1 MPa (201 mM). A regression analysis of germination time against salt concentration with a Kruskal-Wallis rank sum was significant ($P < 0.0001$). Germination time increased, with a weak positive correlation of 0.34, produced using a Kendall's rank.

GI showed similar outcomes to germination, with a significant ANOVA result of $P < 0.001$ and a strongly negative PPMC (-0.87). The proportion of seed germinated at the end of the test drops approximately 12.8% per MPa.

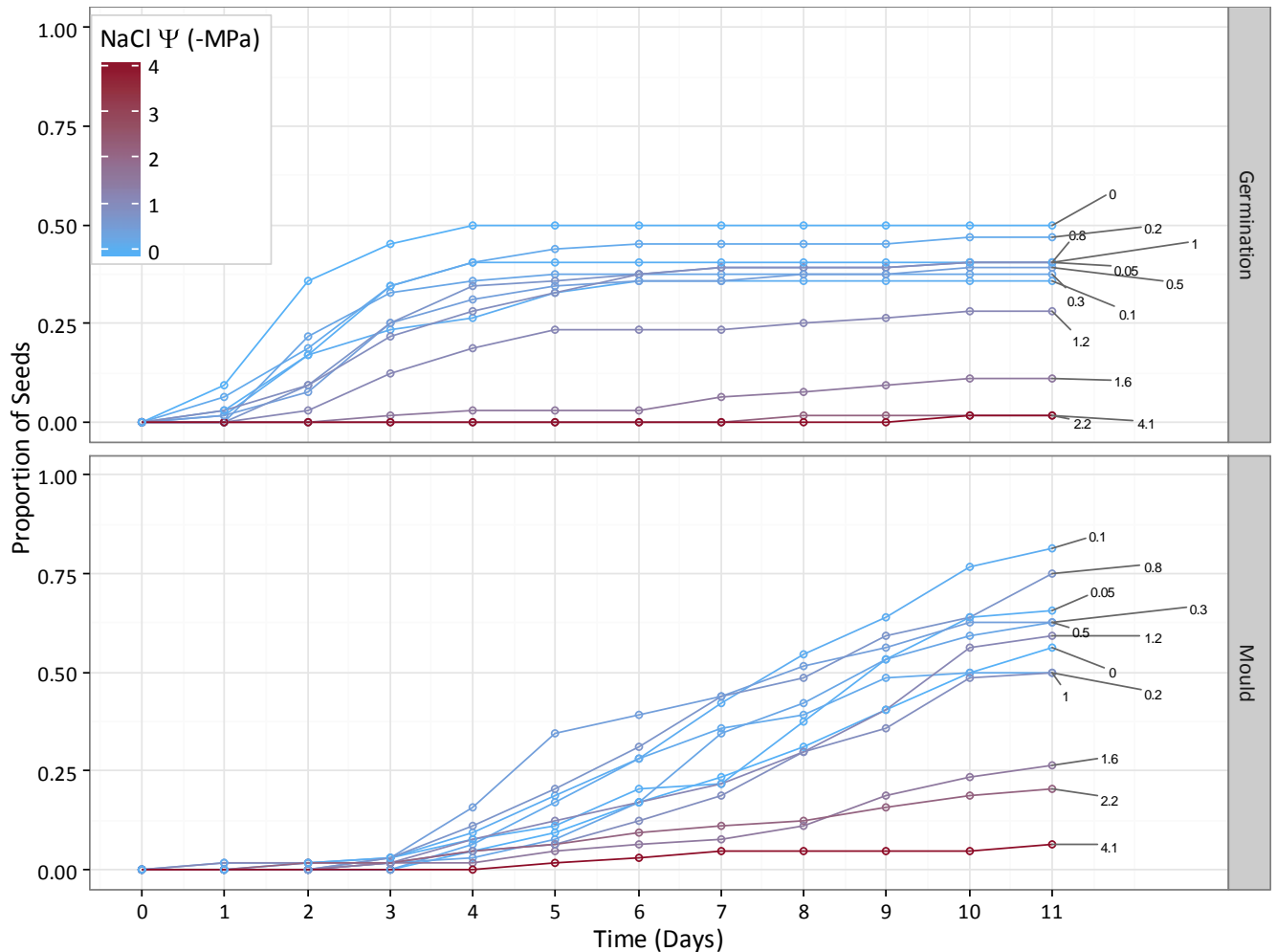


Figure 4-24: Germination and mould over time, traces represent proportion of seeds germinated. Water stress for each trace is represented by colour and annotation. These are not mean values and just show a trend.

The time required for mould to take hold is also increased by the higher salt concentrations (Kruskal-Wallis rank sum, $P < 0.01$) (Figure 4-24). While peak mould proportion is at -0.1MPa (Figure 4-24), and ANOVA showed a significant result ($P < 0.001$), and the proportion of seeds with mould was strongly negatively correlated with level of NaCl (PPMC, -0.86).

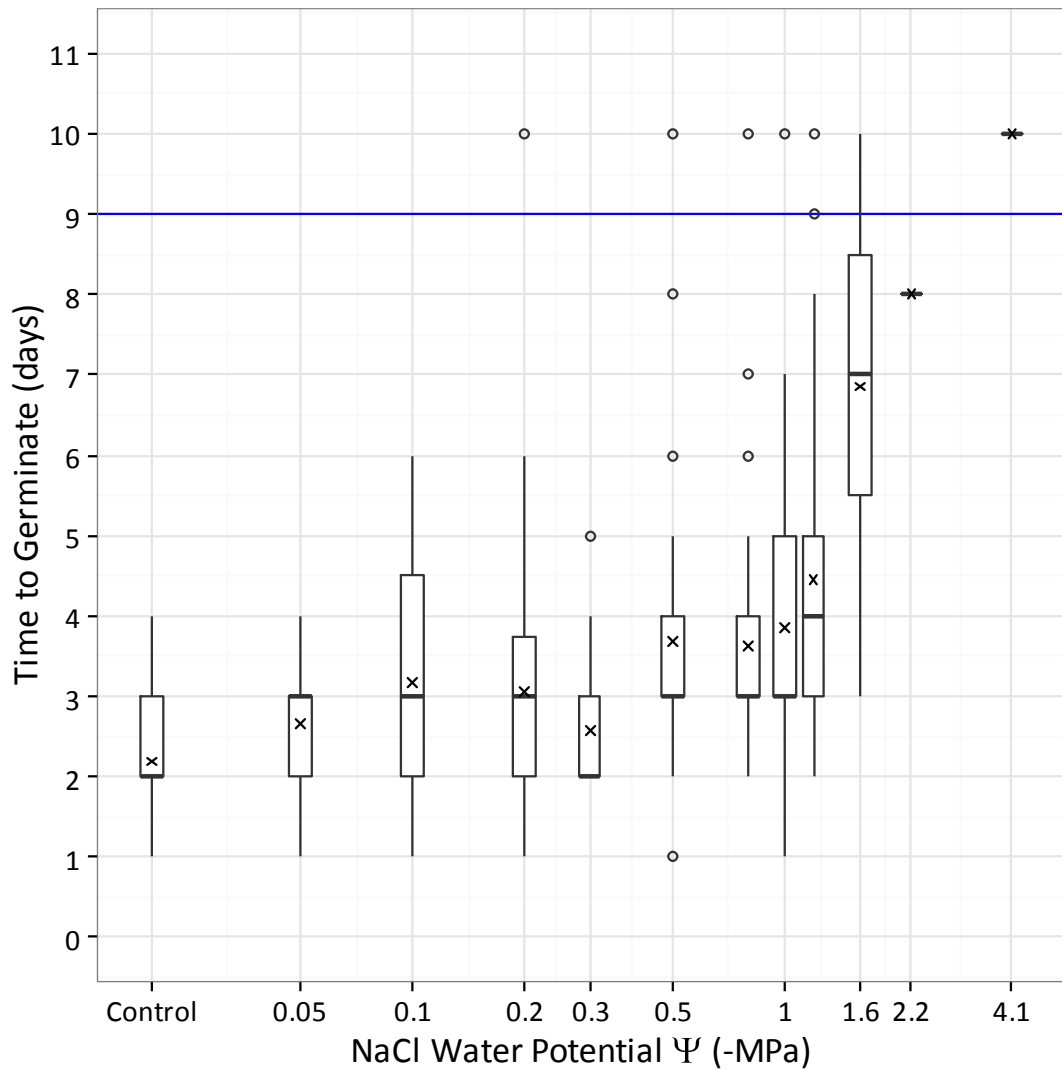


Figure 4-25: Number of days for each seed to germinate while in a solution of NaCl (\log^{10} x-axis), the horizontal line represents the day extra SDW was added. Each box represents a dish (up to 64 germinated seeds). The control has been added on the left.

The higher salt concentrations, at -2.2 and -4.1MPa (444-827 mM), have little germination as seen in Figure 4-24, even after water is added on day 9 (also visible in Figure 4-25).

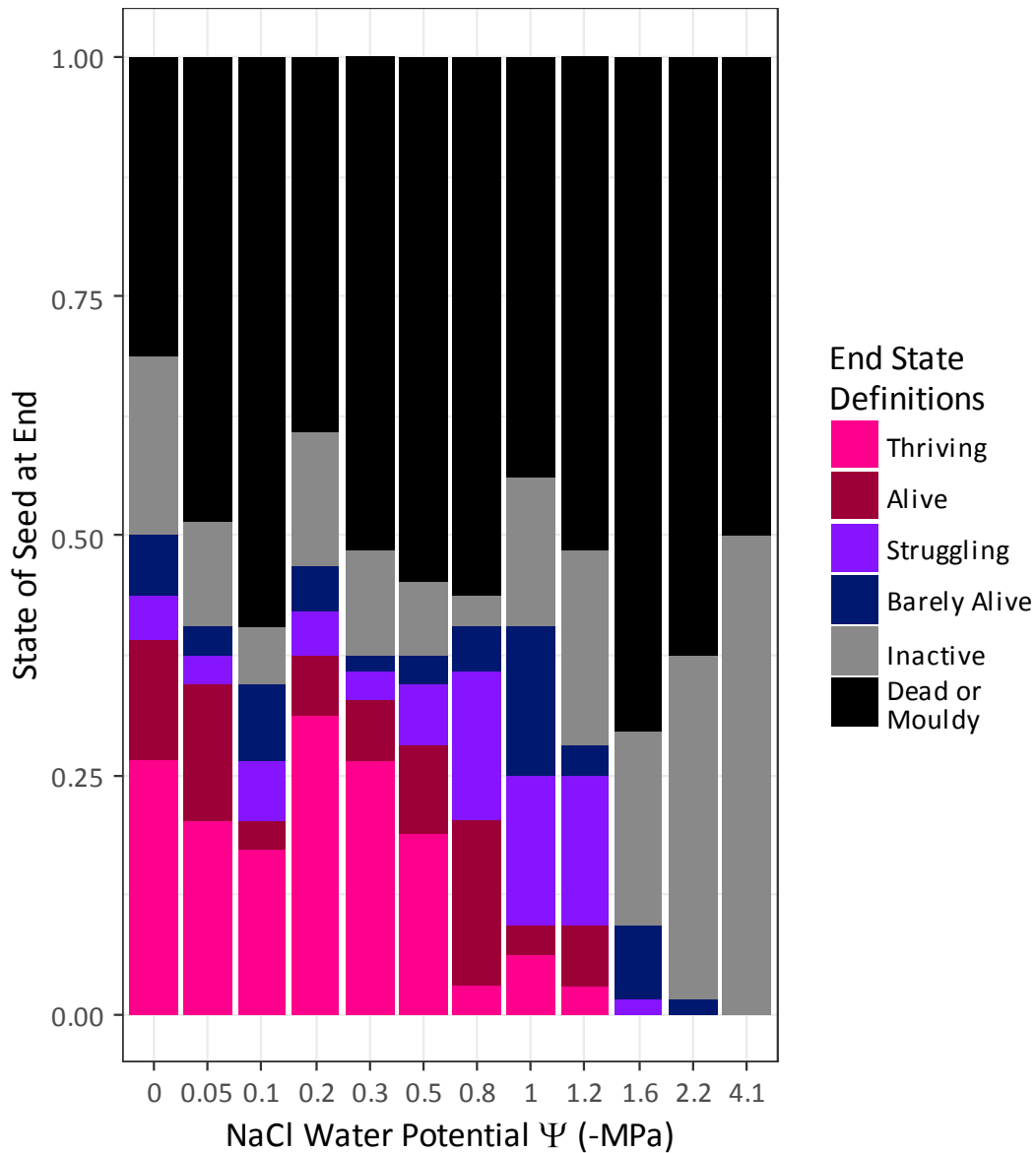


Figure 4-26: Colour coded stacked histogram representing the proportion of seed at each end state as assessed visually and by chlorophyll fluorescence at the end of the 11 day experiment.

The x-axis is categorical, representing the concentrations of salt in MPa.

The effect of lower concentrations of salt on the state of the plants appeared to have little effect on the proportion of 'Thriving' seedlings until -0.8 MPa (161 mM) when final seed states started to drop (Figure 4-26). There were also still 9.4% of seedlings 'Struggling' (one seedling) or 'Barely Alive' (five seedlings) at 1.6 MPa.

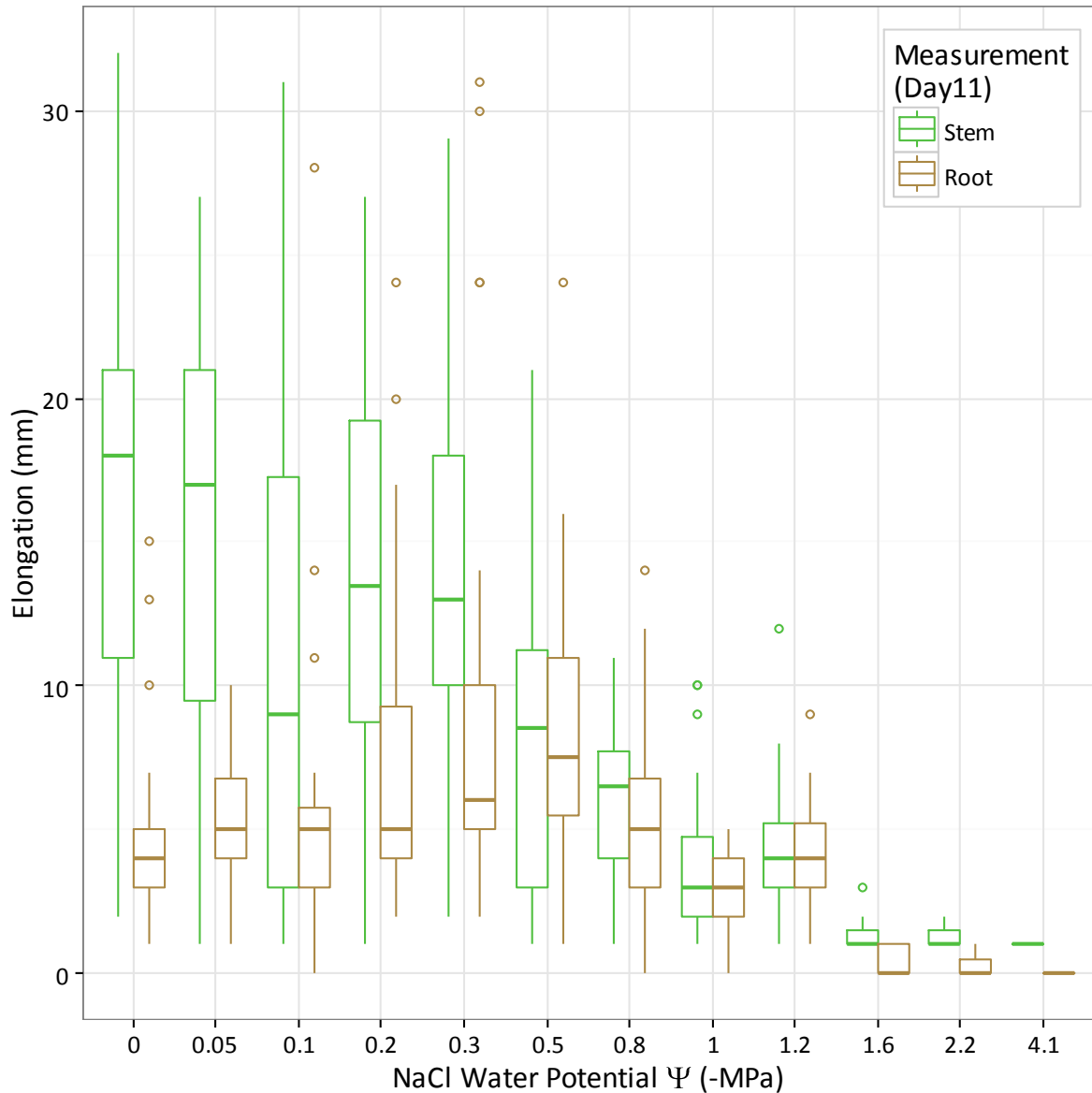


Figure 4-27: Root and stem elongations for seedlings grown under a range of NaCl concentrations, as measured on the last day of the experiment, excluding un-germinated seed. Each dish up to 64 seedlings is represented by two boxplots, one for root elongations, and one for stem elongations. The range of NaCl concentrations has been drawn on a categorical scale.

Stem elongations did not follow a normal distribution, so they were square root transformed. Stem elongations decreased with salt when this data was tested with a one-way ANOVA ($P < 0.0001$), this had a correlation of -0.58 when a PPMC was calculated. Figure 4-27 shows stem elongation linearly decreasing with the nonlinear x-axis (Ψ). Before -0.8 MPa (161 mM) the increase in salt had

a positive effect on root elongation (Figure 4-27), after that the effect is negative in line with the effect of salt on stems. Due to this there is a small negative Kendall's rank correlation of root length with Ψ (-0.16), with a significant Kruskal-Wallis rank sum of $P < 0.0001$.

POLYETHYLENE GLYCOL

Stem elongations, and to a lesser extent root elongations, are clearly seen declining with water potential (Ψ) as induced by PEG (Figure 4-28). This starts at 0.05 MPa in both cases but decreases more rapidly after a Ψ of 0.3 MPa for stem elongation and after a Ψ of 1.2 MPa for root elongation. These trends were tested with four Kruskal-Wallis rank sums. Roots in PEG 4000 were significantly affected by Ψ ($P < 0.001$), while roots in PEG 8000 were not ($P = 0.059$). Stems in both PEG 4000 and 8000 were significantly affected ($P < 0.01$, $P < 0.001$). Two extra Kruskal-Wallis rank sums were used to test the overall difference in elongation between the PEG 4000 and 8000, for both roots and stems; both of these tests were significant ($P < 0.001$ & $P < 0.0001$ respectively). In both cases, seeds were more affected by the PEG 8000 than the PEG 4000, as seen in the more consistent drop for PEG 8000 in Figure 4-28.

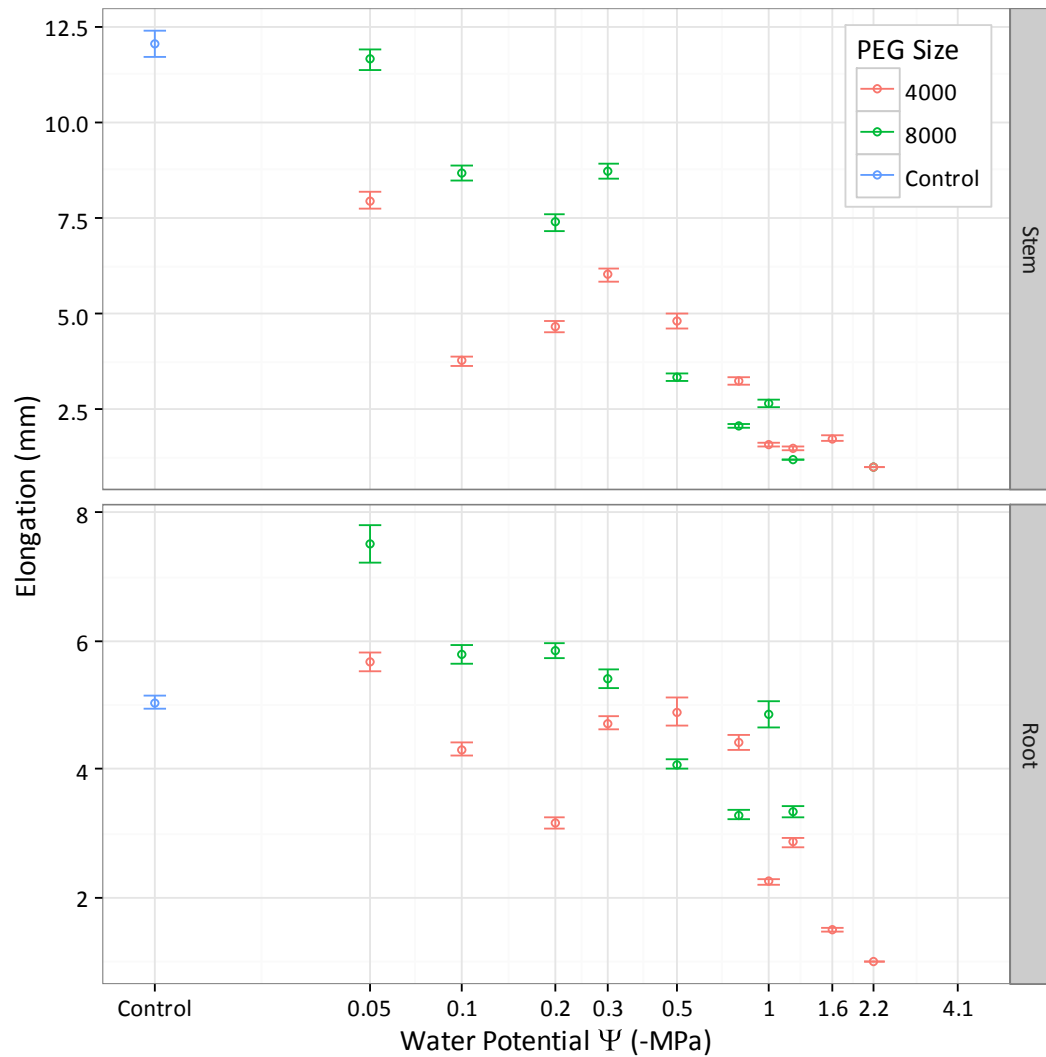


Figure 4-28: The elongations of the stems and roots at the end of the experiment (on day 11). Standard error bars have been added around each mean for the elongation of seeds that germinated (up to 64).

Figure 4-29 demonstrates the sharper decrease of seedling stem elongations in PEG 8000. This effect is exaggerated by the increase in ‘Thriving’ and ‘Alive’ seedlings at a Ψ of -0.05 MPa in PEG 8000. PEG 8000 also does not have any living seedlings at the end of the test in a Ψ of less than or equal to -1.6 MPa, whereas PEG 4000 has living seed down to a Ψ of -2.2 MPa (Figure 4-29).

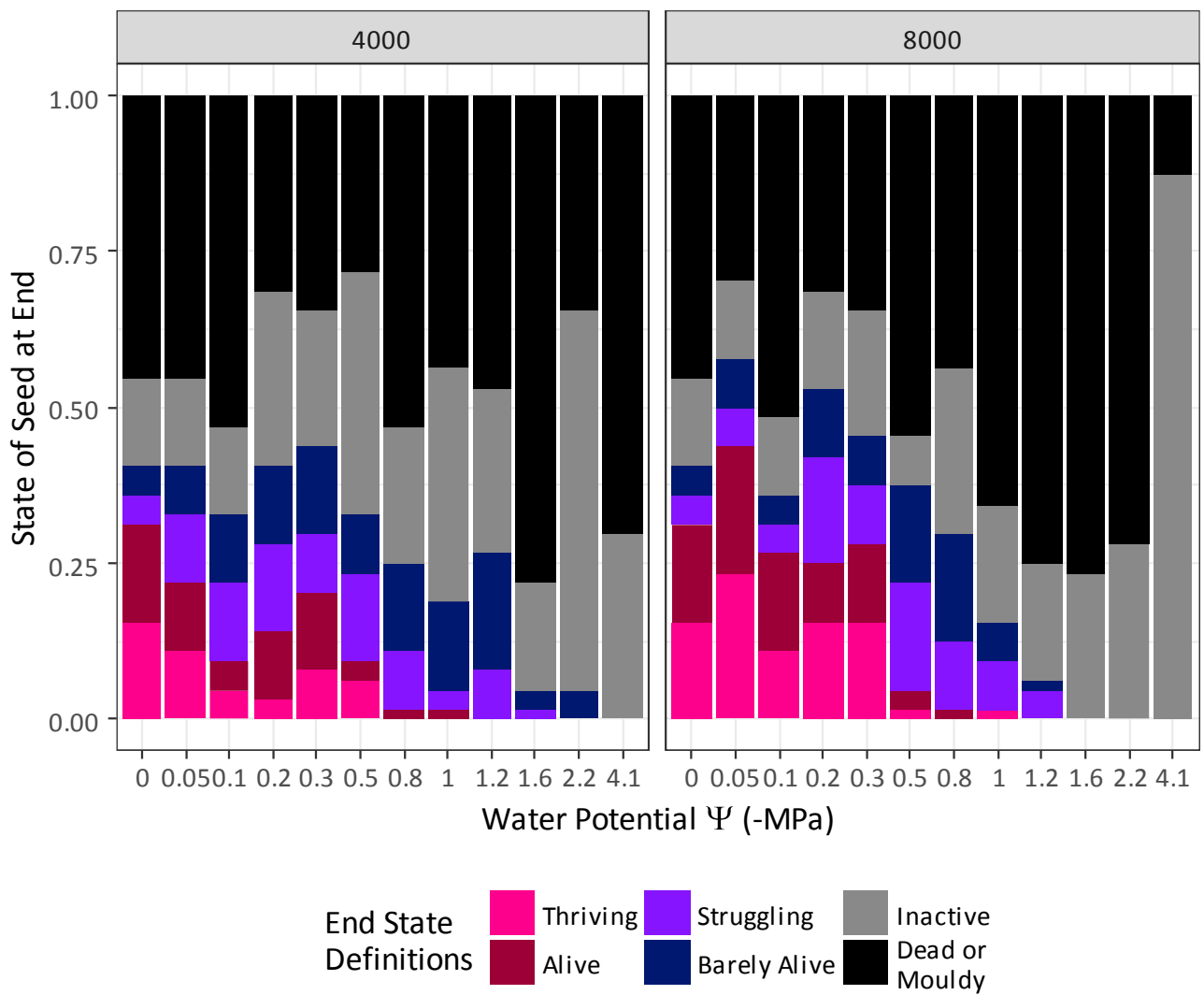


Figure 4-29: Colour coded stacked histogram representing the proportion of seed at each end state as assessed visually and by chlorophyll fluorescence at the end of the 11 day experiment. Both PEG sizes are shown separately over the same water potentials (MPa). The x-axes are categorical.

A two-way ANOVA was performed to test the effect of PEG size and Ψ against the proportion of mouldy seeds. The total proportion of mouldy seeds did not significantly differ between PEG 4000 and 8000 ($P = 0.44$). The Ψ did have a significant effect ($P < 0.01$) on the proportion of mouldy seeds in the same test. This effect was negative with less mouldy seed when Ψ was higher, as seen in Figure 4-29. PEG 8000 has a consistent level of 'Thriving' seed until -0.3 MPa. PEG 4000 has a slowly dropping level of 'Thriving' seed until -0.5 MPa. PEG

4000 had over a quarter of seeds as living seedlings at -1.2 MPa, whereas PEG 8000 dropped below a quarter at -0.8 MPa (Figure 4-29).

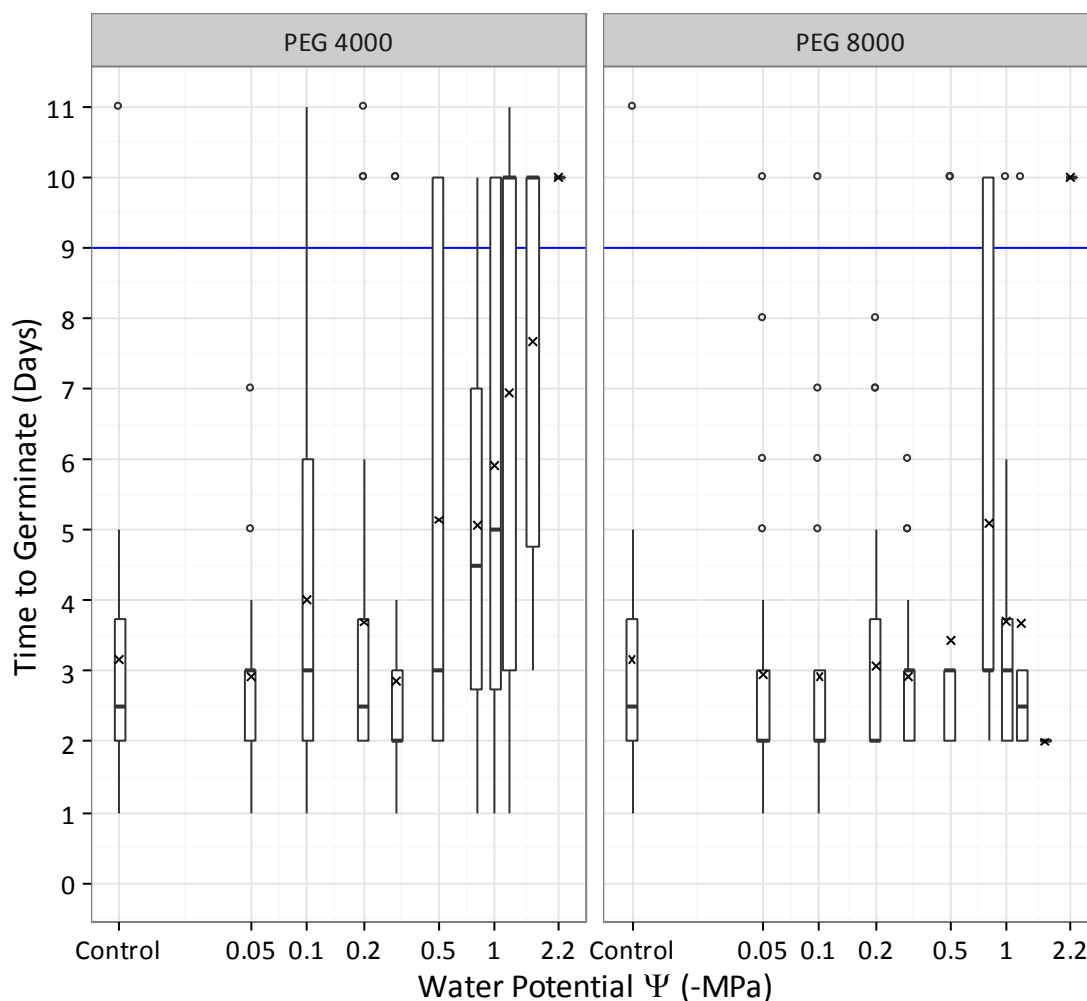


Figure 4-30: The time seed germinated at in the PEG solutions, with Ψ shown on a \log^{10} scale. The two PEG sizes are shown separately. The horizontal line represents when water was added. Boxes show the variation in germination time between seeds (up to 64). There were no germination time results for -4.1 MPa.

Time taken for seeds to germinate rises sharply around -0.5 MPa for both PEG 4000 and 8000 (Figure 4-30). The difference in germination time between the PEG types was tested with a Kruskal-Wallis rank sum, which resulted in a significant difference ($P < 0.01$). Seed in PEG 4000 germinated more slowly and over an inter-quartile range of 2 to 6 days, compared to PEG 8000, where they

germinated over an inter-quartile range of 2 to 3 days. When tested with Kruskal-Wallis rank sums, PEG 4000 showed a significant change in germination time over the levels of Ψ ($P < 0.0001$), while PEG 8000 did not ($P = 0.13$).

Time taken for seeds to become mouldy (Figure 4-31), was tested for the two types of PEG against levels of Ψ with a Kruskal-Wallis rank sum. This resulted in a significant effect on the rate of mould visibility for PEG 8000 ($P < 0.001$) but not for PEG 4000 ($P = 0.4$). However, when time taken to become mouldy was tested between PEG 4000 and 8000 there was no overall difference between the two sides of the experiment (Kruskal-Wallis rank sum, $P = 0.79$). The mould percentage peaks at -0.05 MPa in PEG 4000, and at -0.1 MPa in PEG 8000.

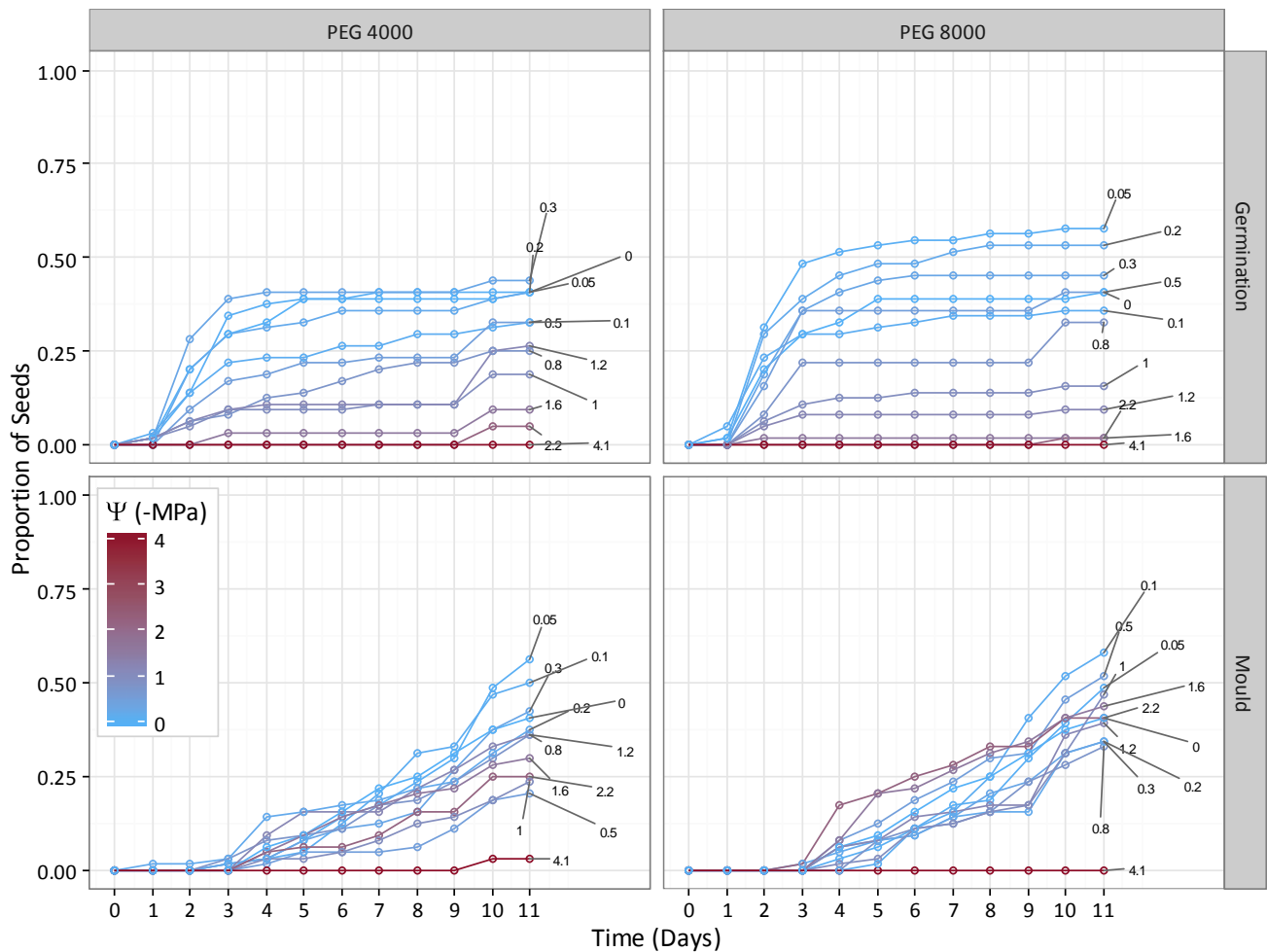


Figure 4-31: Germination and mould of seeds over the 11-day experiment. PEG 4000 and 8000 are separated for clarity, and the water potentials of PEG the seeds were grown at are coloured to show trends.

A two-way ANOVA was used to analyse the effect of PEG size and Ψ against total proportions of seed germinated. The total proportions of seed that germinated during the experiment were not significantly affected by the type of PEG ($P = 0.45$), but were significantly affected by the Ψ ($P < 0.001$). However, seeds at levels of -1.6 and -2.2 MPa had a germination proportion of ~ 0.1 in PEG 4000 and ~ 0 in PEG 8000 (Figure 4-31). The comparable trends of PEG 4000 and 8000 are not clearly represented in Figure 4-29 and are better seen in Figure 4-31, because end states do not show seed that germinated then died. GI follows the same pattern as proportion of seeds germinated; when tested with a

two-way ANOVA there was a significant difference in GI by Ψ ($P < 0.01$) but not by type of PEG ($P = 0.44$).

COLD STORAGE

The effect of cold storage on seed germination and mould was tested using two sets of seed: Freshly threshed seed and seed stored at 5°C for two weeks. When tested using a t-test neither mould nor germination were significantly affected by cold storage at the chosen time points, with a value of $P = 0.15$ for germination at 7 days and $P = 0.06$ for mould at 15 days (Figure 4-32).

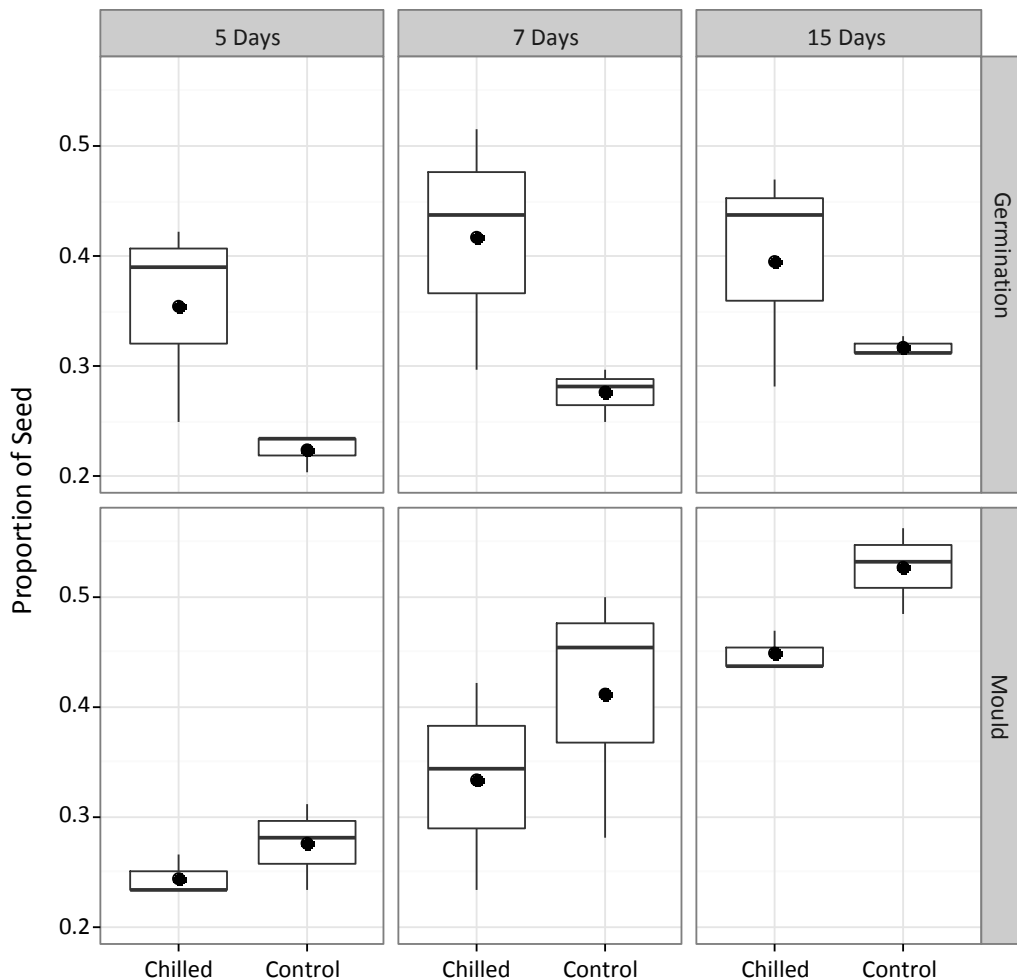


Figure 4-32: Mould and germination as a proportion of seeds, over three time points. Each box-plots shows the variation within the three replicates of 64 seed cooled at 5°C for 2 weeks and within the replicates of seed threshed and not chilled. Dots show the means.

7 days germination and 15 days mould were chosen in the methods as the key time points for measurement. Figure 4-32 shows that 7 days germination and 15 days mould were the clearest examples of mould and germination results, showing the least overlap of range, which supports this choice. The germination variance is greater in chilled *Miscanthus* seed at all time points (Table 4–2).

Table 4–2: Standard deviation in germination between the three replicates for chilled and control seed across three times.

	Day 5 germination (SD)	Day 7 germination (SD)	Day 15 germination (SD)
Treatment			
Chilled	5.86	7.09	6.43
Control	1.15	1.53	.58

STRATIFICATION

Shown in Figure 4-33, wet only stratified seeds' end (day 11) stem elongations (averaged across all wet stratified dishes) appear to be consistently higher (mean of 8.3 ± 0.27 mm [SD]) than those in the control dish (6.6 mm). The dried stratified seed appear to have a similar increase in stem elongation when stratified for one to seven days (mean of 8.3 ± 1.4 mm [SD]), yet only reach at 7.8 mm at 14 days. However, when tested with a two-way ANOVA on stratification levels and drying regimes (control, dried, & wet only), there was no significant difference between the levels of stratification ($P = 0.07$) and there was none between the regimes ($P = 0.33$).

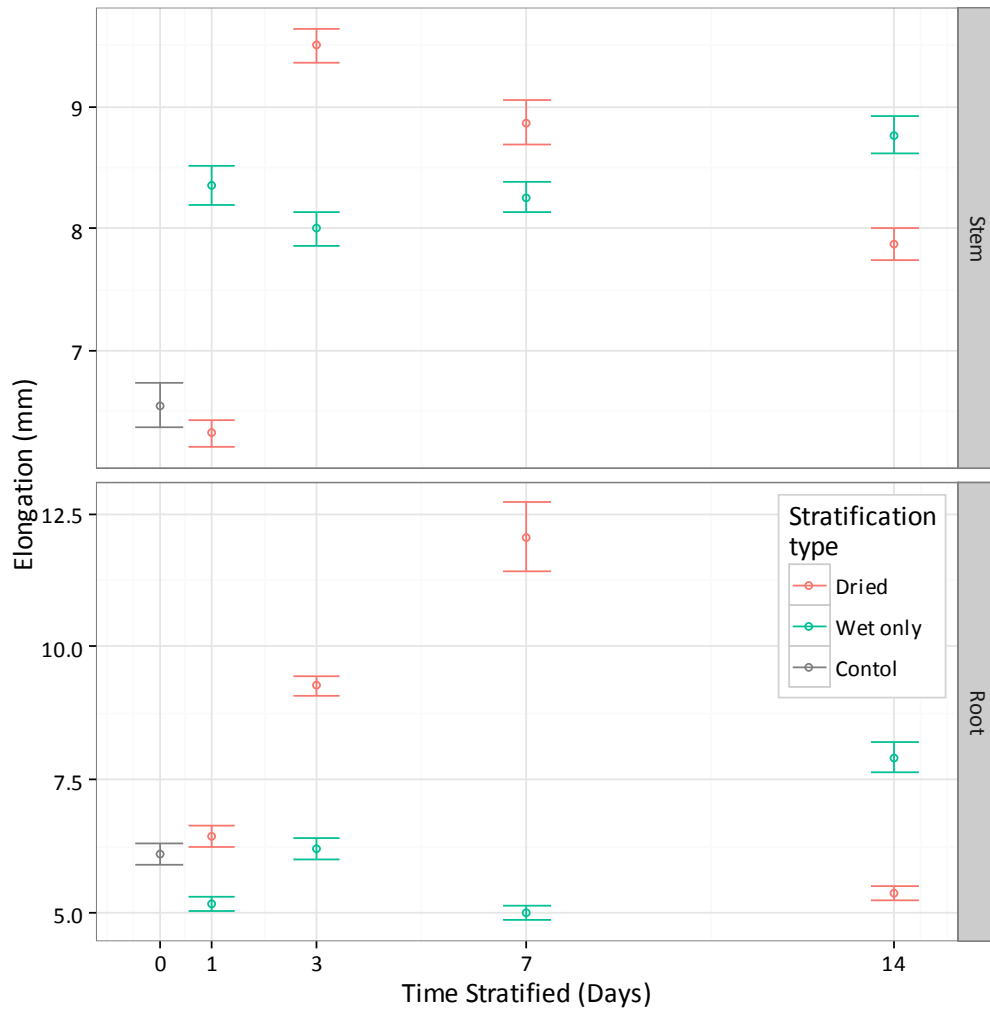


Figure 4-33: Root and stem elongations after wet and dried stratification at 5°C over four time points, plus control. Standard error bars have been added to show the differences between individual seedlings (up to 64 seeds).

The root data appears in Figure 4-33 to be more mixed. The control dish has a mean root elongation of 6.1 mm, with the mean root elongation of wet only also being 6.1 ± 1.2 mm [SD], whereas for dried seed this was 8.3 ± 2.6 mm [SD]. The root data was not transformable to a normal distribution; therefore, the levels of stratification were tested with two Kruskal-Wallis rank sums for wet only and dried regimes, both including the control as 0 days in that regime. This gave a result of $P = 0.47$ for wet only and $P = 0.07$ for dried. Another Kruskal-

Wallis rank sum found significance when testing for a root elongation difference between the three regimes ($P < 0.05$).

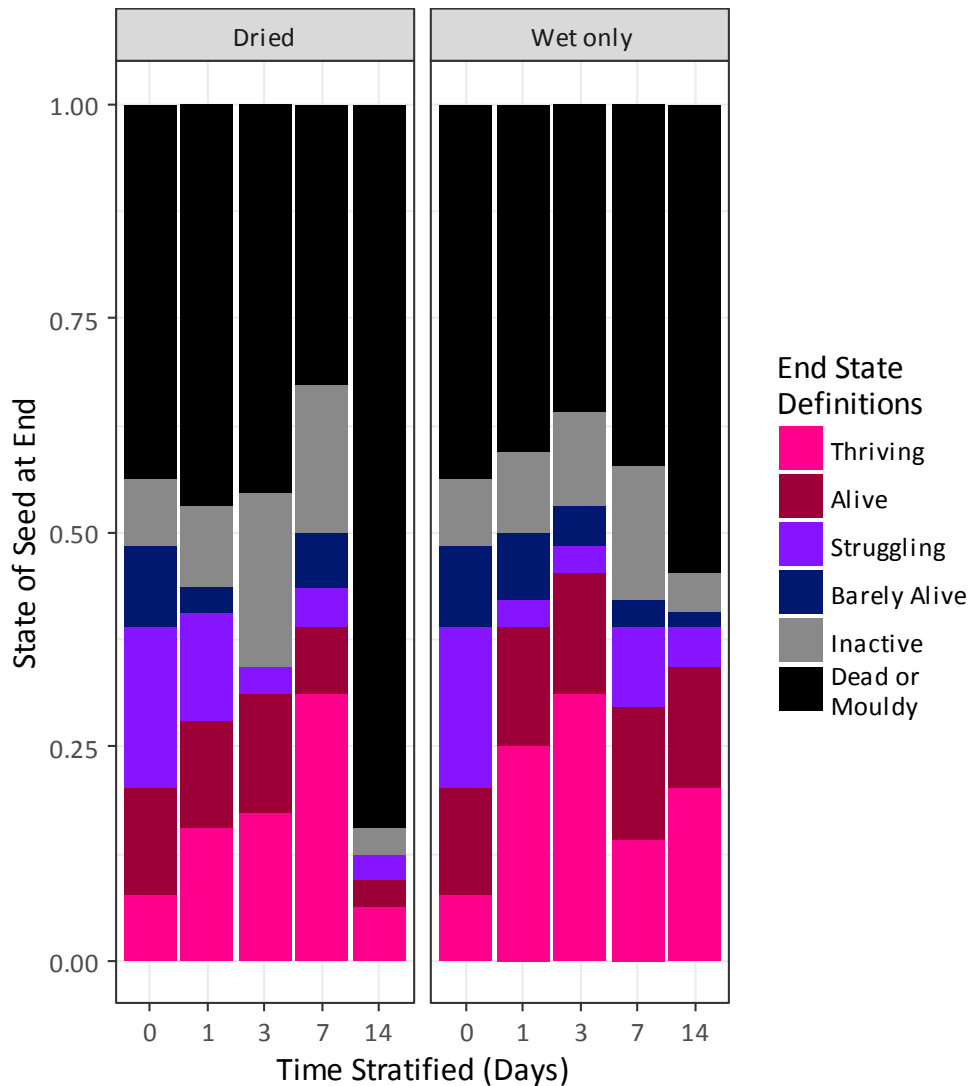


Figure 4-34: The proportions of seed in each end state of dead to thriving, as assessed visually and using chlorophyll fluorescence, at the end of the test (day 11). Seed have been separated into columns by how long the seed was stratified for and separated into seed that was dried back and seed that was not. Control (0 days stratified) seed has been placed in both panes for convenience.

With the exception of seed dried after 14 days of stratification all the stratified seed plates had higher proportions of 'Thriving' seed than the control, a median of 16.4% for dried and 20.3% for wet only compared to 7.8% in the control (Figure 4-34). The total proportion of mould when tested with a two-way ANOVA

was not significant for either level or regime, with results of $P = 0.16$ and $P = 0.99$ respectively.

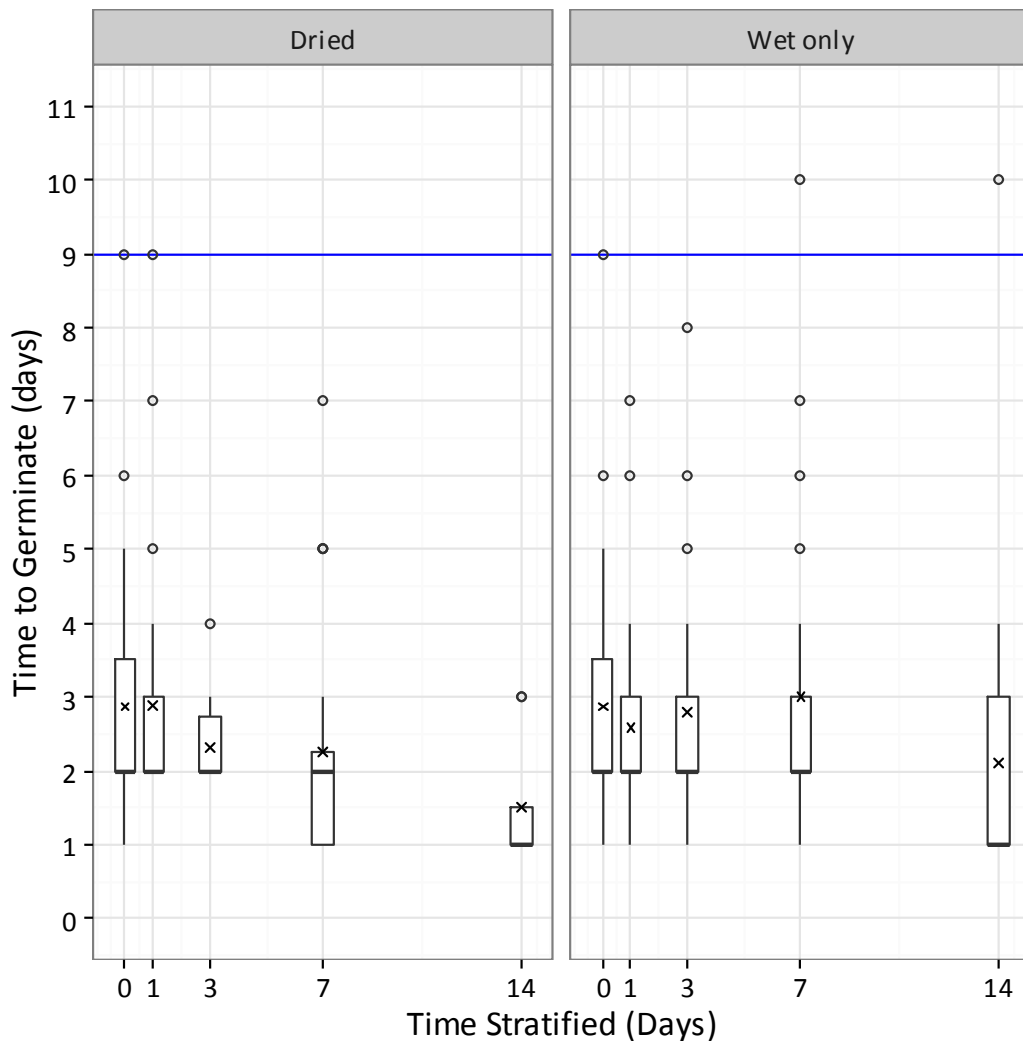


Figure 4-35: A boxplot of the time seeds took to germinate; each box represents the variation between all seeds stratified for that amount of time. The horizontal line represents day nine where extra SDW was added. Control (0 days stratified) seed has been placed in both panes for convenience.

Time for seeds to germinate (Figure 4-35) was not significantly different between dried, wet only, and control groups when tested with a Kruskal-Wallis rank sum ($P = 0.26$). However, as seen in Figure 4-35 the time required for seeds to germinate did decrease with stratification time, when tested with a Kruskal-

Wallis rank sum this was significant for both wet only ($P < 0.05$) and dried seed ($P < 0.01$).

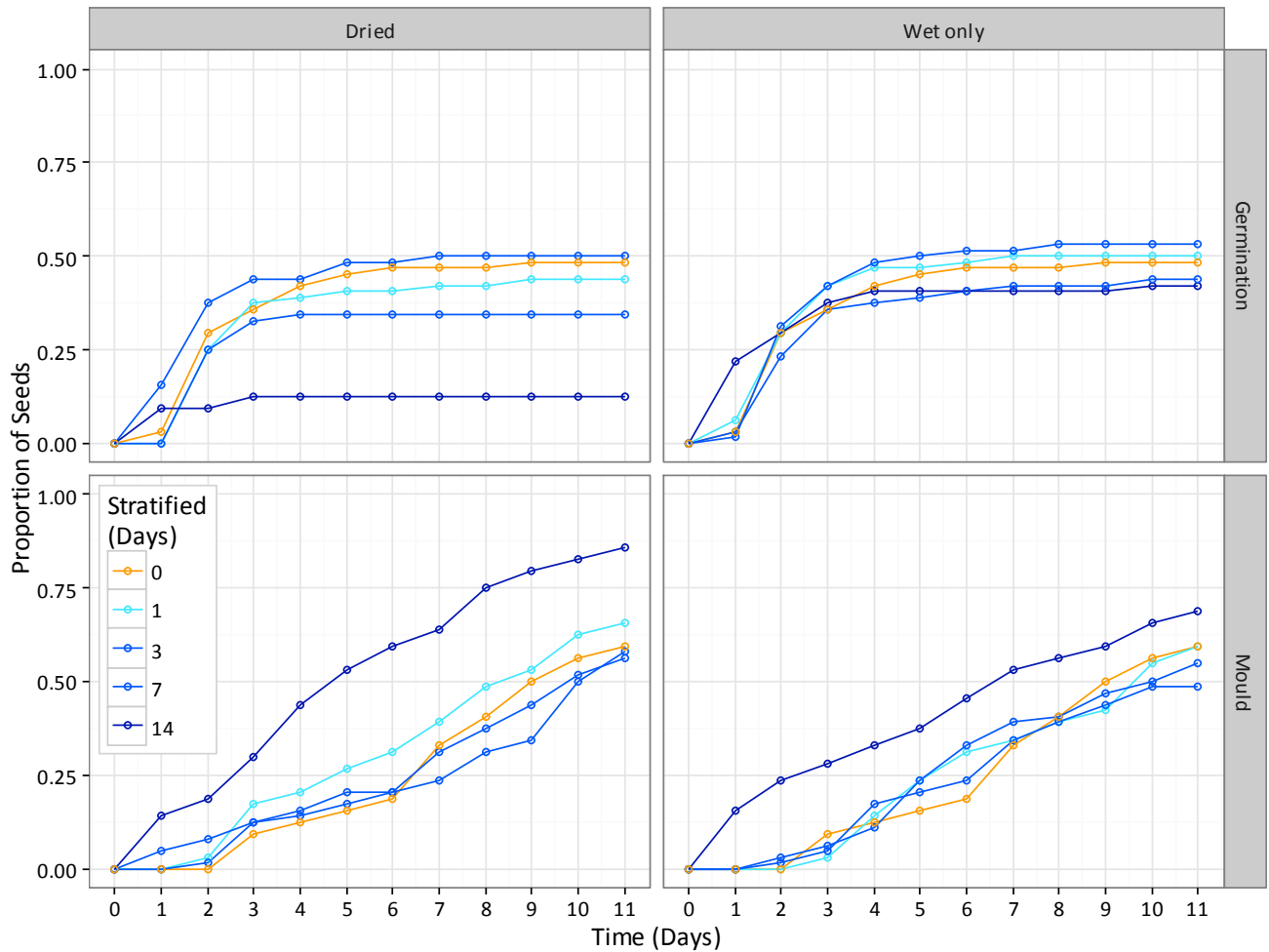


Figure 4-36: Proportion of seed that germinated and/or became mouldy over time. Separated into panes for seed that was dried back and seed that was not, and coloured for time spent stratifying.

The same reduction in time required to germinate was seen for the seed that went mouldy. There was no significant difference between wet only, dried, and control for how quickly mould took hold (Kruskal-Wallis rank sum, $P = 0.21$); however, when tested with a Kruskal-Wallis rank sum there was a significant difference between levels of stratification for both wet only ($P < 0.05$) and dried seed ($P < 0.01$). In Figure 4-36, the proportion of mould can be seen to increase

faster at 14 days of stratification on seed that were dried back, and to a lesser extent 14 days wet only stratification.

The total proportion of seeds germinated (Figure 4-36) only appears noticeably lower for 14 day stratified seed that were dried. A two-way ANOVA was used to test the effect of time stratified and regime (wet only and dried) on germination proportion. No significant relationship was found for time stratified ($P = 0.38$) or regime of stratification ($P = 0.78$). GI behaves the same as germination proportion, which was also tested with a two-way ANOVA and resulted in a value of $P = 0.45$ for length of stratification and $P = 0.78$ for regime.

These results show that germination was not affected by stratification, but root growth, stem growth, and seedling health were, particularly for seed that were dried back. However, these benefits for dried seed were lost at 14 days stratification.

PRIMING

The results that were obtained from the preliminary priming experiment are outlined in Table 4-3. Stem elongation is the only significant result; control (not primed) seed produced longer stems at 17.1 mm compared to 13.3 mm for primed seed (Table 4-3).

Table 4–3: Primed seed vs control, tested using the standard germination technique outlined in section 4.2b. The statistical test used is included with significance symbols where necessary. Proportion of seed germinated and percentage of mould are single scores with no repetition and thus do not have associated stats.

	Control	SD	Primed	SD	Statistic
Proportion of seeds germinated	.5		.344		
Proportion of seeds mouldy	.563		.688		
Time to germinate (days)	2.19 ± .86		2.59 ± .96		Kruskal-Wallis
Time to become mouldy (days)	7.86 ± 2.17		7.11 ± 2.31		Kruskal-Wallis
Final stem elongation (mm)	17.10 ± 7.63		13.29 ± 8.89		*** t-test
Final root elongation (mm)	4.90 ± 3.11		4.90 ± 6.32		Kruskal-Wallis

Priming seed was further investigated and the results for the second priming experiment to test over time fluorescence and end growth are below (Figure 4-37).

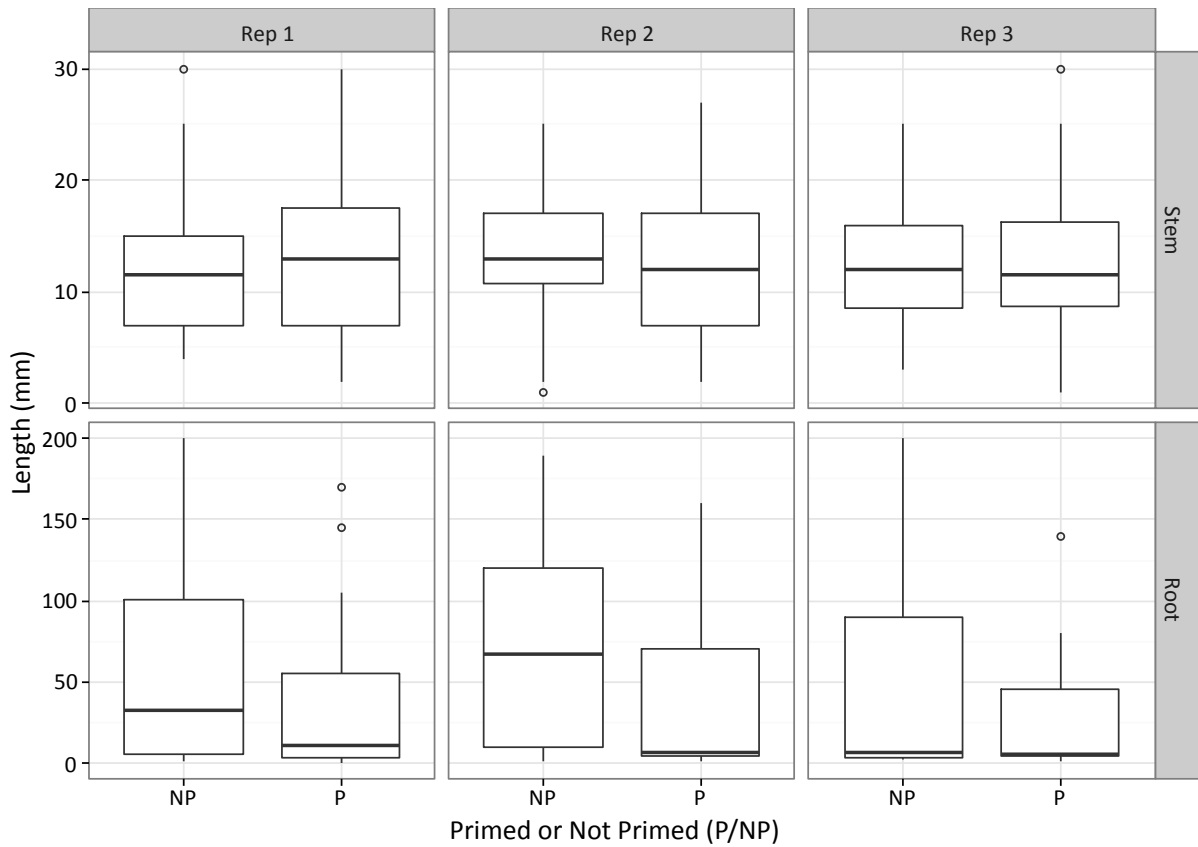


Figure 4-37: Boxplot of the root and stem elongations of the germinated seed out of the 50 seed sown in the petri dish of SYN55 (NP) and primed SYN55 (P) after 37 days sown on a petri dish. All three replicates are shown separately.

The primed and not-primed mean root elongations were significantly different when tested with a t-test ($P < 0.05$). The stem elongations in each replicate were not normally distributed and therefore were tested with a Kruskal-Wallis rank sum, finding no significant difference ($P = 0.83$). Number of seeds germinated in each section was also not normally distributed and was not significantly different when tested with a Kruskal-Wallis rank sum ($P = 0.18$).

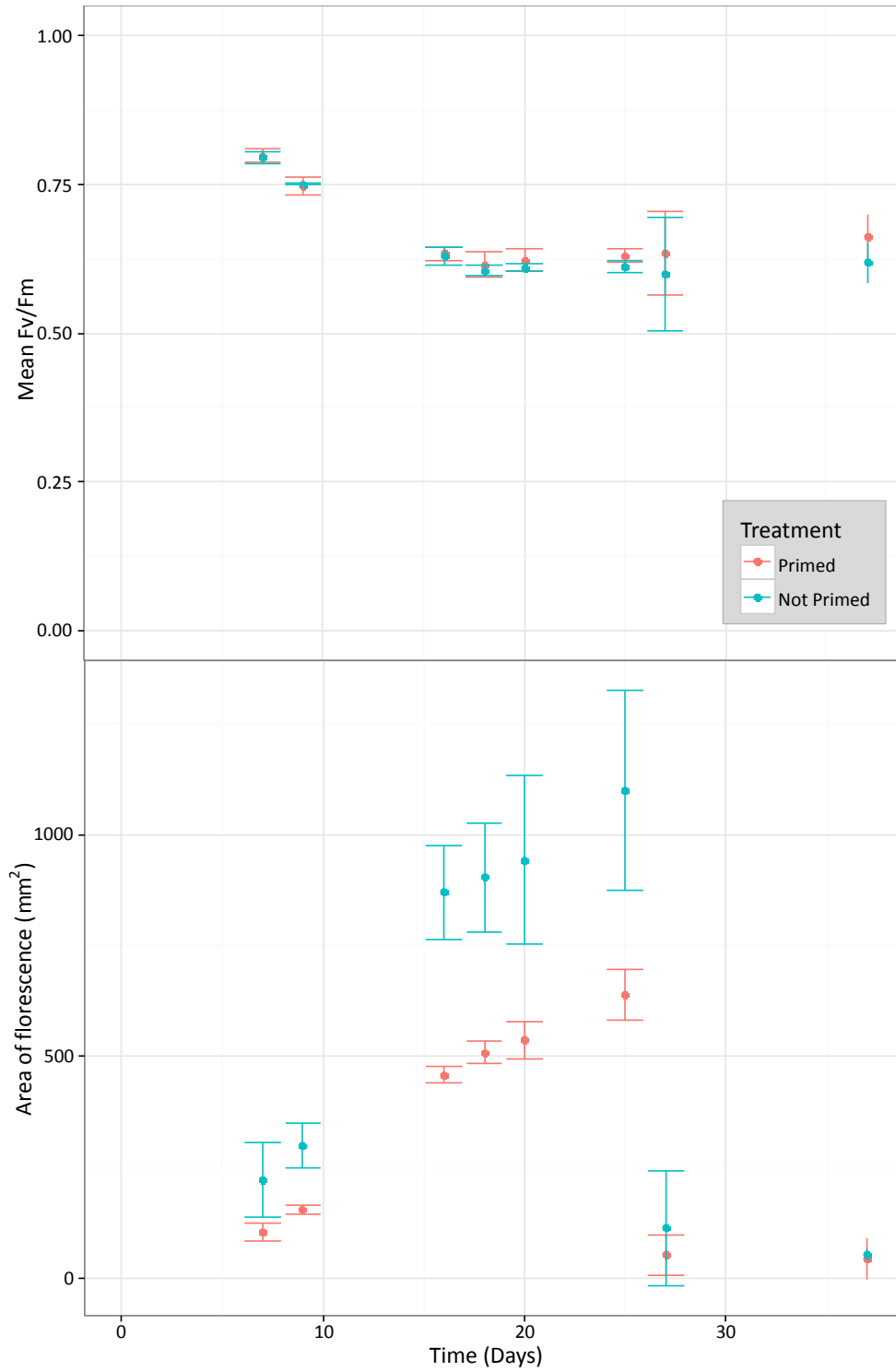


Figure 4-38: *Fv/Fm* and florescence area results for primed seed tested side by side on the same plants and imaged over 37 days. Standard deviation error bars have been added, representing the error between the three replicates.

The mean F_v/F_m dark-adapted chlorophyll response was significantly higher in primed seed when tested using a Wilcoxon signed rank ($P < 0.01$) (Figure 4-38). A Wilcoxon signed rank was used because the data was not normally distributed and the primed and control readings at each time point needed to be paired. The mean total fluorescence area was tested with a t-test and was significantly different between primed and not primed seed ($P < 0.01$) indicating not-primed seed had a larger fluorescence area (Figure 4-38).

4.4C LIGHT AND DARK

The results for the effect of light on seed germination, how long the seed can grow without light, and how the size of the seed affects this, are set out below.

SEED MASS MODEL

Modelling seed mass using MARVIN was impractical because the results were too clustered. The largest seed lot and the smallest seed lot were at the top and bottom; however, the others were indistinguishable (see Figure 4-39).

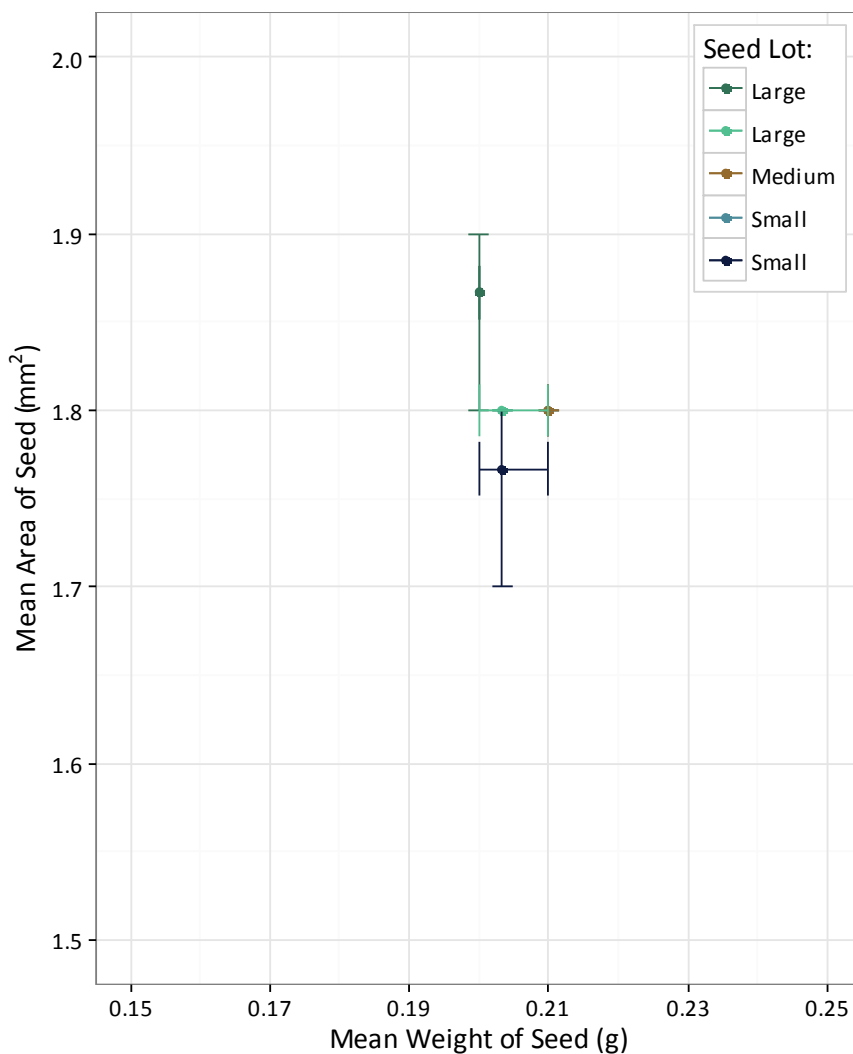


Figure 4-39: Chart of the average repeated weights and measures of the five differently sized seed batches of SYN55, using MARVIN. Error bars represent the minimum and maximum values of the repeated measures demonstrating the problems with MARVIN and variable seed. The scale has been increased to make the clustering of the data apparent.

The alternative, manual testing method (Section 4.3c) did reveal differences between SYN55 and MX300 and the seed were modelled separately (Figure 4-40), producing R^2 results of 0.96 and 0.87 respectively (Figure 4-40). This model was then used on seed for the 'Dark Burnout' experiment below to estimate the masses of the seed.

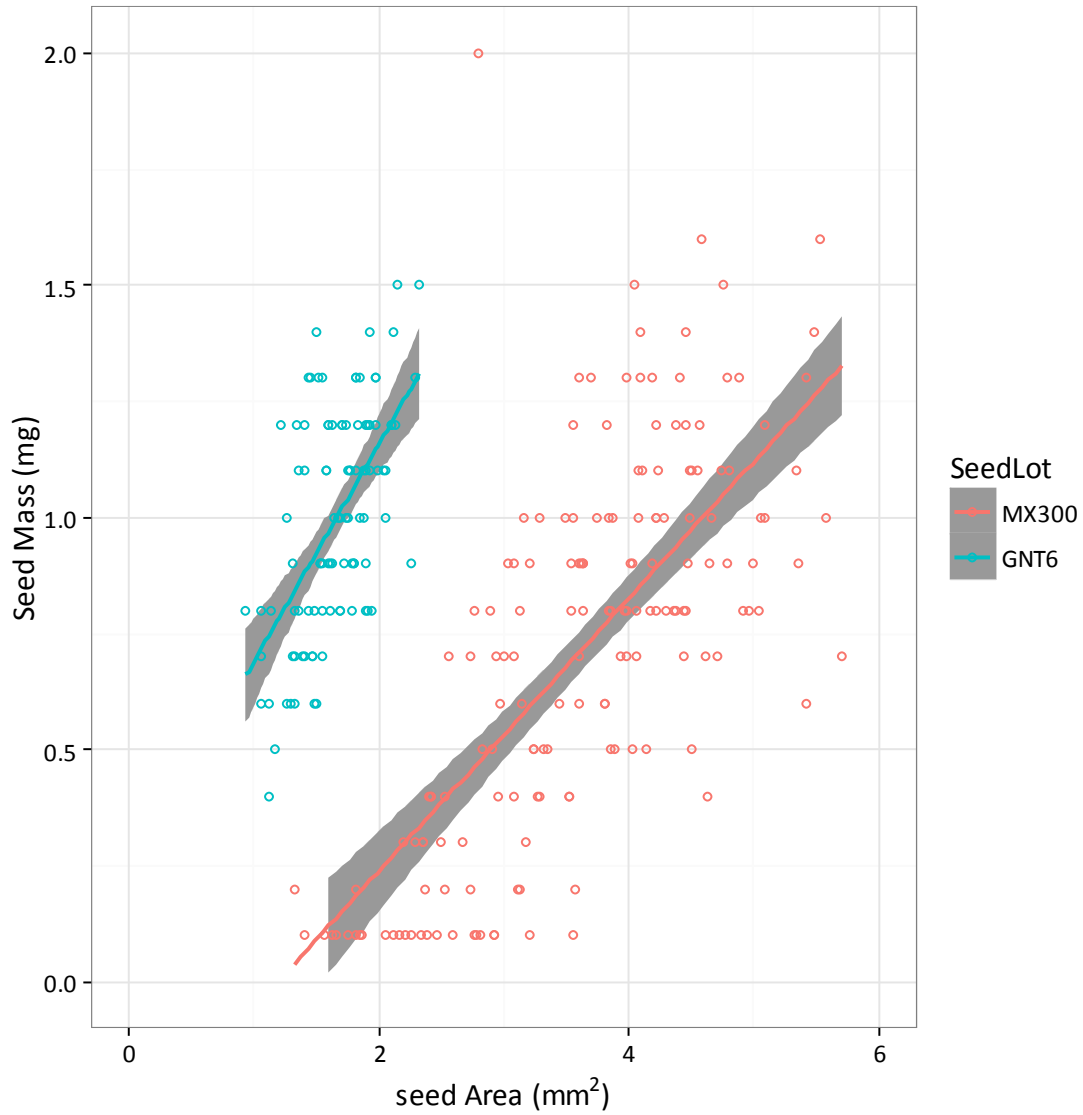


Figure 4-40: A comparison of seed size and area for two seed lots SYN55 and MX300, with linear models of the relationship between seed area and mass. The grey areas are a 95% confidence region.

Both SYN55 and MX300 are *M. sinensis* interspecific hybrids and are shown in distinguishable clusters in Figure 4-40. There is substantial visible variation in the clusters of weights to size; however, both clusters fit linear models, with R^2 values of 0.34 and 0.52 respectively. When tested with a Kendall's rank correlation both were significant ($P < 0.0001$). The variability in SYN55 is seen in Figure 4-40 to be less than in MX300.

DARK BURNOUT SEED TESTING

This experiment was designed to test the growth, health, and survivability of seedlings when germinated without light, and to examine the relationship between these factors and seed size.

To test if seed germination benefited from time in the dark (TiD), the number of seeds germinated per replicate when each dish was removed from the dark was correlated with TiD. As this was not simply transformable to a normal distribution a Kruskal-Wallis rank sum was used to examine the effect of TiD on germination this did not produce a significant result ($P = 0.22$). However, this measurement is problematic because each dish's removal time was at a different time since the beginning of the experiment. Some dishes therefore had more time to germinate before this measurement was taken, e.g. the early (less than 5 day) germination would have been far from complete. Some dishes also had far more time before being removed from the dark, which caused other problems, e.g. later (after 15 day) germination had mould that could render seeds unrecognisable. Therefore, a mid-point was needed; this was chosen at the first point where the maximum number of Petri dishes was screened on the same day. This was time eight (14 days after the start of the experiment), where seed exposed to 0 - 336 hours of darkness were all assessed for germination (see Figure 4-41). Under these parameters there was also no significance of TiD against germination ($P = 0.61$).

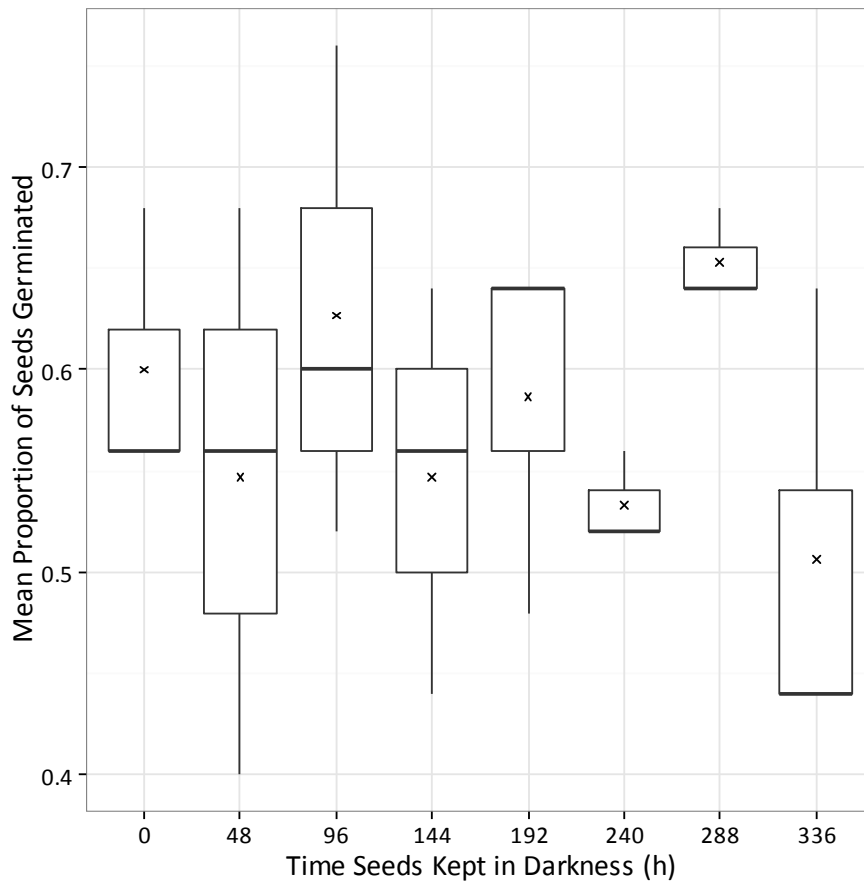


Figure 4-41: Germination variation against time kept in dark, boxplots representing the variation of the three replicates. All were assessed at time 8 (16 days) for simultaneous comparison across eight different times in darkness.

There was more variability of the germination level after 192 hours TiD and there was a decrease in germination at 336 hours TiD, as seen in Figure 4-41.

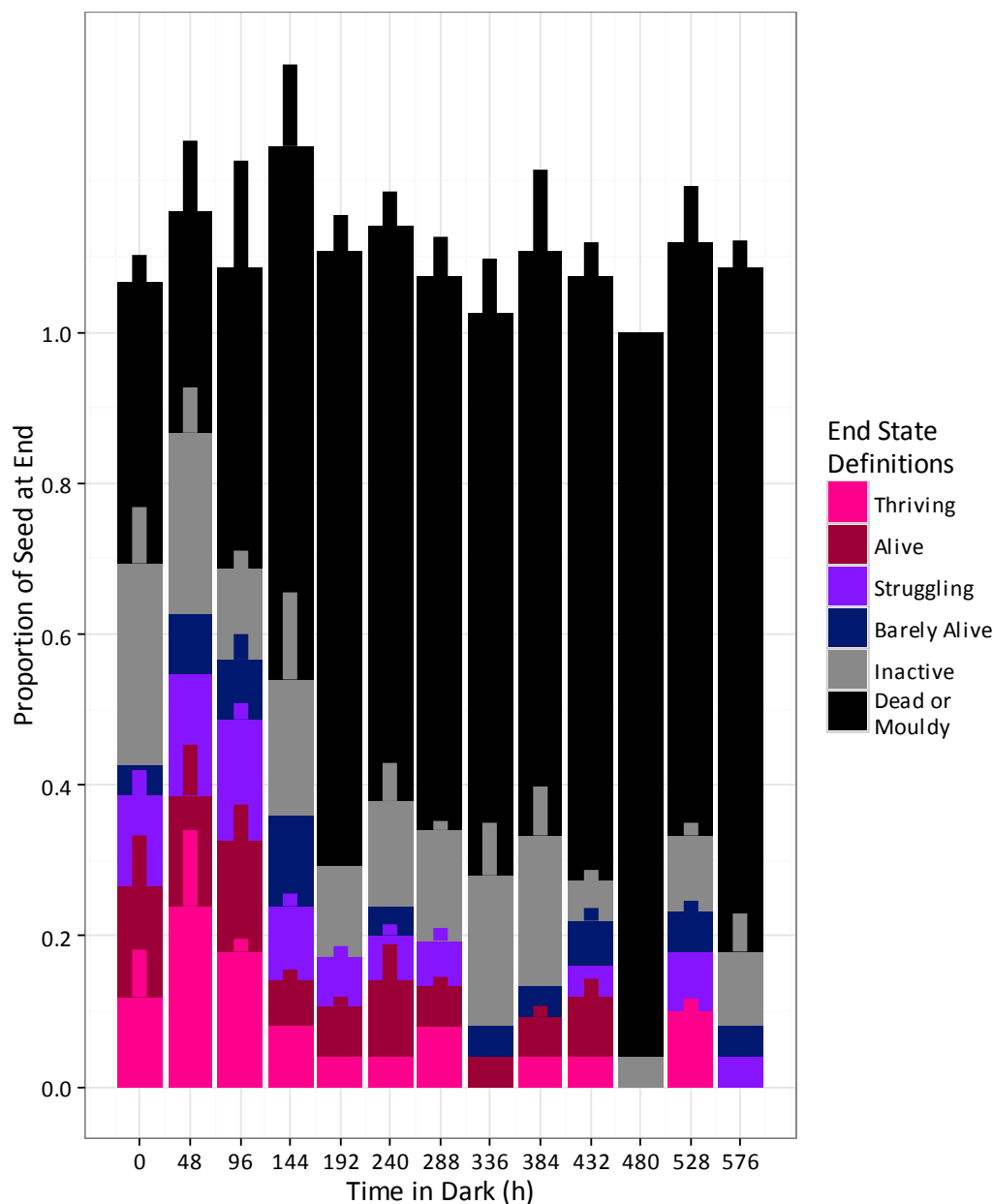


Figure 4-42: The proportions of seedlings in each end state for the average of the three replicates, the small bars show the standard error. These were assessed 12 days after being germinated for n hours in darkness.

The end state of the seedlings is not improved by being germinated in the dark (Figure 4-42). The proportions of seedlings at each end state per dish were tested using a one-way ANOVA. For 'Thriving' seedlings, a normal distribution was achieved by logging the data. The log proportion of 'Thriving' seeds did change significantly with hours in darkness ($P < 0.001$); however, a Tukey's

HSD did not reveal any distinct groupings. The number of seeds that were ‘Alive’ at the end of the test was also significant ($P < 0.0001$), and the Tukey’s HSD found significant groupings at the extremes of TiD (Table 4–4). The number of dead seed was also tested in the same way, and was significant ($P < 0.0001$). The Tukey’s HSD for dead seed also found significant groupings (Table 4–4).

Table 4–4: The Tukey’s HSD result for the proportion of dead seeds at the end of the experiment grouped by the number of hours in darkness. This was tested on the three replicates at each time point.

Proportion of Seeds (Tukey’s HSD)		
Time (h)	Alive	Dead
0	a	bcd
48	a	d
96	a	cd
144	ab	abc
192	ab	abc
240	ab	abc
288	ab	abc
336	ab	abc
384	ab	abc
432	ab	abc
480	b	a
528	b	abc
576	b	ab

The effect of TiD on the final dry weight of the seedlings was tested with a one-way ANOVA. Log transformed seedling dry weight per dish was used, as it did not follow a normal distribution. The result of this was significant ($P < 0.0001$). Dishes beyond 384 hours were excluded because the mould growth on dead seedlings after that time made weighing more difficult and less accurate. The dry weights are shown with the resulting groupings from a Tukey’s HSD in Figure 4-43 below.

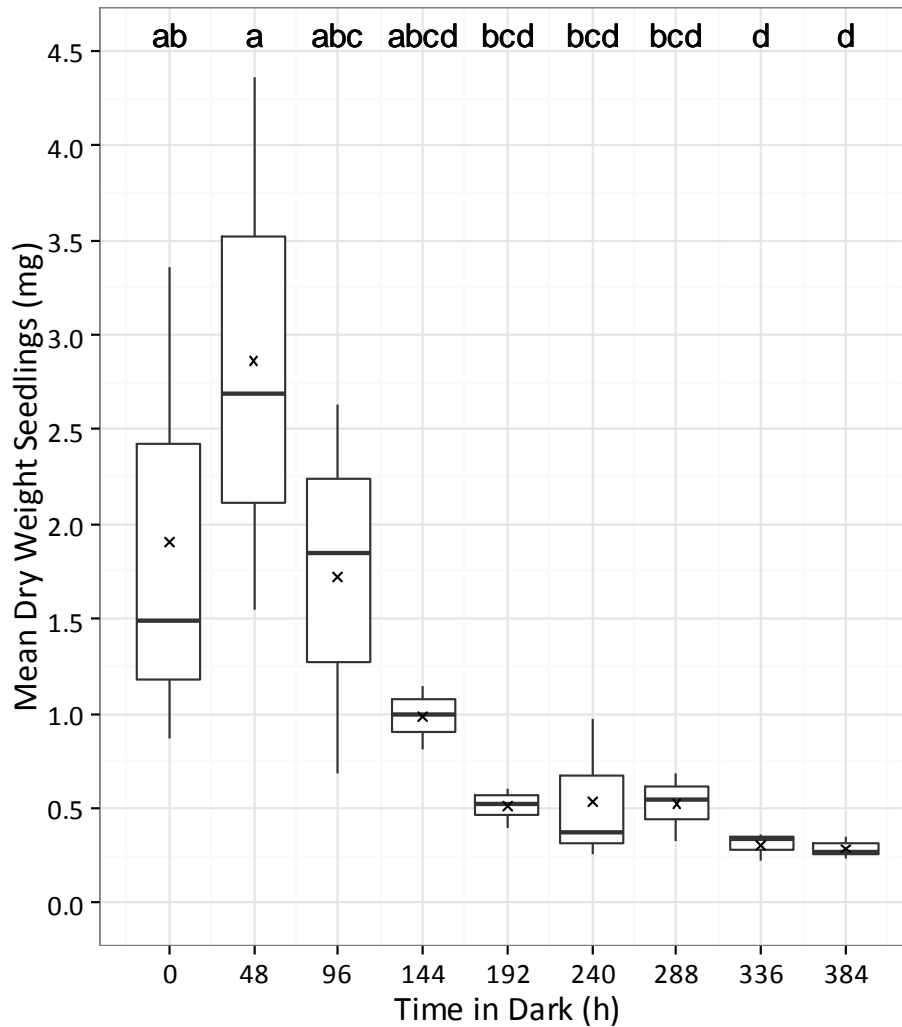


Figure 4-43: Mean seedling dry weight at the end of the experiment by TiD. The Tukey's HSD result has been added along the top of the graph. Boxes represent the variation in the three replicates. Data shown for the first nine sets (later sets excluded because mould growth on dead seed hindered accuracy).

The stem elongation the *Miscanthus* seedlings could reach without light can be seen in Figure 4-44. The mean stem elongation per dish levels out at around 192 hours onwards, the mean at more than or equal to 192 hours is 25.7 ± 9.8 (SE) mm.

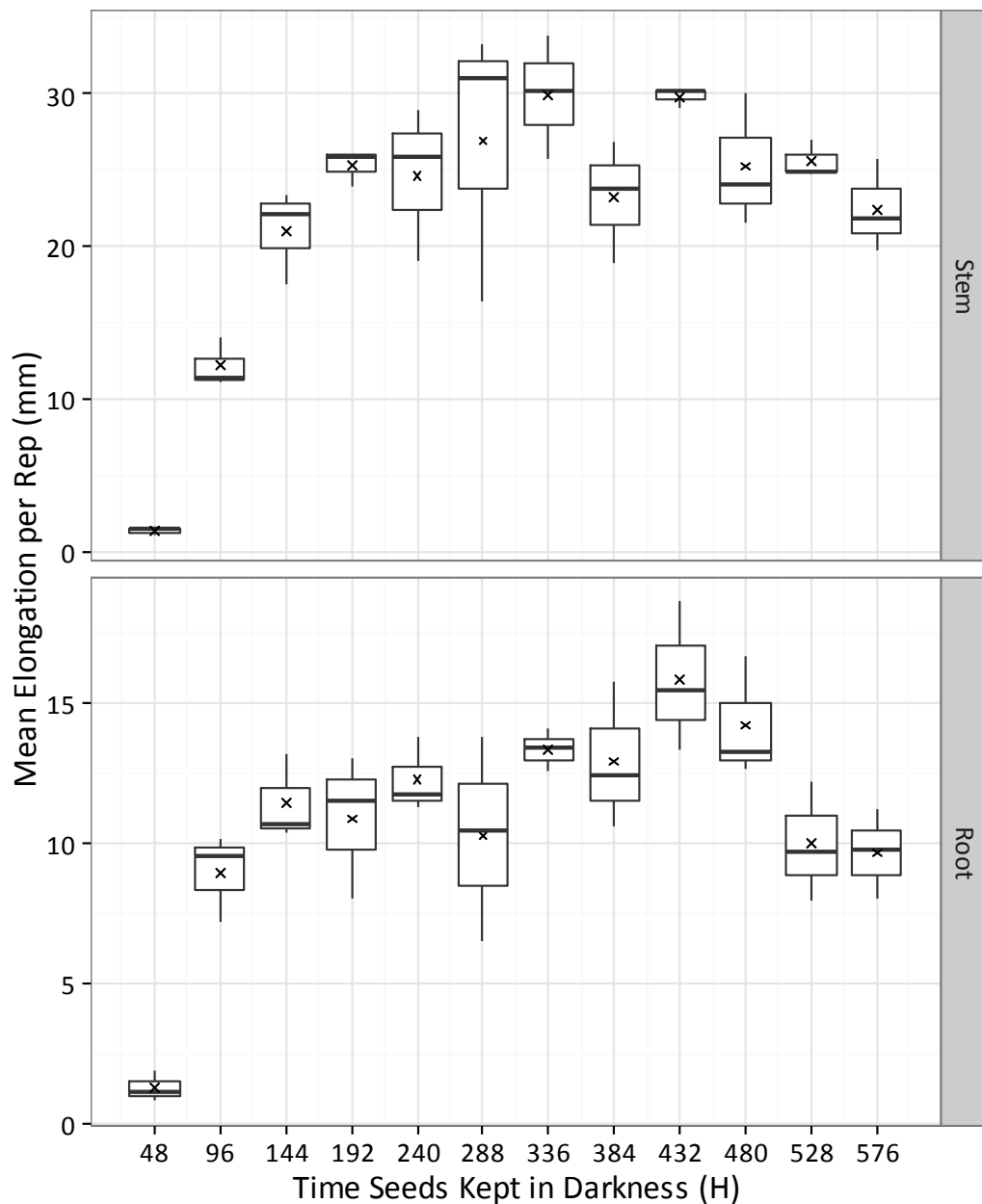


Figure 4-44: The total mean elongation of seedlings (stem and root) when removed from the dark, boxes show the difference between the three replicates; means are marked with an x.

The root elongations also levelled out around 192 hours, yet remained more variable (Figure 4-44 (bottom)), with a bump around 400 hours. Only data from the seeds immediately after removal from the dark was used, because seedlings that survived after 288 hours TiD which were observed and subjected to light, often regrew from the base of the green tip of the stem forming new roots there.

This resulted in the seed and long white stem dying, and had a negative impact on the ability to accurately measure the stem and leaf elongation.

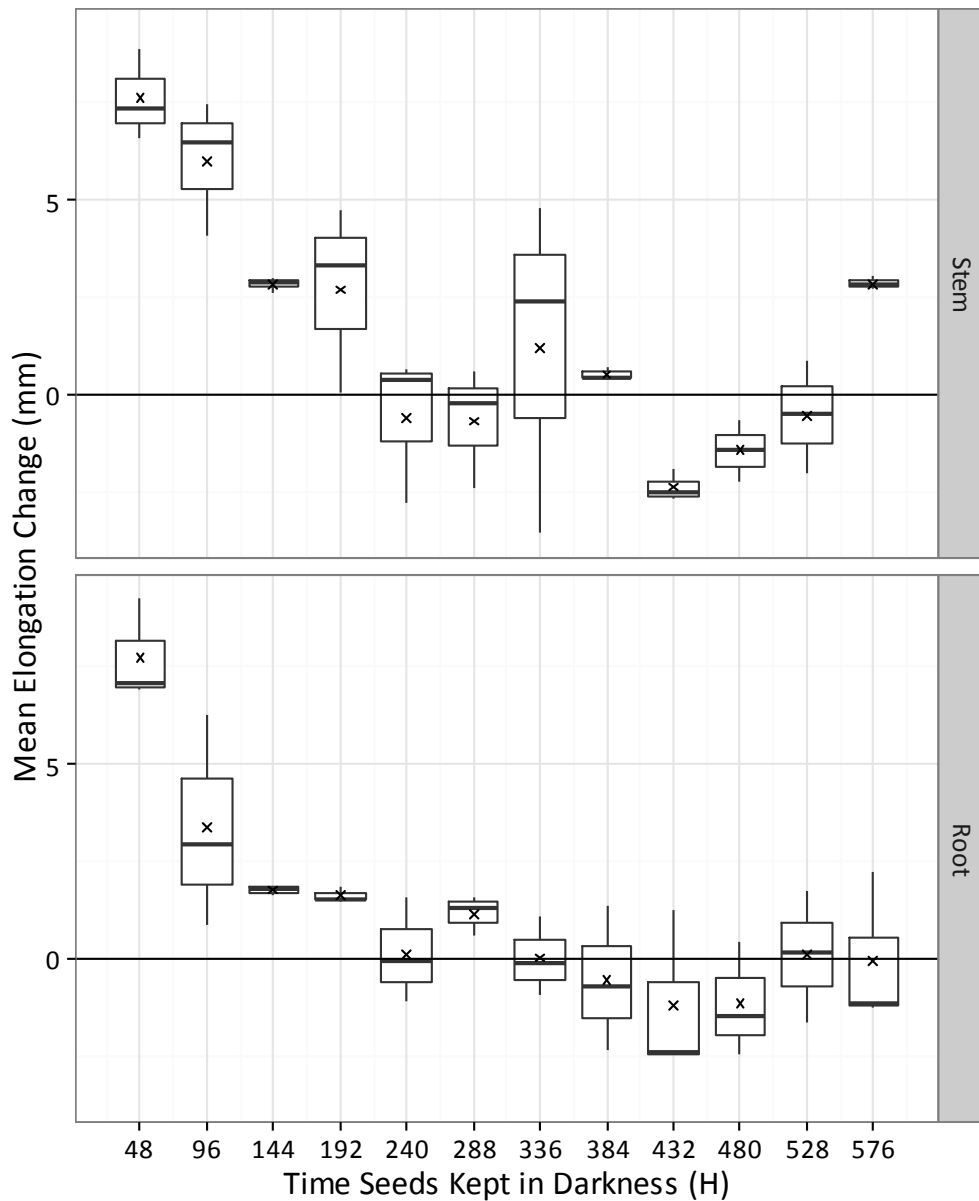


Figure 4-45: The change in mean elongation of the stems and roots of the seedlings from the first time exposed to light until 12 days later, when they were last measured. Boxes show the variation in the three replicates.

It was necessary to determine how long the seedlings could remain viable without light. The state of the seedlings, as assessed after 12 days observation, (Figure 4-42) appears to show that the seedlings struggle to survive between

336 – 480 hours of TiD. The other way to assess this is by examining the seeds' ability to remain growing. This was done by assessing the mean change in stem and root measurement per dish from the time the seeds were taken out of the dark until the end of the 12 day observation period (Figure 4-45).

The exact time seedlings died is unknown but as shown in Figure 4-45, the seedlings show near zero to negative growth in the 12 days they were monitored after being removed from darkness, between 240 and 576 h. The negative growth is due to stems that were previously measurable becoming immeasurable due to mould, and thus skewing the average.

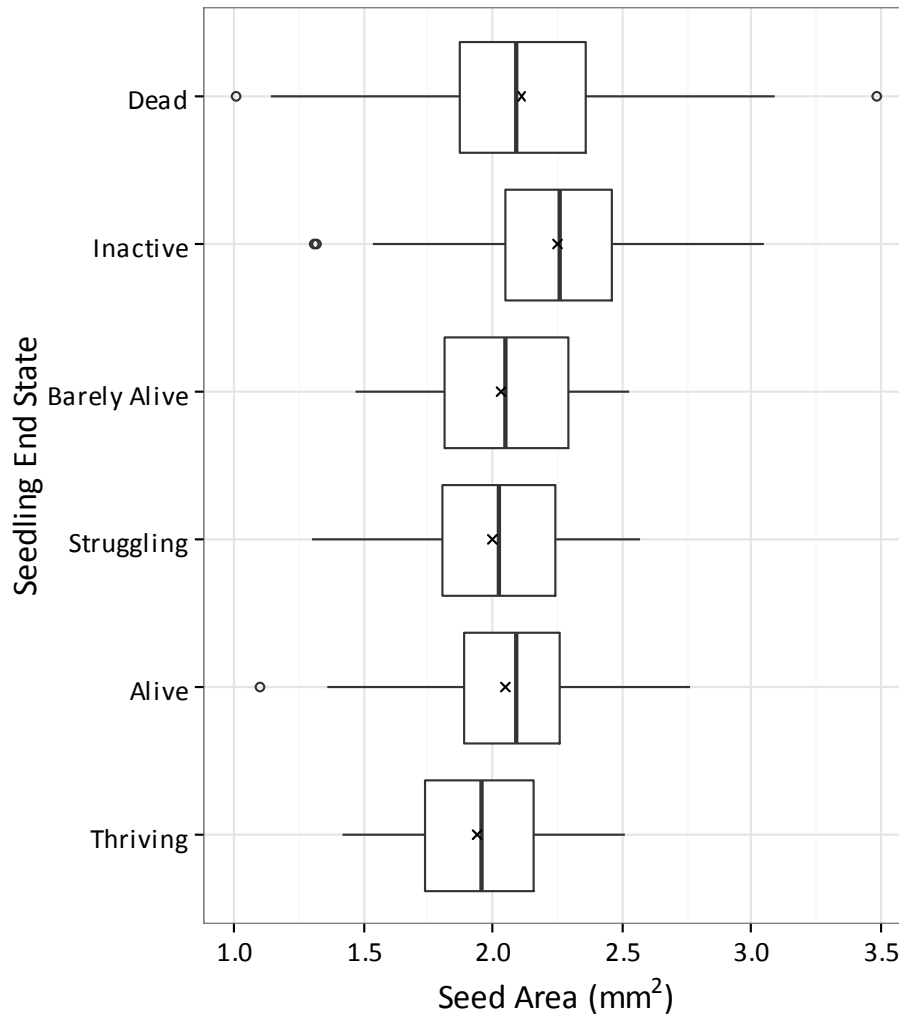


Figure 4-46: A boxplot showing a comparison of the size of the original seeds, grouped by end category state, for all 975 seeds in the experiment.

To determine if seed size affected the state of the seeds at the end of the experiment, a one-way ANOVA was performed on seed size by end category. This produced a significant difference of $P < 0.0001$ (Figure 4-46). The residuals were normally distributed when tested with a Shapiro-Wilk normality test ($P < 0.05$) and the results of the ANOVA were tested with a Tukey's HSD result in Table 4-5. The only unique group was 'Inactive' seeds, which were from larger seeds than other end states (Table 4-5).

Table 4–5: Result of a Tukey's HSD on the ANOVA result for seed size vs status classification at the end of the test, across the 975 seeds in the experiment.

	Mean Size (mm)	
Thriving	1.941	c
Barely Alive	2.034	bc
Struggling	1.999	bc
Alive	2.050	bc
Inactive	2.248	a
Dead	2.112	b

To determine if there was a relationship between elongation and seed size, only data from each dish when it was removed from the dark was used; this was restricted to seeds measured at more than or equal to 192 hours to only show fully elongated seedlings. This was because this appears in Figure 4-44 (top) to be the point that elongation by time in the dark levels off. When tested with one-way Wilcoxon signed rank tests, there were significant effects of seed size on stem and on root elongation (both $P < 0.0001$); however, the correlation was low and negative for both stem (-0.09) and root (-0.09) elongation, when correlated using Kendall's rank correlations. This was due to clustering of more elongated seedlings around the mean seed size.

To test whether seed size had an effect on seedling dry mass at the end of the test, an ANOVA with TiD as the an extra factor was attempted based on hours in darkness and seed area; however, it was not possible to transform the data to meet the prerequisites of an ANOVA. Therefore, the results from three sets of seed were chosen as a representative sample; these were from 48, 144, and 288 hours. When tested against seed size with Wilcoxon rank sums, the 48 hour sample was not significant ($P = 0.98$) but the later time points were highly significant, both being $P < 0.0001$. However, when correlated the strongest

correlation at 288 hours TiD was only -0.11, showing while seed size affected dry weight, bigger seeds did not equal bigger plants.

DARK VS. RED LIGHT GERMINATION EXPERIMENT

This experiment aimed to test the effects of darkness and red light on multiple genotypes of *Miscanthus* seed. The two genotypes of *M. sinensis* (SYN55 and MX300), were not significantly different for germination percentage with or without light (Figure 4-47) when tested using a t-test, with a result of $P = 0.77$ for SYN55 and of $P = 0.91$ for MX300. However, the *M. sinensis* × *M. sacchariflorus* (GNT14) was showed a significant improvement in red light over no light when tested with a t-test ($P < 0.05$). The t-test for *M. sacchariflorus* (SYN70) seed germination showed a more significant improvement in red light ($P < 0.001$) than GNT14.

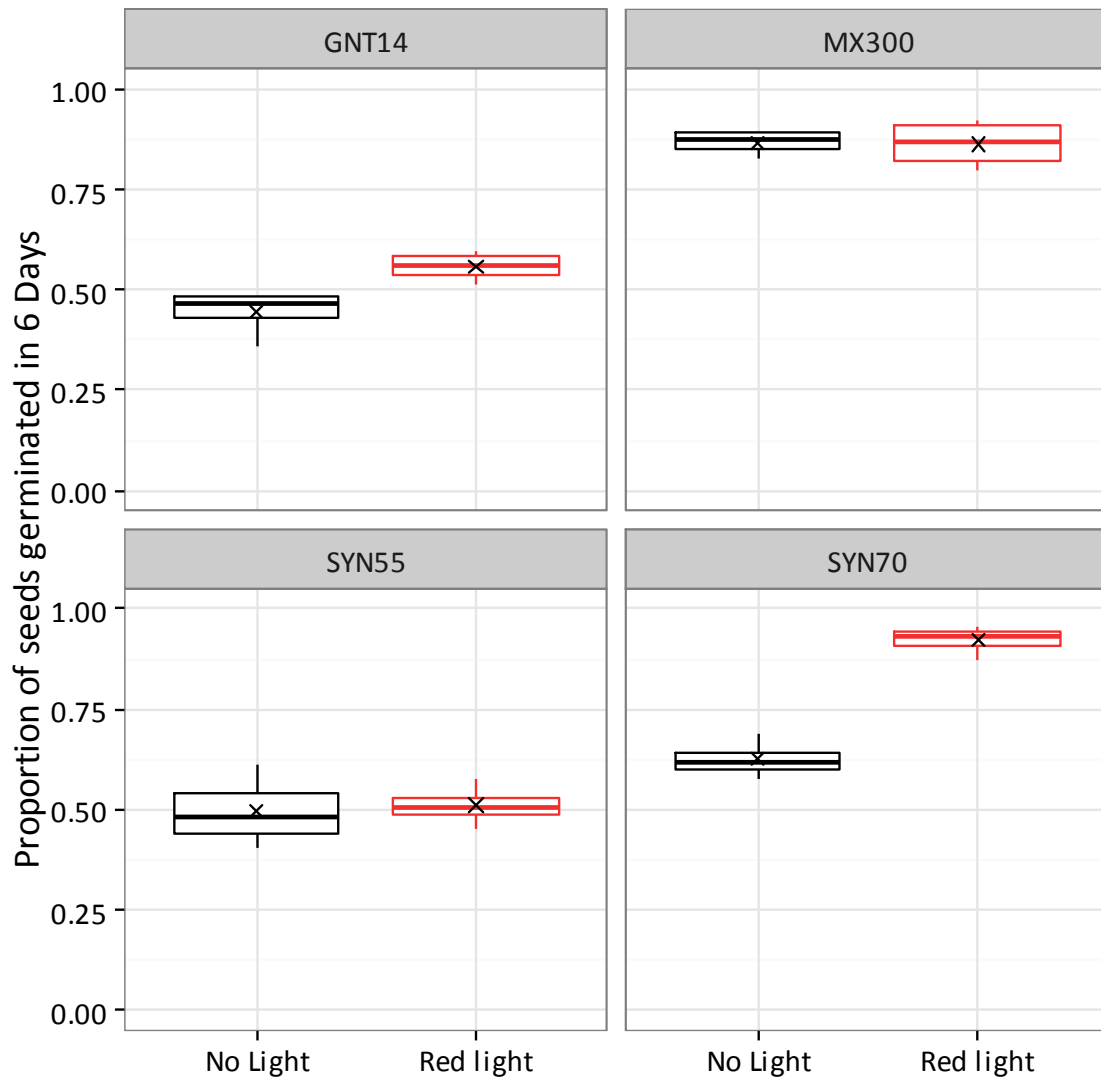


Figure 4-47: The differences in 6-day germination with red light and without light for four *Miscanthus* seed lots. *M. sinensis* synthetic (SYN55), *M. sinensis* open cross (MX300), *M. sinensis* × *M. sacchariflorus* (GNT14), and *M. sacchariflorus* synthetic cross (SYN70). The boxes show the variation in the four replicates, and means are represented by x's.

The variation between the replicates was low for all of the results (see Figure 4-47). The vigour of the seeds growing in the dark was observed to be different dependent on genotype, with the *M. sacchariflorus* seed growing less (observed stem elongation) in 6 days and appearing to have frequently died during the test, while *M. sinensis* did not. The results suggest that *M. sacchariflorus* germination is heavily influenced by light, whilst *M. sinensis* is not.

4.4d TAGUCHI RESULTS

The Taguchi analysis was performed by Dr Sreenivas Rao Ravella of Aberystwyth University.

When the results of the Taguchi 16_L experiment were analysed, water stress had the biggest effect of any factor, followed by ABA, with the other factors all similar in impact overall but varying in which output they affected most (Figure 4-48). The raw results from the germination testing that went into the Taguchi analysis are shown in full in Appendix L.

Starting with the lowest weighted outputs, stem and root elongations were both similarly affected by water stress, though stem elongation slightly more so; root was affected by 28% and stem affected by 38%. Stem elongation was also more affected by ABA than root elongation, being affected by 36% to root elongation's 21.5%. GA had little effect on either, though the effect was particularly small for stem elongation (2.2%), while root elongation was affected by 4.9%. Priming only had a limited impact on root elongation (9.2%).

Next, germination rate as given by $1/T_{50}$ was analysed (Figure 4-48). This was affected most by light levels (37.4%), followed by BR (22.9%). There were also effects from GA (13.7%) and ABA (16.1%). Germination percentage at 7 days differs from germination rate mostly in that there was a much larger effect on germination percentage from priming (22.6 to 0.02%) and water stress (24.2 to 2.7%), with the reverse being true for light, where germination rate experienced the larger effect (3 to 37.4%).

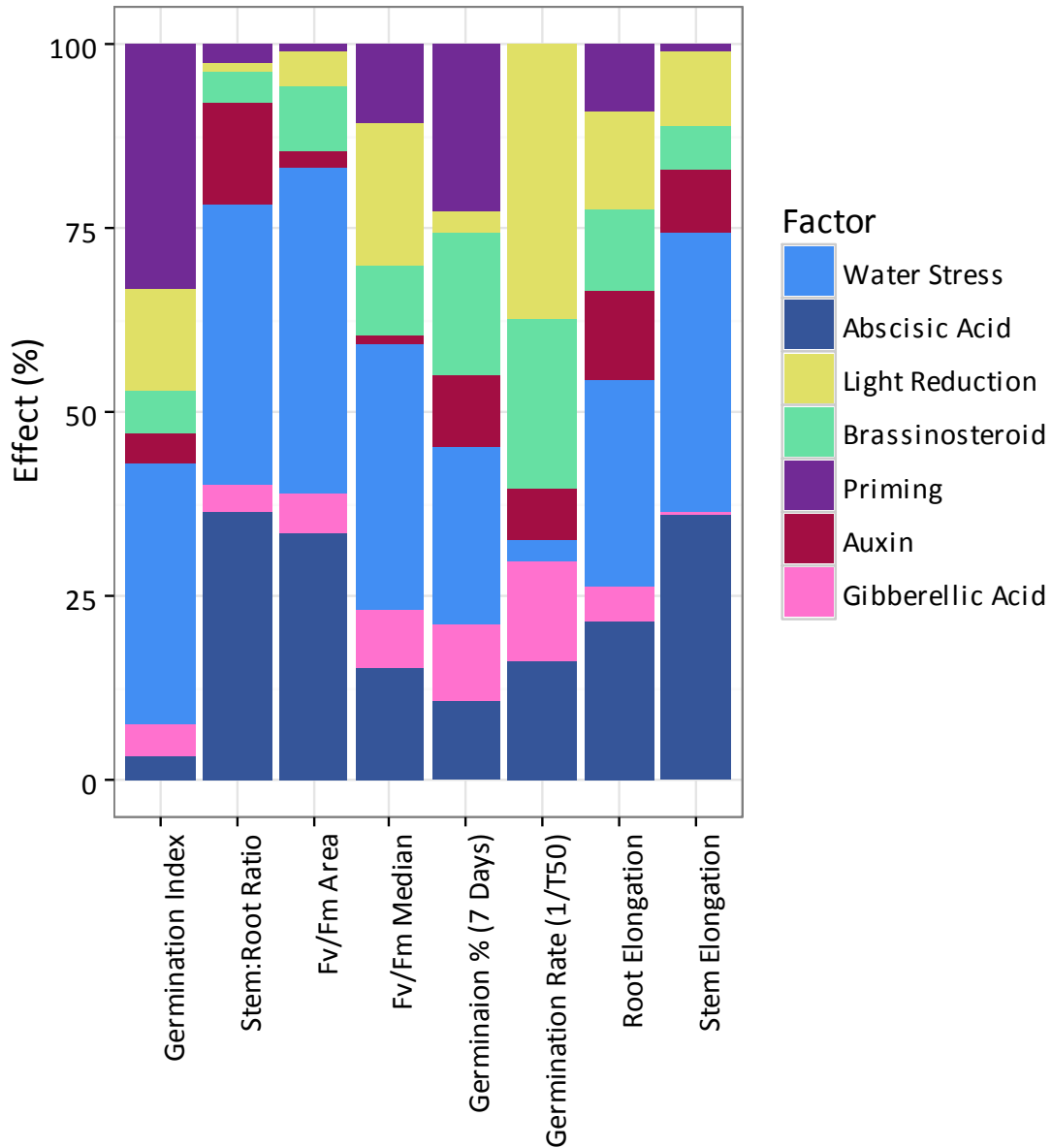


Figure 4-48: The percentage effect of each factor on each output, from the ANOVA result of the Taguchi. All numerical values are shown in full in Appendix L.

GI was used as a combined measure of germination (Section 2.1a). The results for GI show that it was less affected by ABA (3.1%) than germination percentage (10.9%) or germination rate (16.1%). This was also the case for BR, where GI was affected by 5.5% compared to 19.4% for germination percentage and 22.9% for germination rate. These results can be seen in Figure 4-48.

Fv/Fm median values and total area of photosynthetic activity were used as measures of seedling health. Median values describe the average photosynthetic activity over each treatment, while total area gives an indication of the total amount of photosynthetic activity occurring in the treatment. The primary difference between median and area values was for ABA, with area being affected by 33.6% and median by 15.1%, less than half. The effect of priming was greater for median level (10.8%) than for area (0.7%). This was also true for light, where the median Fv/Fm was affected by 19.3% whereas total area was affected by 4.9%. Fv/Fm total area was also more affected by water stress than Fv/Fm median, at 44.3% to 35.9%. Figure 4-48 illustrates these differences.

Lastly, stem:root ratio was analysed. This was most affected by water stress (38.1%), followed closely by ABA (36.3%), then less so auxin (13.9%) (Figure 4-48).

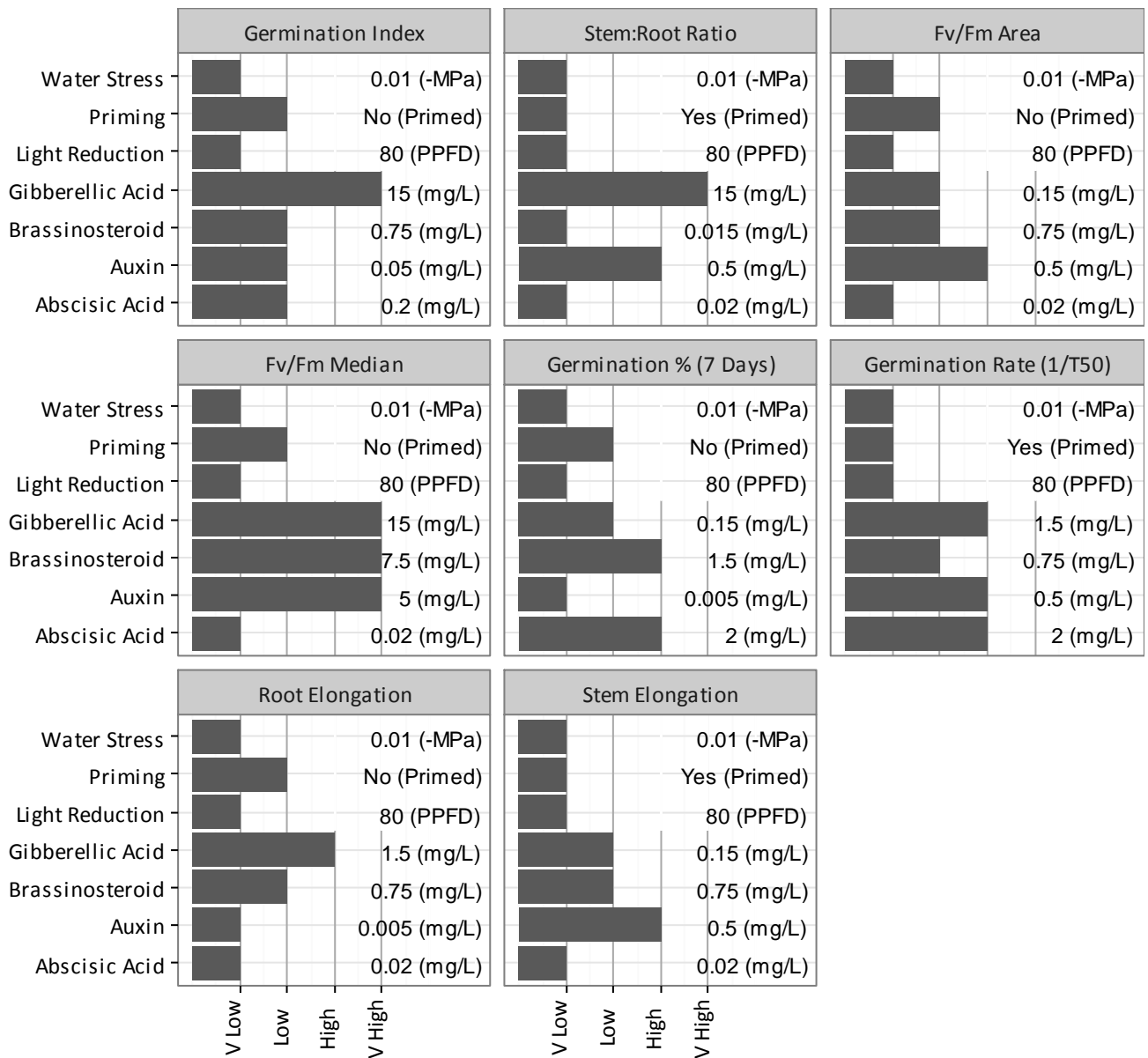


Figure 4-49: The Taguchi calculated optimum level for each factor, in each output of the test. The first three factors have only two levels, here listed with the others as V Low and Low; however, these could be stated as low and high. The optimum level has been given next to each (units have been abbreviated: $\mu\text{mol m}^{-2} \text{s}^{-1}$ as PPFD and mg L^{-1} as mg/L). All values are shown in Appendix L.

The Taguchi also calculates the best levels for each factor at each output (see Figure 4-49); the usefulness of these is dependent upon how high the percentage effects were (Figure 4-48). For example, the effect of GA on stem elongation

was best at 0.15 mg L⁻¹ (low), but the percentage effect of GA on stem elongation was only 2.2% (Figure 4-48), so clearly the effect of GA was limited at any level.

For light, which had only two levels, low light (PAR of 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$) was an improvement over high light (PAR of 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$) in all performance outputs (Figure 4-49). Light was most relevant in speed to germinate, followed by percentage germination, having a 2.5% effect on the percentage of seeds germinated (Figure 4-48). It also had a 0.3 mm effect on mean root elongation. Light had a large overall effect of 13% (Figure 4-48).

Water stress had a mean effect of 31% on outcomes, with effects over 30% in GI, stem:root, Fv/Fm area and Fv/Fm median, e.g. making an 86.8 mm² impact on Fv/Fm area (Figure 4-48). Water stress was best at a low level of -0.01 MPa for all outcomes (Figure 4-49).

The presence of ABA was calculated by the Taguchi to have affected the germination percentage by 6.8% (Figure 4-48). ABA was optimal for most measurements at its almost zero (very low) level of 20 $\mu\text{g L}^{-1}$ (Figure 4-49). It had some effect on GI at 200 $\mu\text{g L}^{-1}$ but the percentage effect of this was low (3%) (Figure 4-48). It also had optimal performance at 2 mg L⁻¹ (Figure 4-49) for germination rate ($1/T_{50}$) and total germination percentage (effect percentages of 16.1 & 10.9%, shown in Figure 4-48). There were no points where 20 mg L⁻¹ was the best treatment; the average best treatment for ABA was 0.54 mg L⁻¹ (Figure 4-49).

GA presence is never optimal at its near zero (very low) level of 15 $\mu\text{g L}^{-1}$ (Figure 4-49). The optimum level varied over the 150 $\mu\text{g L}^{-1}$ to 15 mg L⁻¹ range. GA had the biggest effect on germination rate, at 13.7% (Figure 4-48), for which the optimal level was calculated as 150 $\mu\text{g L}^{-1}$ (low) (Figure 4-49). The effect of GA on germination percentage was optimal at 1.5 mg L⁻¹ (high) (Figure 4-49);

however, the percentage effect was low at 10.2% (Figure 4-48). GA had a low average effect of 6% (Figure 4-48).

Auxin (1-naphthaleneacetic acid) was optimal for root elongation and germination percentage at the near zero, very low level ($5 \mu\text{g L}^{-1}$) (Figure 4-49). A level of $50 \mu\text{g L}^{-1}$ of auxin was calculated to be optimal for GI, but auxin only had a 4.3% (Figure 4-48) effect on GI. Auxin normally performed best at a high level of 0.5 mg L^{-1} for stem:root ratio, germination rate, stem elongation, and Fv/Fm area (Figure 4-49); of these, only the first three experienced an effect of more than 5% (Figure 4-48). The very high level of 5 mg L^{-1} (Figure 4-49) of auxin was only optimal for Fv/Fm median, on which it had a low effect (1.2%) (Figure 4-48).

For BR, stem:root ratio was the only measure, which preferred the near zero, very low level ($15 \mu\text{g L}^{-1}$) (Figure 4-49), and this was at a low percentage (4.2%) (Figure 4-48). The level of 0.75 mg L^{-1} was optimum for 5 of the 8 measurements (Figure 4-49), most importantly germination rate with an effect of 22.9% (Figure 4-48). This was followed by root elongation (11.3%) and Fv/Fm area (8.8%). BR was optimal at the high level (1.5 mg L^{-1}) for germination percentage (Figure 4-49); on which it also had a high effect (19.4%) (Figure 4-48). At BR's maximum level of 7.5 mg L^{-1} , it was only most effective for Fv/Fm median (Figure 4-49); on which it had an effect of 9.5% (Figure 4-48).

In priming, which had an average effect of 10%, the largest effects were on GI (33.1%) and germination percentage (22.6%) (Figure 4-48). Un-primed seed were optimal (Figure 4-49) in all measurements that experienced an effect of more than 10% (Figure 4-48). However, primed seed is shown as optimal in Figure 4-49 for stem:root ratio, germination rate, Fv/Fm area, and stem elongation. However, all of these experienced a low percentage effect, the highest being

stem:root ratio at 2.5% (Figure 4-48). The Taguchi calculated that priming changes germination percentage by 7%.

Unexpectedly GI and germination percentage had no interactions above a sensitivity index of 70%; however, they are related measures. The first combination with four relevant interactions was GA and BR, which interacted in stem:root ratio, Fv/Fm median, stem elongation, and root elongation. The other pair of factors that interacted at four points was the combination of GA and auxin, which interacted in the measurements of stem:root ratio, Fv/Fm area, stem elongation, and root elongation. Two other pairs of factors interacted. Firstly, water stress and priming interacted in the germination rate, which appears to show that not primed seed would germinate faster under water stress than primed seed. Secondly, light level and priming interacted in the stem:root ratio, this shows primed seed growing with a bigger stem:root ratio under high light than unprimed seed.

The OEC calculated weighted combinations (seen in Table 4-6) were used in a Taguchi analysis to determine the optimal conditions. This resulted in an optimal level of $80 \mu\text{mol m}^{-2} \text{s}^{-1}$ for light (low), an optimal Ψ of -0.01 MPa (low), and with unprimed seed being optimal (Table 4-7). The optimal hormone levels were 0.02 mg L^{-1} for ABA (very low), 0.75 mg L^{-1} for BR (low), 0.5 mg L^{-1} for auxin (high), and 15 mg L^{-1} for GA (very high).

Table 4–6: Each experimental setup in the Taguchi experiment ordered by the OEC statistic. The combination of factors to get the optimal weighted result is at the top of the table.

ABA	GA	Water stress	AUX	BS	Light	Primed	OEC
Low	Low	Low	High	V High	Low	YES	84.886
V Low	Low	Low	Low	Low	High	NO	83.049
V Low	V Low	Low	V Low	V Low	Low	YES	77.302
High	High	Low	V High	Low	Low	YES	74.090
Low	V High	High	V Low	Low	Low	NO	70.203
Low	V Low	Low	V High	High	High	NO	68.634
High	V High	Low	High	V Low	High	NO	66.477
V Low	High	High	High	High	Low	NO	62.291
V High	V High	Low	Low	High	Low	YES	54.014
V High	High	Low	V Low	V High	High	NO	46.004
High	V Low	High	Low	V High	Low	NO	41.693
V Low	V High	High	V High	V High	High	YES	33.249
V High	Low	High	V High	V Low	Low	NO	21.395
Low	High	High	Low	V Low	High	YES	10.220
High	Low	High	V Low	High	High	YES	5.296
V High	V Low	High	High	Low	High	YES	4.594

The lowest percentage effect was from auxin (1%); the hormones GA and BR, despite being optimal at the higher concentrations, also had low percentage effects (under 4%) on OEC (Table 4–7). As with other metrics, the strongest interactions in the OEC were GA with auxin and GA with BR, with both scoring over 70% sensitivity. Table 4–7 shows effect of ABA’s very low optimal level was second only to that of water stress. There was also a notable effect of light level (7.2%), where low light was better.

Table 4–7: The percentage effect of each factor in the Taguchi and the level at which it was optimal.

Factor	Percentage Effect OEC	Optimal Level OEC
Absciscic Acid	21.2	V Low
Gibberellic Acid	1.5	V High
Auxin	1	High
Brassinosteroid	3.8	Low
Water Stress	50.1	Low
Light	15.2	Low
Priming	7.2	No

4.5 DISCUSSION

4.5a HORMONES

ABSCISIC ACID

Overall Absciscic acid (ABA) had the expected effect with germination reduction and seedling end state being lower with increasing concentrations. However, while the ABA should have perpetuated seed dormancy the proportion of inactive seeds did not change throughout the experiment. This could be because the warm wet conditions made the seed too susceptible to mould when not germinating. Levels of mould were lowest at the five smallest concentrations of ABA.

The positive effect small quantities (0.05 to 5 mg L⁻¹) of ABA has on stem elongation may have been due to preventing weaker seeds from germinating first. The mean and median times for individual seed to germinate do not decrease considerably from low amounts of ABA (3-4 to 6 mg L⁻¹). This may be due to the seeds that would have germinated last failing to germinate, thus being held in a

dormant state. However, because the drop in thriving seedlings came before the overall drop in living seedlings the ABA is having an effect on seedling performance even at low levels. Therefore, the increase in stem elongation at the end of the test may have been due to seeds that would have been smaller and classed as 'thriving' growing longer, less healthy stems.

GIBBERELIC ACID

The hormone Gibberellic acid (GA) was expected to have a positive effect on germination time by down regulating the seed dormancy and promoting germination (Yaldagard et al., 2008). There may have been a small positive effect on germination but germination percentage, time, and GI results were not significant. However, there was an effect of GA concentration on mould levels, which were higher with the highest concentrations (more than 75 mg L⁻¹); this should have caused a drop in germination numbers because high mould levels in a dish spread to weak seeds. Therefore, this drop not happening may also imply there is a small benefit to germination, and that there may be some benefit to mould from GA. Therefore, unlike in the Christian et al. (2014) study, here GA does not seem to contribute much to dormancy breaking in *M. sinensis*. There was no dormancy found in *M. sinensis* in this study and while Christian et al. (2014) found an effect of GA the triphenyl tetrazolium chloride dormancy test they used showed few dormant seeds. It was therefore it was unsurprising that GA did not affect the germination. Further investigation would be needed to determine if GA has a more positive effect on germination in *Miscanthus* species or if the effect of GA for dormancy breaking in *Miscanthus* is dependent upon other hormones.

GA was also expected to have a positive effect on stem growth, possibly disproportionately (Aso, 1976). It was found that even tiny concentrations of GA

(0.15 mg L⁻¹) produced longer stemmed seedlings. However, these seedlings were not judged at the end of the test to be of a different state than the control group. The effect on elongation of seedlings stabilised around 1 mg L⁻¹ of GA. Whether these longer seedlings would have been vigorous if growing had continued is not known, but observationally this seems unlikely, because at least some of them appeared overstretched, pale, and weak. This confirms the *Miscanthus* observations by Aso (1976), that the seedlings' growth was promoted unevenly; however, Aso also saw increased germination in *Miscanthus* which was not seen here.

AUXIN

Auxin was predicted to have a positive effect on root growth (Müssig et al., 2003); however, the effect was small and mostly concentrated in the auxin concentration of 0.05 mg L⁻¹. Auxin had a more consistent, but not as positive as GA, effect on stem elongation. For both root and stem elongations, the positive effect from auxin was lost by 0.1 and 100 mg L⁻¹ respectively. The lack of a clear effect on roots, with a decrease in elongation at most concentrations, may have been because auxin's effect on the roots was not to make them longer. It was observed that the roots seemed thicker and fluffier with high concentrations of auxin. This may be due to a change in regulation of root hairs, because Pitts et al. (1998) expected auxin to be required for proper root hair growth. This effect could be further investigated more with high-resolution root imaging. Pitts et al. (1998) and Wang et al. (2011) suggested high concentrations of auxin could limit root growth, and the short roots would ultimately have an effect on stem lengths.

There was no impact from auxin on time for the seeds to germinate or germination percentage, but auxin may have more of an effect when interacting with

other hormones, particularly those it was previously proposed to interact with (BR and ABA) (Bao et al., 2004; Wang et al., 2011b); this was tested in the Taguchi.

Seedlings in low concentrations of auxin (0.1 to 0.5 mg L⁻¹) had an end state recorded as thriving at higher proportions than in the control. This appears to be part of a bi-staged effect where auxin concentration affects the end state of seedlings. There is again a positive effect at low concentrations before a negative effect after ~1 mg L⁻¹ of auxin.

The mould readings were spread out; mould peaked at the same time 'Thriving' seedlings did at low auxin levels. This may be due to a complex effect on the prevalence of mould by the auxin, but that would not be possible to prove here. Further study should try to eliminate any crosstalk with mould, auxin, and germinating seeds.

BRASSINOSTEROID

The decrease in root size at higher levels of brassinosteroid (BR) suggests that findings by Steber & McCourt (2001) of BR inhibiting root elongation may apply to *Miscanthus*. The slight decrease in stem elongation may have been due to hormone signalling or just smaller roots.

The proportion of seedlings 'Thriving' in the concentrations, would require further investigation to determine if there was a drop in success of seeds with low as well as high levels of BR, with a stable point at around 0.05 mg L⁻¹ where BR performed as well as the control. High (above 1 mg L⁻¹) BR seems to kill the seedlings; however, at these concentrations, the seeds germinate more slowly, though a similar proportion of the seeds do germinate. These seeds do not often become 'Thriving' and go mouldy at a higher proportion than seeds at lower levels.

Low levels of BR do not appear to produce a useful effect on time to germination or germination percentage. However, this may be because BR works at an intermediary step in hormone pathways, and BR could increase the effectiveness of GA (Kucera et al., 2005; Steber & McCourt, 2001) as tested in the 'Taguchi'.

4.5b PHYSICAL STRESSES AND PRE-TREATMENTS

WATER STRESS

Time to germinate appeared to increase slightly at -0.1 MPa in PEG 4000 and NaCl but not PEG 8000. This gives some indication that PEG 4000 and NaCl may inhibit germination before the effect of water potential. An ion toxicity effect of NaCl is well established (Bajji et al., 2002; Zhang et al., 2010), less so is the possibility of PEG 4000 toxicity as reported by Lagerwerff et al. (1961) and Lawlor (1970). However, due to the design of this experiment there was no detectable significance of either toxicity effect.

PEG Ψ more than any other test increased the time to germinate which sometimes increased beyond the time extra water was added. The main increase in time to germinate for NaCl and the PEGs came at around -1 MPa. This suggests that the Ψ needed to slow germination is -1 MPa, with Ψ below -2.2 MPa stopping germination entirely. The salt stress on *Miscanthus* seed was noticeable after -1 MPa, this is a similar level (≈ 200 mM) that Stavridou et al. (2016) found to affect adult *Miscanthus*' below ground biomass.

Germination percentage and GI in NaCl dropped significantly with lower Ψ . This also occurred with PEG induced water potential. Proportions of seed that germinated were not significantly affected by the type of PEG used. However, high PEG concentrations did have somewhat better germination in PEG 4000

than 8000, and overall germination was better in PEG 8000. The biggest drop in germination percentage is after -1.2 MPa NaCl, -1.2 MPa in PEG 4000 and -0.8 MPa in PEG 8000. Here PEG 4000 is behaving closer to salt than PEG 8000.

Time for mould to take hold resembles time to germinate, and is significantly increased by NaCl and PEG 8000, though not for PEG 4000. However, total mould was not significantly different between the two PEG sizes. Mould growth also has the problem of Ψ so mould levels go up before they go down, because the deteriorating condition of the seeds due to salt toxicity boosts mould growth.

Seedlings could survive in higher concentrations of NaCl and PEG 4000 than PEG 8000, possibly showing a harsher droughting effect of PEG 8000. However, while the effect on stem elongations was significant and correlated negatively with Ψ for all three treatments, root growth had a significant negative reaction to Ψ from NaCl and PEG 4000, but not PEG 8000. This may be because PEG 8000 killed the seeds more abruptly once the Ψ was strong enough. In NaCl root elongations increased until -0.8 MPa as seedlings attempted to recover more water from the environment. After -0.8 MPa this effect was not seen because seedlings were growing so poorly. This effect was also seen to a lesser extent in PEG 4000 and PEG 8000.

The two PEG sizes were different in the elongation of stems and roots. This is the only time there was a significant difference between PEG 4000 and PEG 8000. If there is a difference between the PEGs, it is in how the seedlings grow while under water stress. Once again, it could be concluded as in germination speed and germination percentage above; that PEG 4000 better represents salt and PEG 8000 represents just the effect of Ψ on the seeds.

STRATIFICATION AND COLD STORAGE

The cold storage test showed no significant differences in either mould or germination percentage; however, the control germination had a lower variance (Figure 4-32) than the chilled. If the seeds' average germination were moving gradually to a higher percentage, this would be what would be expected when the seeds had not been chilled for long enough. In addition, if the chilled seed had been more tightly clustered around the mean the higher germination would have been significant.

The fifteen-day mould was close to a significant difference. This also could have had more of an effect on the mould in the seed coat if the cold treatment was longer. This could be expanded into a range of cold treatments for a range of times if possible, to examine if there is a ceiling on cold treatment length, because there is some anecdotal evidence that *Miscanthus* seeds having been stored at 5°C for 2-3 years have higher germination rates.

Unlike Christian et al. (2014) stratified seed germination and GI were not affected; however, time taken for seed to germinate decreased with days stratified for both. This was most distinct for dried seed, where the mean time to germinate halved from 2.87 days for the control to 1.5 days at 14 days dried.

Stratification had no significant increase in stem and root elongation. However, there was a significant difference between dried stratification, wet stratification and no stratification in root elongation. This was because seven and to a lesser extent three day dried stratification had much longer roots than the other treatments.

Most stratified dishes had higher proportions of 'Thriving' seedlings than the control (dried peaked at 7 days, wet only at 3). This implies there may be some benefit to seed vigour from stratification in *M. sinensis* seed.

Observations of the seed appeared to show specks of mould on or around the seed that had been stratified for a long time; however, there was not a significant increase in mould. Mould growth did occur significantly faster in stratified seed; this was a particular problem because the seed was not sterilised. Therefore, specks seen around the seed may have been mould growth but the seed did not undergo more mould damage, even though the mould had a head start. Consequently, if the seed had been sterilised stratification may have given a boost to germination percentage.

Both stratification and cold storage would benefit from further investigation, specifically to assess seed performance in stratification and to increase the time and possibly the temperature range in which seed is stored.

PRIMING

The results of the first replicated test gave a set of not significant negative effects from priming. The first test's only significant result was a reduction in seedling stem elongation from priming that was not found in the second, replicated, experiment. Therefore, the first test can be viewed as the impetus for the replicated test.

In this second experiment, there was a significant negative effect from priming on root elongation, mean F_v/F_m , and total fluorescence area, but not germination or stem elongation. It therefore appears that priming has a negative effect on the seed, and seedling growth. However, this could be due to priming speeding up the strongest seed and weakening the weakest seed (Sathish et al., 2011), which in the variable *Miscanthus* seed could lead to a drop in the average responses. This negative effect was confirmed in Section 6.3e below, when primed seed did not outperform control seed in the thermal gradient plate experiment and in SYN55 when primed seed did less well in field conditions.

The disappointing lack of results from the first experiment may be mitigated by two factors. Firstly, the effect on total fluorescence area implies an effect on stem growth that may not have been captured in the stem elongation measurement. Secondly, the stem and root measurements took place at 37 days, far longer than in the first test at 11 days. At this greater temporal difference, the elongations are not directly comparable measurements.

4.5C LIGHT AND DARK

SEED MASS ESTIMATION

When determining the seed mass from the model there was an inherent amount of variability. In both tests to determine seed mass from size, the scales could not be accurate enough for more than an estimate of the seed mass. When imaging them the exact orientation of the seed would greatly affect the size and the seed themselves were not necessarily of uniform density. The advantage of MARVIN was by averaging large groups of seed the inaccuracy of the scales and of the imaging was mitigated; however, by testing large groups of seed (200) the differences between groups were lost. This was especially true because it only counted the seeds to 97% accuracy. All the groups except the largest (group 1) had the same area and there was no clear difference in average mass.

The manual technique was much less time efficient, it also suffered much more from the weight inaccuracies highlighted above because it used individual seed. However, this technique did manage to separate the MX300 seed from the SYN55 seed. The increased variability of MX300 over SYN55 may be representative of the increased variability in seed morphology in an open crossed population compared with that of a synthetic crossed population. Further work would be needed to determine if these two seed sets were unusually different in size or if this technique could be used to approximately phenotype seed. A fitted

linear model was used to estimate the mass of each seed going into the dark burnout experiment. However, due to the inaccuracies of the model (R^2 of 0.34), the seed size was used in the dark burnout results too rather than mass, to use a direct observational measurement over a modelled measurement.

DARK BURNOUT

The dark burnout experiment indicated that there was no requirement for light for germination in SYN55. This is confirmed by the 'Dark or Red Light' on SYN55 below. The increased variability of germination visible after 200 hours in darkness may have been due to the difficulty in identifying seeds that had germinated, died, and gone mouldy 10 or more days ago.

While the proportion of 'Thriving' and 'Alive' seedlings at the end of the test did significantly change based on TiD, there was no evidence that this was anything other than seedlings obviously doing less well when they had been in the dark for longer. The number of dead seeds and seedlings showed an exaggerated form of this trend but still it just showed groupings where dead classed seed were more likely after more time in the dark. The effect of TiD on seedling dry weight appeared to show there might be a small boon to being germinated in the dark at around 48 hours. However, this benefit to a short amount of TiD during germination was not continued because seedling dry weight gradually decreased after 48 hours in the darkness.

The information on the average maximum elongation of *Miscanthus* seedlings without light is used in the model (Section 6.2g below).

An indication of how long the seeds' store of energy could last, would also be useful for the model (Section 6.2g). Seeds were determined by seed state to be able to survive while germinating for 14 to 20 days without light. Determining this limit with post light growth had a range of 10 to 24 days; however, the

reliability of this method may have been inferior. The problem of seeds that survive the dark re-growing from nearer the top of the stem may have inflated the regrowth of the few seeds between 192 and 336 hours that survived. In addition, seedlings that died may have gone mouldy enough by the end of the test to be non-measurable, leading to negative growth values.

At the end of the experiment, the largest seeds were statistically more likely to be classed as inactive while the smaller seeds were more likely to be living, so smaller seeds may overall have better germination. There was evidence of light leaking into two of the replicates of the last two time points, because they showed more life than the prior time points.

The significant effect on stem elongation of seedlings and seed size for the seeds that had grown in darkness for over 200 hours was small and inverse; this may be due to larger seeds having an increased chance of damage in the threshing process that then led to more mould. This higher level of mould and/or damage could have inhibited seedling growth. Another possibility for the larger seeds tending slightly towards shorter elongation while in the dark was that the seed was from a large synthetic cross. The variability of the synthetic cross may suggest that one of the parent plants had larger seeds and shorter elongation. Further study would be required to determine whether this was a real effect and its cause.

There was a significant effect of seed size on end dry weight of seedlings for any of the TiD tested, but only for normal sized seeds to do better. Overall, seed size, as far as bigger seeds surviving and growing better, seems to have little impact on seed survival and growth in darkness.

DARK OR RED LIGHT

The red-light/no-light experiment expands upon the dark burnout and indicates a genetic component to light dependant dormancy. The *M. sinensis* seed lots (SYN55 & MX300) showed no significant difference in germination proportion (Figure 4-47), this corroborated the findings of Christian et al. (2014). However, seed of an interspecies cross of *M. sinensis* × *M. sacchariflorus* (GNT14) showed a significant difference in germination dependent on lighting (Figure 4-47). This could be a heritable trait for light dependent germination present in *M. sacchariflorus* but not in *M. sinensis*. Further study of this could reveal if the presence of this trait would have an impact on field sowings of interspecific hybrids. *M. sacchariflorus* is more sensitive to flowering time signals (Jensen et al., 2011), which includes photoperiod; possibly showing *M. sacchariflorus* is generally more sensitive to photoperiod. Alternatively the effect could be due to the depth of primary dormancy of the seed being affected by harvest time leading to a light requirement (Ellis et al., 1985a), as Acosta et al. (2013) found the light requirement for germination decreased with storage time.

4.5d TAGUCHI INTERPRETATION

In order to produce an optimal condition for seed germination and early growth a Taguchi method experiment was carried out. This experiment entailed compromises of what to test and at what levels, because the number of factors and levels was limited by the basic design and practicalities of a Taguchi experiment. Levels of each factor were chosen to cover the full spectrum of effect; however, these limitations of the method did not allow for testing of light and water stress at as many points as would have been preferable.

The percentage effects of each input give different results depending on the output. This is as expected as different combinations of treatment should effect

seedling germination and growth differently. Stem elongation was slightly more affected by water stress than root elongation; this was expected because in Section 4.5b above roots were positively affected then negatively affected by water stress, yet stems were only negatively affected. The large impact on germination rate by light levels was odd given the result of the 'Dark vs. Red Light Germination Experiment' above, where light was not found to influence the germination of *M. sinensis*.

The large effect of priming in germination percentage that was not present in germination rate implies that priming does not influence the rate of germination, despite lowering the percentage of germination. The other main differences between germination percentages were water stress and light; both of these effects may be due to the seed adapting to the situation. Therefore, low light or low water germination causes germination speed to be more changeable, but it does not stop seeds germinating. This may be because light is an optional trigger for germination in this *Miscanthus* seed.

GI as a combination of the number of seeds germinated each day over seven days should mirror a combination of percentage and rate of germination; however, GI has a much lower effect from ABA and BR.

As F_v/F_m measured both total area photosynthesising and median photosynthetic activity per test, differences between the two should indicate the difference between size and health of the plants. The area was much more affected by ABA and water stress, demonstrating these factors affected the size of the seedlings much more than their health. As a hormone that negatively regulates growth, it is not surprising that ABA affected the seedlings' total photosynthetic area more than their median photosynthesis. This is particularly interesting for water stress, because it seems that the water stressed plants

were growing smaller or fewer of them were growing, rather than that median photosynthesis was reduced.

The median Fv/Fm level was more affected by priming and light level. This conveys the reverse effect to above; that priming and light impacted photosynthesis more than they did total area. For light, this is probably because the seedlings reacted much more to the test light due to growing in low light levels. Primed seeds and unprimed seeds are behaving differently for median Fv/Fm; this is the same as in Section 4.4b above. This is harder to explain but may suggest priming weakening the seedlings.

Stem to root ratio was most affected from water stress, which is understandable because this may force the plant to change the deployment of resources between above and below ground. ABA also had a large effect, which is expected as a growth hormone. Whilst not a large effect, it was the measurement most affected by auxin, which again as a growth hormone it is not surprising that it changed the ratio of root to stem.

It seems counterintuitive but lower light levels had a beneficial effect on all seed measurements; however, this was not zero light as in the preliminary experiments, here high light levels could have increased the evaporation rate and led to an interaction with water stress, this may need more investigation to resolve.

It is unsurprising that plants with low water stress performed better in all measures. Water stress had an important effect on outcomes particularly in the health and growth measurements.

ABA affected the most measurements. As expected, lower concentrations proved more effective; the positive effect on GI at 200 $\mu\text{g L}^{-1}$ can be discounted due to the low percentage effect. However, for germination rate and total germination percentage it had best performance when at 2 mg L^{-1} , suggesting that medium

concentrations of ABA may have positively influenced germination. ABA did not interact with GA on germination to a high sensitivity index; this could have been due to not enough levels being used, or a lot of noise in the germination data.

The greatest effect of GA was on rate of germination, for which a high level was best. This could be because GA is promoting germination but only to a certain extent, after which it has filled the capacity of the hormone channels and has no further effect. However, a higher level was optimal for germination percentage indicating that more GA was maintaining a positive effect. There was little to no effect of GA on stem elongation unlike in the range finding experiments, as stem elongation was controlled largely by the physical factors and ABA. GA did also interact with auxin in four measurements on the seed, this was expected as auxin can counteract GA in the control of dormancy in other seed (Shu et al., 2016).

Auxin's best performance in root elongation and germination percentage at the very low level can be explained because auxin may improve growth of root hairs, but not improve total root elongation, possibly hindering it (as in Section 4.4a). Auxin's good performance at a high level for stem to root ratio, germination rate, and stem elongation is because moderate to high levels positively affect the stem elongation, and negatively affect the root elongation; thus positively affecting the stem to root ratio for the germination rate. Auxin may have a stimulating effect that increases germination rate but does not increase the total percentage of germination.

Between 0.75 and 1.5 mg L⁻¹ of BR, three measurements were optimally affected; root elongation, Fv/Fm area, and germination percentage. This suggests that there is an optimal level for stimulating *Miscanthus* seedling germination and growth around 1 mg L⁻¹. BR did interact with GA to a high level in many

metrics; this supports Shu et al. (2016) who suggested that BR may enhance the effect of GA. BR influenced about 10% of the photosynthetic median, and was optimal in this at its very high level; this suggests that BR can increase the activity of the plant if the plant is oversaturated with it. This increase in activity may not be good for the plant because the level best associated with most measurements was 10x less concentrated.

Seed priming was added as an extra binary factor; the most interesting outcomes of priming were unprimed seeds triumphed in GI and germination percentage. Both of these outcomes indicate that priming negatively affects germination.

The OEC Taguchi analysis showed the optimal conditions for seed germination and early growth. Four of the factors studied had more than a 5% effect on the OEC Taguchi. The greatest of these was water stress induced by PEG, which as expected was best when low. The next largest effect was ABA and as expected from both the range-finding tests and the literature, ABA was optimal when at its lowest level. The third largest effect was from light level, which was optimal when reduced with the muslin, as was clearly shown in the individual metrics Taguchi analysis. Lastly, priming did have a notable negative effect on the OEC in the Taguchi analysis. The fact that auxin, GA, and BR had notable interactions may have prevented them from contributing a clear percentage effect, rendering their optimal levels less reliable.

Further investigation would be required to test each of the interactions on the Taguchi and the explanations of the reactions individually; this study should provide a multidimensional framework as a basis for further study.

5 CLUSTER SOWING TO IMPROVE ESTABLISHMENT

5.1 INTRODUCTION

In a perennial crop establishment, gaps influence yield for the lifespan of the crop: when *Miscanthus* is planted from rhizome, there are gaps in fields (Anderson et al., 2015; Caslin, Finnan, & Easson, 2011; Clifton-Brown, Breuer, & Jones, 2007). Once a gap is formed, it can expand over the first three years, because replanting is not a good commercial option, it is critical to establish a complete and uniform field at the start. Switchgrass (*Panicum virgatum*) is often oversown to account for low establishment (Vogel, 1987). *Miscanthus* seed is known to have low establishment and high thermal requirements (Clifton-Brown et al., 2011) leading Anderson et al. (2015) to suggest it was impossible in much of northern Europe. Therefore, in this study UK direct sowing of *Miscanthus* seed was oversown in rows at 300 seeds per meter (see 'Direct Sowing Agronomy Trial' and 'Multi Genotype' trials (Chapter 6 below)); this resulted in uneven emergence, visible in Figure 3-14. Therefore, a method of sowing the seed in tightly spaced groups (referred to as clusters), was postulated to avoid the problem of inconsistent rows by having individually spaced plots, but sowing clusters of seed in each. Two things needed to be determined: If inter-seed competition affected germination and establishment, and the number of seeds per plot required to produce a plant (referred to as strike rate).

This chapter covers the investigation of the effects of seed proximity and grouping on germination and early growth in *Miscanthus*. This was undertaken to determine if high density sowing could overcome germination and establishment problems. This investigated both how many seeds need to be sown to produce a successful established seedling, and if the seeds compete adversely affecting the seedling germination and establishment. This is relevant to direct sowing of seeds in the field in particular, but also the sowing of seed in tray modules for plug planting where often more than one *Miscanthus* seed is used to ensure at least one seedling (thus cutting the cost of redundant tray space).

Miscanthus is known to be competitive with other species, Chou & Chung (1974) found that in clearings between bunches of *Miscanthus* most seedlings were still *Miscanthus*.

5.1a CLUSTER SOWING RATIONAL

Sowing *Miscanthus* seed in clusters of multiple seeds could alleviate some of the issues associated with low germination rates. In the sowing trials (Sections 6.3a & 6.3b below), and in the laboratory (Section 6.3c below), sowing the seeds in a line resulted in very uneven emergence and growth as visible in Figure 3-14 and Figure 6-2. This irregularity would be a problem for commercial sowing because uneven emergence leads to a drop in harvest yield; this is most likely due to inter-plant competition and exposure to differing levels of environmental stress. This effect could be exacerbated due to the interaction of wind with canopy formation; shorter stems allow wind to hit neighbouring tall plants causing field edge effects at multiple points in the field. For example, in corn it has been suggested that replanting would improve yield on a very uneven crop (Nafziger, Carter, & Graham, 1991) but this is unlikely to be suitable for perennial crops such as *Miscanthus* that have a long growth season and in which slight differ-

ences in early growth may be amplified over many years growth. Sowing seed in clusters, where more than one seed are sown together to establish a single plant, would therefore provide an increased likelihood of synchronised emergence and establishment. Direct sowing of extra seed is used in Switchgrass (*Panicum virgatum*) to produce a full yield from seed with a low germination rate (Vogel, 1987). By oversowing in batches within a small area, the plants will be evenly spaced, and by picking the right number of seed to sow, it may be possible nearly to guarantee a plant establishes at each sowing position, even for a low germinating cross.

5.1b EFFECTS OF SEED COMPETITION

Seed clusters need to have enough seeds that each plot will have at least one plant for a given germination percentage of the seed lot, under field conditions; some batches in a location may have many plants in them. When several seed germinate, traditional wisdom is that the seedlings will compete with the strongest seedling eventually winning out. However, it is not known if in *Miscanthus* the strongest seedling will outcompete the others and produce a better plant or if all seedlings will grow competing for resources and leading to a smaller crop. Even if the strongest seedling wins out the period of competition may slow the plant compared to seedlings that did not have to compete. *Miscanthus* plants have separate root systems with dominant *Miscanthus* plants extending their root systems into a larger territory (de Kroon, Mommer, & Nishiwaki, 2003, p. 228). This may have an effect on root growth during establishment because they may need to expand sideways below ground, while above ground upwards is the primary direction of growth. This competition for root space during establishment might make a difference to successful overwintering.

ALLELOPATHY AND ALLELOCHEMICAL EFFECTS

Allelochemical effects are chemical effects between plants. These can be positive (Hussain et al., 2007) as well as negative chemical effects from one plant to another. These effects are often used to reduce competition as in the novel weapons hypothesis where exotic invaders take advantage of native susceptibility to allelopathy (Bais et al., 2003). Many plants adopt allelochemical effects, importantly in *Miscanthus* Chang-Hung & Yi-Feng (1991) found samples from *M. transmorrisonensis* had significant effects on germination and growth on many other species, suggesting an allelochemical effect. Before this Chou & Chung (1974) found that chemicals such as acetic acid leached out of the stems and roots of *M. floridulus* and affected growth in lettuce. This is exhibiting interspecific competition; but any chemicals that limit germination and / or growth in other species may also affect *Miscanthus* seed, or *Miscanthus* could have explicit autotoxicity. Autotoxicity would negatively impact a plant's own progeny but it may be useful to ensure spatiotemporal distribution of seedling establishment Ervin & Wetzel (2000). For instance Giant Parramatta grass has been observed to inhibit its seedlings as well as those of other species, but this was not proven in germination tests (Andrews, Jones, & Whalley, 1997). Ervin & Wetzel (2000) found autotoxicity to seedlings from tissues of the common rush (*Juncus effuses*).

There is a diverse range of potential chemicals for allelopathic effects that have been located all over the world in very different species. In wheat (*Triticum aestivum*) Siddiqui et al. (2009), found potential allelochemicals resembling flavonoids, waxes, tannins, and phenolic acids inhibited seedling growth. Bais et al. (2003) found catechin (a flavonoid) to be used for interspecific allelopathy in *Centaurea maculosa*. Acetic acid was found in plant tissues and soils of *M. floridulus* as well as and low concentrations that did not give significant

results of coumaric, ferulic, hydroxybenzoic, syringic, and vanillic, acids (Chou & Chung, 1974).

5.2 METHODS

The testing of the effects of seed clusters on germination and growth started with laboratory tests carried out in water, leading to soil testing in a controlled environment then finally a field trial.

5.2a TESTING SEED FOR ALLELOPATHY IN WATER

For the entirety of the laboratory testing, a Fitotron 120 Plant Growth Chamber was used. The temperature was kept at 25°C with constant fluorescent light at 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (measured with a Skye SKP 215 sensor).

PRELIMINARY SEED CLUSTER GERMINATION INHIBITION EXPERIMENT

To test for germination inhibition from allelopathy in *Miscanthus* seed, a simple, well-replicated laboratory experiment was conducted using 12 5 × 5 25 compartment 100 mm Petri dishes. Each compartment of each dish was filled with 2 ml of sterile distilled water. In 24 of the 25 compartments in each dish, one MX300 seed was placed, and in the one random remaining compartment, twenty-four MX300 seeds were placed. The MX300 seed was used unsterilised because sterilising the seed with bleach removes colouring from the seed coat and it is possible that it might also remove allelopathic chemicals, and because field sown seed would not be sterilised, unsterilised seed were a fairer approximation to field sowings. Germination was then observed every 24 hours for 96 hours.

The results of each time point were analysed for a normal distribution with a Shapiro-Wilk test. If they were distributed normally, the proportion of seed

germinated in the twelve-clustered sets of twenty-four seed was compared with the twelve sets of twenty-four single seed in a Two Sample t-test. If the distribution was not normal, a Wilcoxon rank sum was used instead. Each time point was treated independently because over time effects were not the purpose of the investigation, but at what time any possible effects would be seen was unknown. Germination Index (GI), a combination of germination speed and number as described in Section 2.1a above, was used to highlight differences in germination over the whole experiment.

DETERMINING SEED CLUSTER GERMINATION INHIBITION WITH STERILISATION

Mould had taken hold in some of the compartments during the 'Preliminary Seed Cluster Germination Inhibition Experiment', potentially introducing an additional source of germination inhibition. Another experiment was planned which used sterilised MX300 seeds (technique in 2.4 above), to limit the effect of mould. The same setup was used but with five replicates instead of twelve, because the effect of batches of seed sown together was significant ('Preliminary Seed Cluster Germination Inhibition Experiment' in Section 5.3a below). Germination was observed every 24 hours for 96 hours and at 168 and 264 hours. Longer germination periods were used to test if germination had stopped because in the 'Preliminary Seed Cluster Germination Inhibition Experiment' there was a decrease in the difference between single seed and batch seed germination at the final time point. This difference in the final time point suggested that the lower germination percentage at early time points could have resulted from a slower germination effect. This was analysed in the same way as the 'Preliminary Seed Cluster Germination Inhibition Experiment' above, with each time point, tested separately for normality then tested for a difference in the means.

DETERMINING THE DENSITY OF SEEDS REQUIRED TO TRIGGER ALLOPATHIC EFFECTS

The two experiments above investigated whether an allopathic effect was present when germinating *Miscanthus*. Further experiments were carried out to determine at what seed quantity the inhibitory effect of seed number occurred. An experiment was performed using a 5 × 5 25 compartment 100 mm Petri dish. This experiment had five replicates of each of 5, 10, 15, 25, and 50 MX300 unsterilised seeds, which were randomly assigned to a compartment. Unsterilised seed were chosen, because the effect of sterilising the seeds had not lessened the effect observed in the prior experiments and the sterilisation added an extra factor. To provide more data on the observed critical range just below 24 seeds a second test used five replicates set up in the same way but the number of seeds per group were 15, 17, 19, 21, and 23. Both these tests were germinated for 72 hours and counted every 24 h. The increased resolution in the second part of the test was to identify if there was a specific concentration of seed that caused a change in the germination inhibition. Statistically the groups at each time point were tested with a one-way ANOVA and if significant and normally distributed, a Tukey's HSD was conducted to test if there was a specific point where the proportion of seed germinating changed.

5.2b SEED COMPETITION SOIL EXPERIMENT WITHIN A CONTROLLED ENVIRONMENT

In previous experiments, seed was germinated in water. An experiment was performed to determine the effect of competition using a realistic substrate. This experiment tested the following: firstly, if germination in soil was negatively affected by increasing the number of seeds in the cluster (oversowing). Secondly, the number of seeds required in a cluster for a strike rate of 100% to be achieved. Thirdly, whether, the root was negatively affected by below ground

competition. Lastly, if there were positive or negative effects on the growth of the plant when sown in a cluster.

This experiment used 3 mm sieved and autoclaved soil collected from the field of the 'Direct Sowing Agronomy Trial' and 'Multi Genotype Direct Sowing Trial' below (Sections 6.3a & 6.3b), this was put into trays 50 mm deep. Four replicates were placed in standard seed trays (360 × 210 mm), with 32 (4 × 8) divots in each. Into each divot was placed 1, 5, 15 or 40 MX300 seeds (8 of each cluster size). This was done randomly for each tray; the trays were placed in a line in a Fisons Fitotron 600H plant growth chamber. Each tray was then covered with mulch film and kept constantly light (from both the tungsten and fluorescent lighting $170 \pm 10 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Caffarra et al., 2011)), at 25°C for 32 days. Germination/tillering and elongation of tallest stem per set of seeds sown *in situ* were recorded at a decreasing frequency (2, 4, 7, 10, 14, 22, 32 days). On day 22, ten days before the end, the film was removed; this was because the seedlings had grown too much and the film in the field would be beginning to degrade by this point (without sun the film was not degrading in the cabinet). On day 32, the seedling clusters were dug up and the elongation of the tallest stem in each cluster was measured. This process was carried out both for total elongation to the end of the leaf, and height to the last ligule. The purpose of this dual measurement was to assess top growth as effectively as possible and the elongation measurement had been used in Petri dish germination experiments in Chapter 4, because it seemed more effective on very small seedlings (see Section 2.1d for details). The length of the longest root in the whole seedling cluster was recorded as a measure of below ground growth. From the roots, the number of individual plants was counted, to get an accurate final germination and a score of tillering. Tillering may decrease with above ground competition. The seedling clusters were dried at 70°C for 48 hours and dry weights were

determined. Then the roots were removed and the roots' dry weights were determined separately from the stems, to give the proportion of mass above and below ground. The environment in the cabinet was measured using a 'Campbell 1000' data logger with two type T thermocouples (tray 2 & 4) and 2 reflectometers (tray 1 & 3), soil moisture was maintained at higher than the real field conditions in Chapter 6 below so water would not be a limiting factor.

The experiment did suffer a power cut [graph Appendix L], that dropped the temperature and light levels for 48 hours, the experiment continued because the effect of the outage was applied evenly across all plants and replicates.

Statistically the controlled environment experiment was also used to test four questions: Firstly, if germination was negatively affected by increasing the number of seeds (oversowing). The strike rate of plots with different numbers of seed was also calculated as a percentage. This was statistically tested over the first 7 days with a generalized linear mixed-effects model using a binomial distribution ('lme4' R package (Bates et al., 2015)). This was then checked with a one-way ANOVA, by testing the change in tillers between 48 and 168 hours; differences were grouped using a Tukey's HSD. The number of plants counted at the end of the experiment was also compared the number of seedlings per seed sown and tested with a one-way ANOVA.

Secondly, the experiment was used to test if the root size was negatively affected by below ground competition when grown in a group. This was analysed for overall dry weight of roots per seedling cluster at the end of the test, the dry weight of roots divided by the number of seeds sown, maximum elongation of roots in each seedling cluster, and the maximum elongation per plant. All these were analysed with a one-way ANOVA and then compared with a Tukey's HSD if significance was found.

Thirdly, if there were positive or negative effects on the growth of the plant based on the size of the cluster. This was measured by comparing elongation rate during the experiment with a linear mixed-effects model from the 'nlme' package (Pinheiro et al., 2016), as well as elongation, height, total tillers, stem dry weight, and overall dry weight at the end of the experiment. These were analysed with a one-way ANOVA, for both total affect and the effect per plant. These were then compared with a Tukey's HSD if significance was found in the ANOVA.

Lastly, the correlation between the height of stems at the end of the test and the elongation to the leaf tip was tested with a one-way ANOVA and a Pearson's product-moment correlation (PPMC). This was done primarily to check the validity of measurements of stem elongation used elsewhere in this study.

When calculating measurements divided by the number of plants, the calculation was done on a per-cluster basis then averaged per rep, to avoid problems with plots that failed.

5.2C FIELD TRIAL OF CLUSTER SOWING SEED

Although more realistic, the controlled environment experiment still does not entirely replicate field conditions. A further experiment was carried out to investigate longer-term growth and survivability over winter, and to investigate properly strike rate in the field, where germination is often low (see Chapter 6). A field near Aberystwyth was prepared by spraying with Roundup™ then power harrowing. MX300 seed was used in the field as well as in the laboratory because of the larger seed numbers available and its higher germination rate (see 6.3e below). The seed was direct sown by hand in clusters of 5, 15, and 40. Single seed clusters were not used because the germination percentage in the field was known to be lower than in the controlled environment; therefore so few

seed would be expected to germinate as to make most or all plots fail, which would not provide useful information for statistical analysis. The field design was four replicate blocks, each block consisting of two rows (covered by a single piece of film) containing 45 clusters each. Thirty of each of the three different cluster sizes (5, 15, and 40 seeds), these were distributed randomly within the 90 cluster locations of seed per block. These were then covered with mulch film. Hand weeding was required intermittently in the summer following the sowing; commercially weed control would have been carried out with a broad leaf herbicide (e.g. Calisto™) but the effect of herbicides on *Miscanthus* post emergence might not be safe, and it was decided not to risk affecting the crop (Anderson et al., 2010). The trial was harvested at the normal *Miscanthus* harvesting time of late winter/early spring (March 2016) (Clifton-Brown & Lewandowski, 2002). At the end of the test, the number of plants per cluster was counted as well as the number of tillers; it was not possible to measure the height of the tillers due to winter senescence, which resulted in a number of tillers snapping, and damage from wind/rabbits. Consequently measuring the effect of sowing density on yield as reported by Vogel (1987) was not possible. The tiller number was counted to include tillers longer than 1 cm; this is shorter than the standard for tiller counts that tend to be multiple centimetres (5-10 cm). The plants were also re-growing tillers for the new season and so all tillers were included as a measure of success. The above and below ground growth from each seed cluster was harvested and washed to remove soil, then the length of the longest root was measured and a visual assessment recorded of the plants' health (zero to four [Dead - Thriving]). The samples were then dried (70°C for 48 hours) and the below ground dry weight of biomass determined.

In most of the results, a mean per block has been used for cluster size, giving an n of four. Occasionally the un-averaged data from all individual clusters are used, if so any significance of the block effect will be stated. The results are separated into three sections as determined by different questions:

Firstly, the data was analysed to determine if the number of seed sown in a cluster affected the presence or absence of a plant (strike rate). Output from this analysis was used to determine strike rate, and if the number of plants in a plot was proportional to the number of seed sown. To analyse the number of plants per cluster, a PPMC was calculated and tested with a blocked one-way ANOVA test. Then the number of plants per seed sown was analysed, this was done in the same way. The strike rate was used to attempt to predict the number of seed required per cluster to achieve a 100% strike rate; this was done by applying several regression models to show the difference in fit and prediction of each.

Secondly, the data was analysed to determine if root growth was affected and presumably limited by the number of seed sown and therefore the number of plants growing. Both the root dry weight and the length of the longest root were analysed with one-way blocked ANOVAs, if significance was detected a Tukey's HSD test was used to determine the effect the three different seed numbers sown; if the ANOVA has a significant effect from blocking it was reported. This was done for the total per cluster size and the average per plant within each cluster size, in order to determine if there was an effect independent of the number of surviving plants after winter. The root dry weights from all individual seed clusters were plotted against the number of plants counted in every clusters plot to give an indication of if at the plot level the roots' mass increased lineally to the number of plants present in the plot. This should be the case if there is no adverse effect on below ground size from more plants in a plot.

Lastly, the mean number of stems per seed sown, and the mean number of tillers per plant was analysed to determine how the above ground growth was affected by the number of seed sown in a batch and consequently the number of resulting plants. Both were analysed with a one-way ANOVA using blocking, if there was significance a Tukey's HSD estimated groupings. The average number of stems per cluster plot was correlated with the number of seed sown using a PPMC. The visually assessed state of the seedlings was graphed with a stacked bar plot to visually represent the relative frequencies of each state for each cluster size. This method was used, because seedling state was a somewhat subjective measure particularly because the seedlings at the start of the second season in the field did not fit into the categorisation tree in Appendix K.

5.3 RESULTS

5.3a TESTING SEED FOR ALLELOPATHY IN WATER

PRELIMINARY SEED CLUSTER GERMINATION INHIBITION EXPERIMENT

The number of seed germinated at 24 and 48 hours followed a normal distribution, so a Welch Two Sample t-test was applied between the single and clustered seed for both times. This resulted in significant differences at both $P < 0.01$ and $P < 0.001$ respectively. A Wilcoxon rank sum test was performed on the 72 and 96-hour germination results, as these were not normally distributed. The results of this were significant for both time points ($P < 0.01$), indicating that a significantly higher proportion of the single seed germinated at each time point. This suggests that the presence of multiple *Miscanthus* seeds had some inhibitory effect on germination (Figure 5-1).

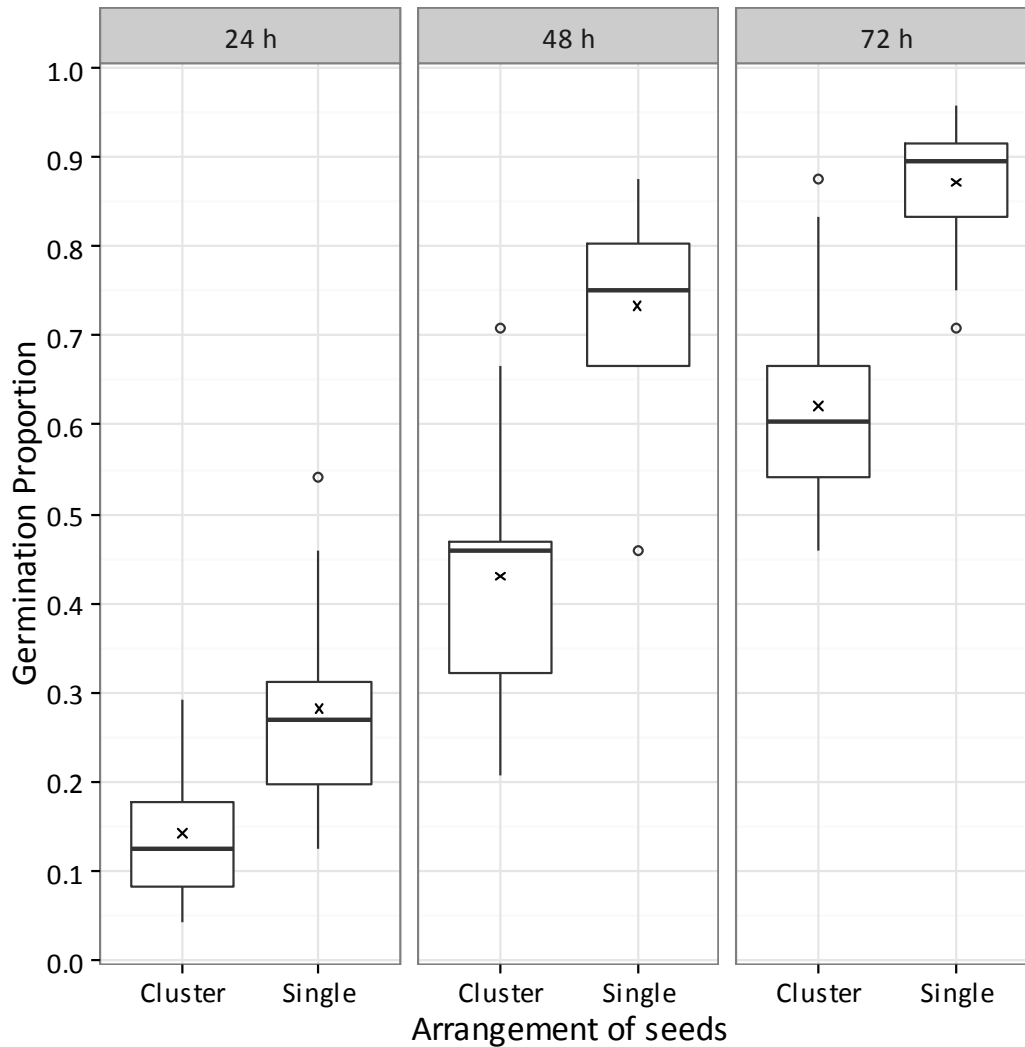


Figure 5-1: Boxplot of the proportion of MX300 seed germinated at 24, 48 & 72 hours comparing germination rates from clusters of 24 seed and 24 single seeds. Each boxplot represents the distribution across twelve replicates; the mean is indicated by an x.

As mould was starting to grow by 96 hours, this time point was discounted. At 72 hours, the twelve replicates of 24 single seed that were germinated in isolation had a mean germination of 87.2 ± 2.3 % (SE) and the twelve replicates of 24 clustered seed had a mean germination of 62.2 ± 3.7 % (SE). The GI (Sections 2.1a & 5.2a above) was calculated from the means of each set over time; for the single seed lots GI was 3.18, and for the clusters of seeds 1.91.

DETERMINING SEED CLUSTER GERMINATION INHIBITION WITH STERILISATION

The follow up experiment used a standard bleach sterilisation (Section 2.4 above), with later time points added to check whether clustered seed continued to have lower germination. It was noted that the germination was levelling off by 96 hours and had levelled when checked at 264 hours (Figure 5-2). When germination responses were compared between sterilised and unsterilised seed, there was more difference between mean levels of germination than was seen in the unsterilised seed, and overall the differences were more significant (see Figure 5-3). The proportion of germination within each time point was normally distributed, therefore a Welch Two Sample t-test was conducted on the data from the time points at 24 h ($P < 0.01$), 48 h ($P < 0.001$), 72 h ($P < 0.001$), 96 h ($P < 0.01$), 168 h ($P < 0.01$), and 264 h ($P < 0.01$). All of these germination proportions were significantly different and all were higher in the single seed than the cluster (Figure 5-2).

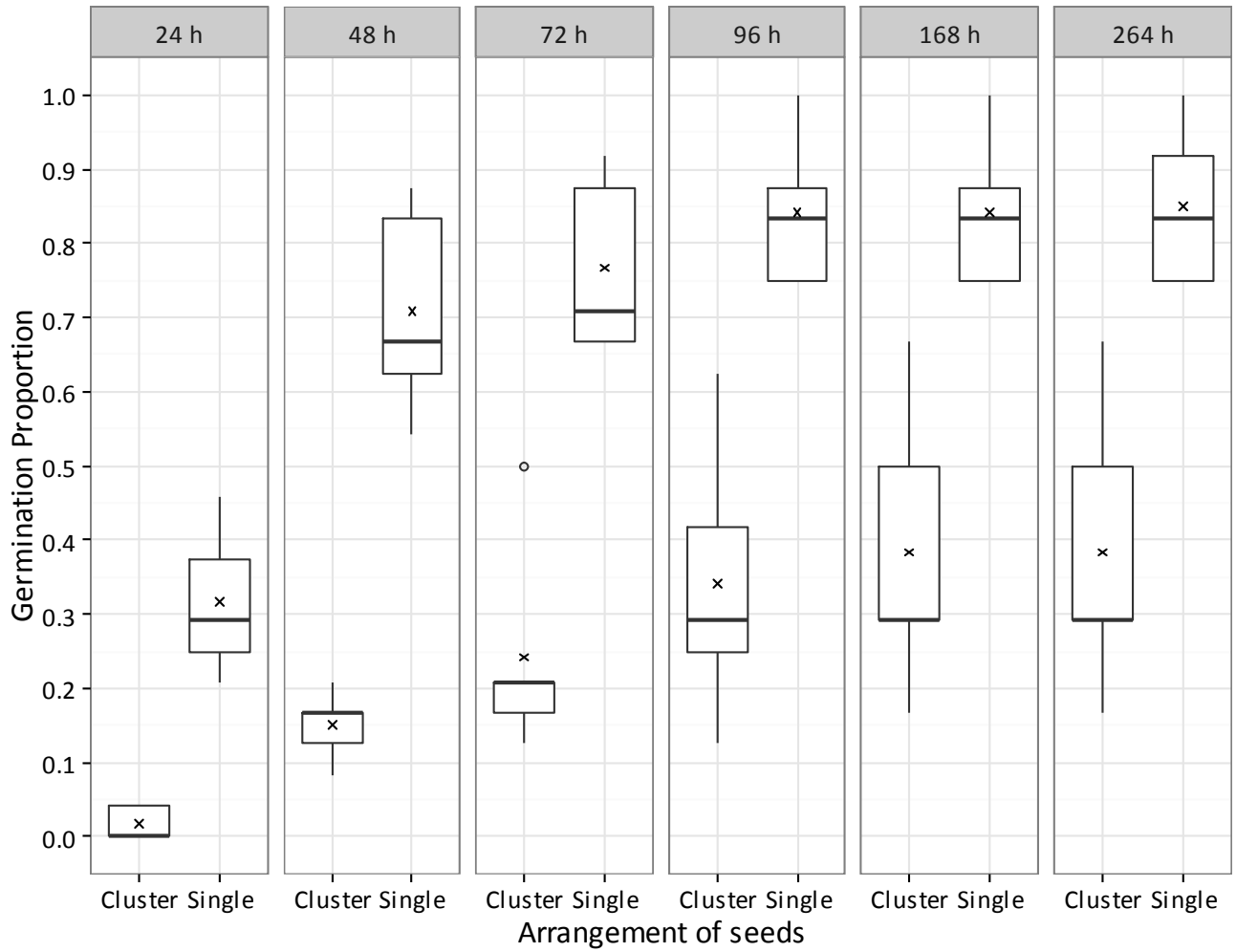


Figure 5-2: Boxplot of sterilised MX300 seeds germination proportion over eleven days, each time point showing single and cluster variation. Each boxplot represents the distribution across five replicates, the means are marked with an x, and the time points are not equally spaced.

There was still mould observed in this test but it was at much lower levels than in 'Preliminary Seed Cluster Germination Inhibition' Section 5.3a above. However, mould still grew later particularly in the compartments with more seed.

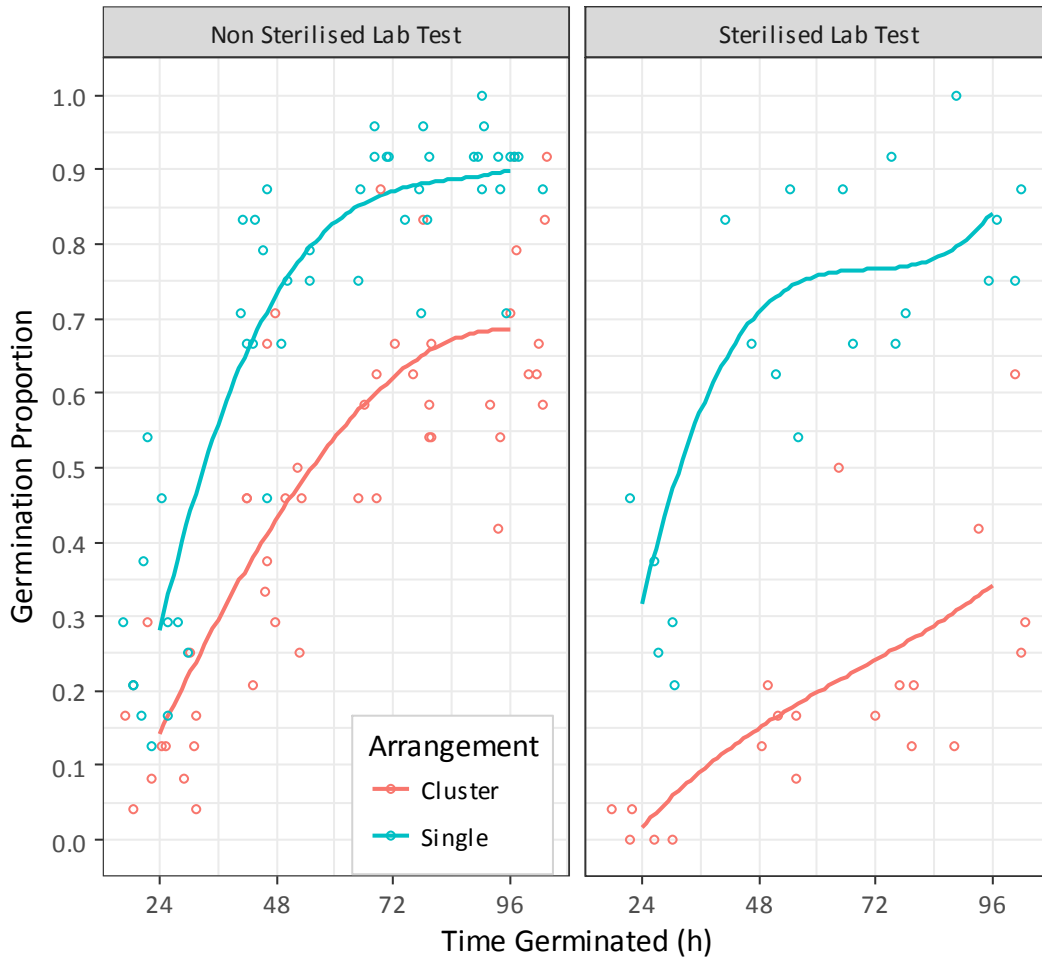


Figure 5-3: Both the sterilised and the first un-sterilised experiment, with a third order polynomial fitted. The points have been moved a little on the x-axis to make overpoted points visible.

The single seed germination did not appear to catch up with the clustered seed over a longer period (Figure 5-3). When comparing the non-sterilised to sterilised seed (Figure 5-3), the germination proportion from clusters of single seed is lower for sterilised seed but is in the same germination range; however, the clusters of 24 seeds are in a lower range when sterilised. If both experiments' data is combined and compared with a two-way ANOVA there is a significant effect of sterilisation ($P < 0.001$) and an interaction of seed number and sterilisation ($P < 0.01$) where sterilisation has more of an effect on clusters of 24 seed.

DETERMINING THE DENSITY OF SEEDS REQUIRED TO TRIGGER ALLOPATHIC EFFECTS

The testing of a variety of numbers between 5 and 50 seeds for germination differences revealed a pattern with higher and lower germinating categories (see Figure 5-4). A broad range test was used to identify approximately how many seeds caused a noticeable decrease in germination, and then a narrow range test focused on the specific amount of seeds that appeared to cause the drop in germination.

In the broad range test (seen in red in Figure 5-4) by 48 and 72 hours the higher and lower categories seemed defined. Five to fifteen seed were germinating at a higher rate; $\sim 80 \pm 15.1\%$ (SD) at 72 hours, while seed in lots of 35 and 50 were germinating around $59.2 \pm 11.8\%$ (SD) at 72 hours. This was significant when a one way ANOVA was used on the numbers of seed per compartment for the broad range test $P < 0.05$.

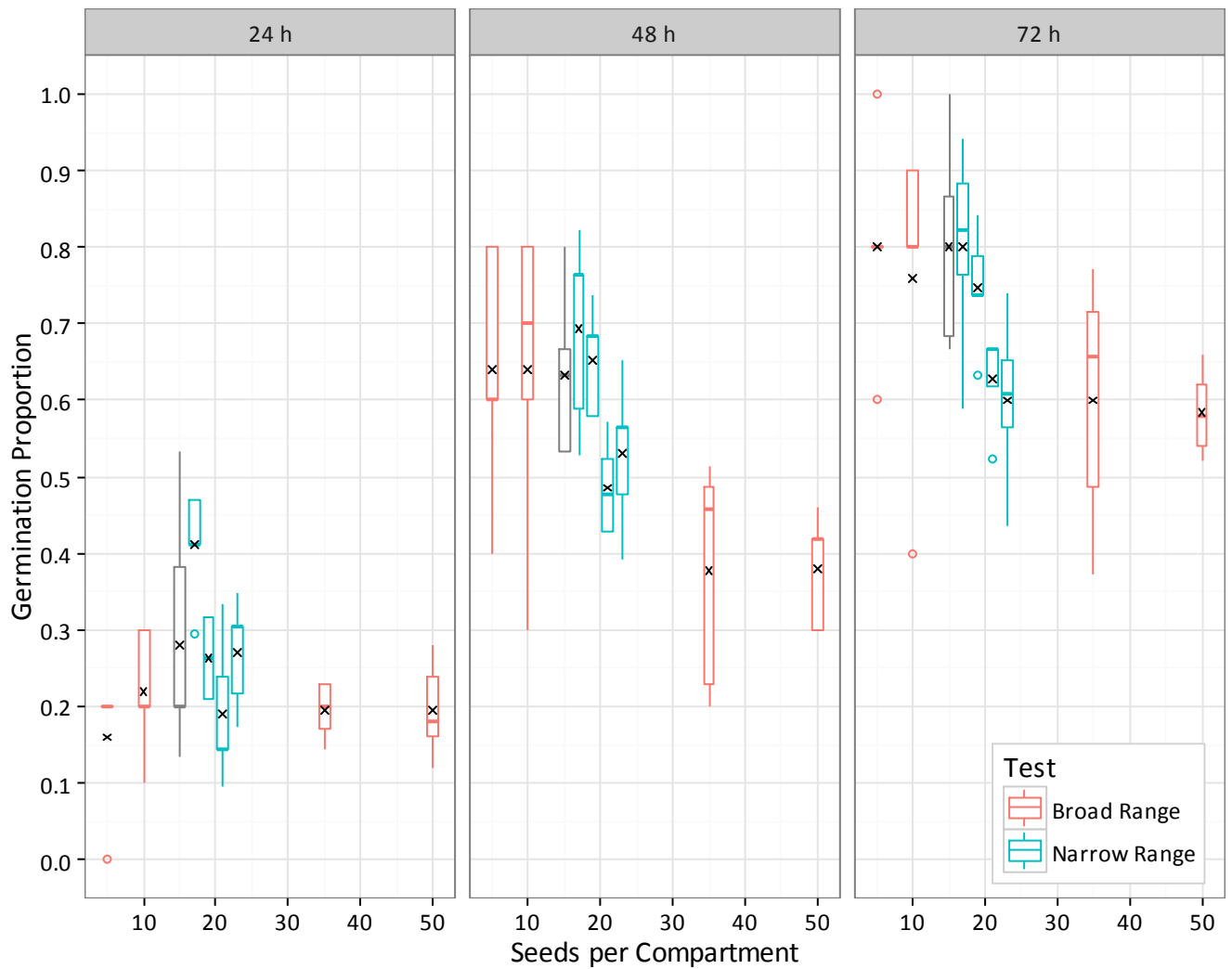


Figure 5-4: The proportion of seed germinating when sown in clusters of varying size. Two tests are shown the broad test in red with 5 to 50 seeds, and the narrow 15 to 23 seeds test in blue, the grey bar represents the time point that was covered in both tests. The data is shown separately for 24, 48, and 72 hours. The boxplots represent five replicates; means are indicated by an x.

When testing the narrow range (seen in blue in Figure 5-4) of 15 to 23 seeds, germination still fell into higher and lower categories. The division occurs at around 20 seeds per compartment, with compartments with more than 20 seeds germinating at $76.9 \pm 11.1\%$ (SD) at 72 hours, and compartments with less than 20 seeds germinating at $61.4 \pm 8.7\%$ (SD) at 72 hours. The number of seeds per cluster had a significant ($P < 0.05$) effect on germination at 72 hours when

tested with a one-way ANOVA, in the broad range test. The point of difference was analysed with a Tukey's HSD but this found no significant groupings. The narrow range test had a more gradual change in germination percentage (Figure 5-4). When tested with a one-way ANOVA it also had a significant effect of cluster size ($P < 0.05$), but a Tukey's HSD did not produce any significantly different groupings. These results suggest that there is a cut off point for the number of seeds in a cluster, after which germination decreases by ~20%.

5.3b SEED COMPETITION SOIL EXPERIMENT WITHIN A CONTROLLED ENVIRONMENT

Testing the seed within a controlled environment on soil as a realistic medium was performed as the next step towards the field trial.

GERMINATION

The strike rate of plants at the end of the test was high, all sowings except those of single seed had a final strike rate of 100%; single seed sowings had an average strike rate of 59%.

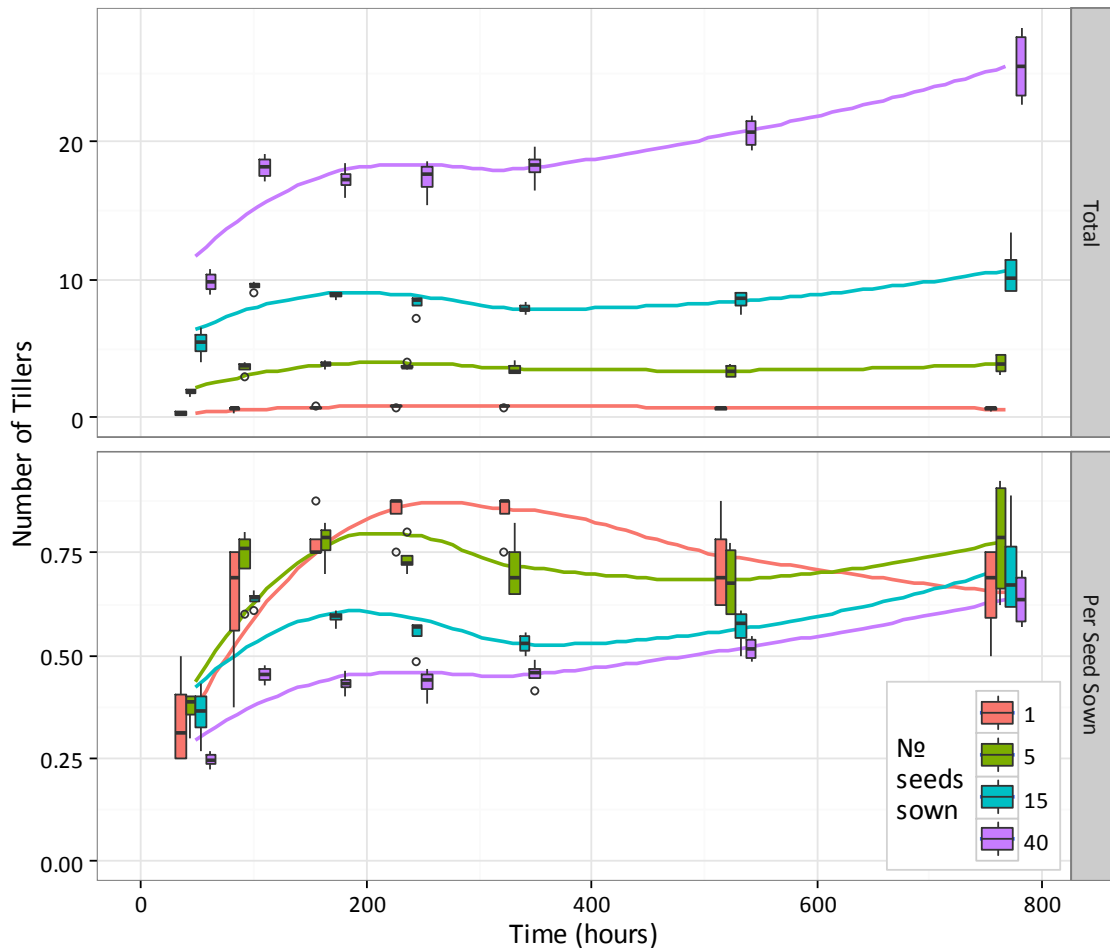


Figure 5-5: The number of stems as counted during the experiment, the tiller count is used as a proxy for germination in the first 200 hours. The total average number tillers per cluster for each treatment in the four replicates (top). The stems divided by the number of seed sown (bottom). A simple loess line has been fitted to show the trend.

The bottom panel in Figure 5-5 shows that single and five seed clusters initially produced more tillers per seed sown, but after 600 hours, all treatments produced similar average tiller numbers. There appears to be three stages of tillering: Firstly, a germination phase, where all cluster sizes increase their tiller number, this occurred at less than 200 hours. Secondly, a reduction phase, where weaker seedlings died, this is particularly notable between 200 and 400 hours. Thirdly, a tillering phase, where the tiller count increased, not through new seeds in the cluster germinating but from the existing plants producing

new tillers, seen at 400 to 800 hours. Therefore, to count germination, tiller counts up to 200 hours were used.

When the tillering per seed sown (Figure 5-5 (bottom)) was tested with a generalized linear mixed-effects model, the cluster size had significant effect for the number of tillers over the first 200 hours ($P < 0.01$), and obviously had a significant effect of time on tillering ($P < 0.01$). To check if the change in tillering over the first 200 hours was cluster size dependent, the change in tillering between days two and seven was tested with a one-way ANOVA, and found a significant difference based on cluster size ($P < 0.01$). A Tukey's HSD showed two significant groupings; the slower tillering group contained 15 and 40 seeds per cluster, while the faster tillering group contained the treatments 1, 5 and 15 seeds per cluster. The faster tillering group had a 44, 41, and 23% increase in tillering between days two and seven respectively, the slower group had a 23 & 18% increase in tillering between days 2 and 7 respectively.

The average total number of plants per cluster (as counted at the end of the experiment) was obviously higher when more seed was sown (Figure 5-6 (top)). This effect can be seen in the total stem count over time (Figure 5-5 (Top)). Total number of plants was tested with a one-way ANOVA and was significant ($P < 0.0001$). A Tukey's HSD was performed; this produced four groups, in which the group with the highest number of plants corresponded to the biggest cluster size, reducing with cluster size.

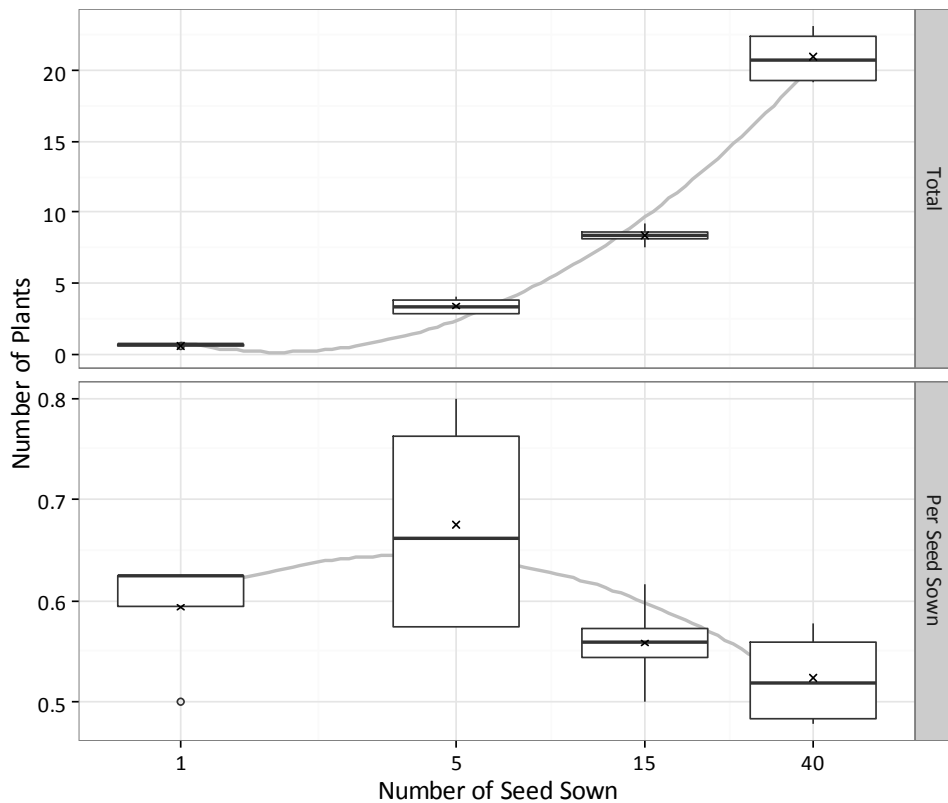


Figure 5-6: A boxplot of the mean number of plants counted at the end of the ‘Seed Competition Soil Experiment within a Controlled Environment’. The x-axis is on a log 10 scale to better show the range and a second order polynomial fit has been added in grey. The top pane shows the total mean plant number over the four replicates, while the bottom pane shows the proportion of plants per seed sown.

The effect of seed number is clearer when the number of plants is divided by the number of seeds sown (Figure 5-6 (bottom)). The number of plants per seed sown at the end of the experiment differed noticeably between cluster sizes but was not quite significant to 5% when tested with a one-way ANOVA ($P = 0.053$). Figure 5-6 (bottom) may imply that at around fifteen seeds per cluster there is a drop in germination causing a lower number of plants. This is a similar level as was seen in the ‘Testing Seed for Allelopathy in Water’ (Section 5.3a above), but this experiment was not sensitive enough to confirm it. From Figure 5-6 it would appear that five seeds produces the highest number of plants per seed sown, but with only four replicates, it is not possible to confirm this. As the

strike rate was 100% for 5 seed clusters and 59% for 1 seed clusters, 2-3 seed would be sufficient in this environment.

ROOT COMPETITION

To determine if there was an effect of below ground competition, the roots from the 'Seed Competition Soil Experiment within a Controlled Environment' were dry weighted and the root elongation per cluster was measured (Section 2.1e above). The dry weight of the roots did have a significant difference between cluster sizes (the data was \log^{10} transformed and a one-way ANOVA was used, producing a significance of $P < 0.01$) (Figure 5-7 (top left)). A Tukey's HSD revealed that clusters of 1 and 5 were in the lower weight group with 5, 15, and 40 in the higher weight group. The effect on dry weight of roots per plant was an exponential decrease (Figure 5-7 (bottom left)). However, this effect was not significant ($P = 0.11$) when tested with a one-way ANOVA.

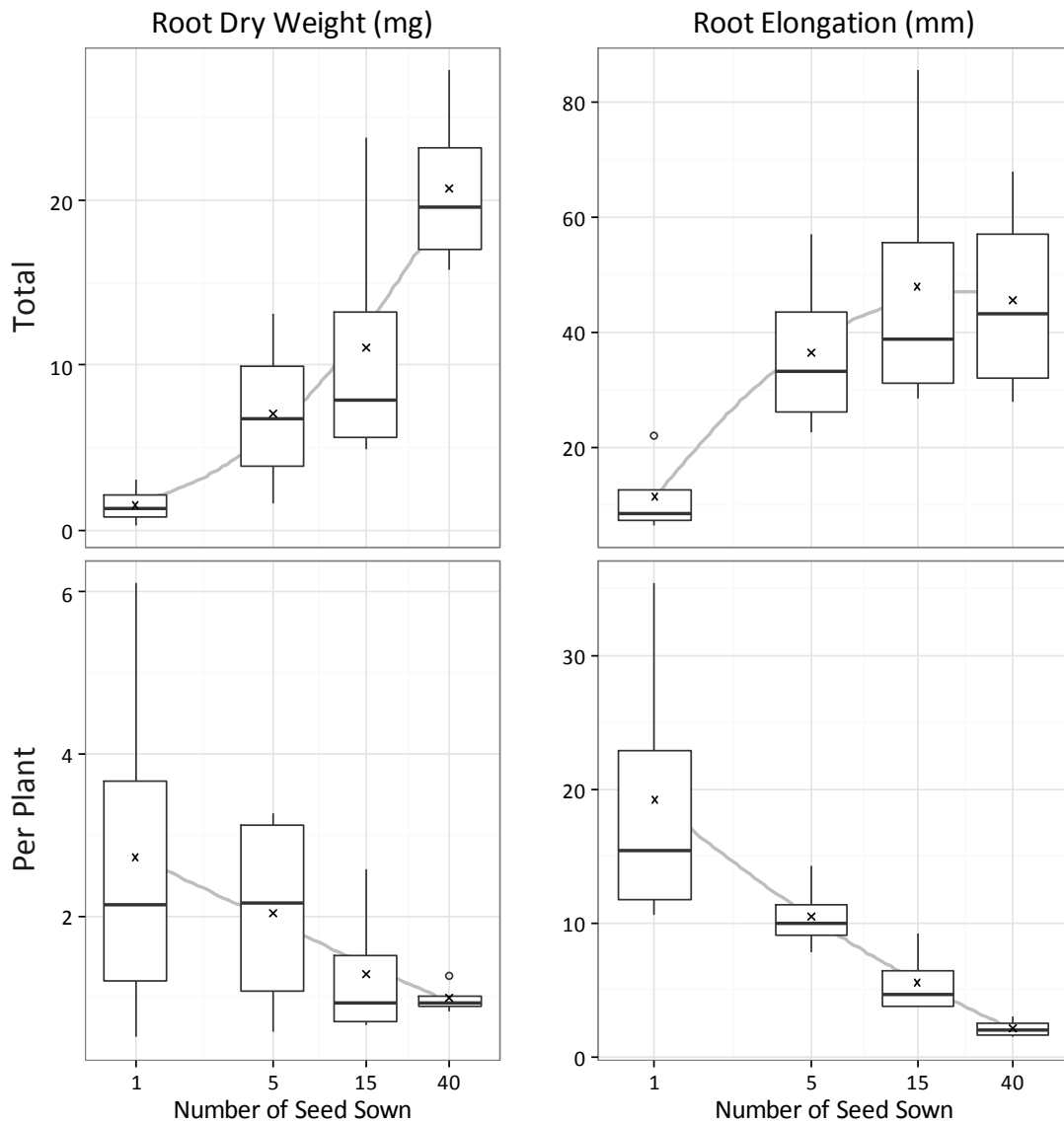


Figure 5-7: Root dry weight (left) and root elongation (right) as measured at the end of the experiment. The top panes show the mean of the four replicates, longest and heaviest roots per rep, while the bottom panes show those values divided by the number of plants. The number of seeds is displayed on a log₁₀ axis and a grey second order polynomial has been added.

The elongation of roots as seen in Figure 5-7 (top right) was not significantly affected by cluster size (a one-way ANOVA produced a significance of $P = 0.08$); however, when log transformed for normality, the length of seedlings per plant sown was significant (a one-way ANOVA produced a significance of $P < 0.0001$) (Figure 5-7 (bottom right)). A Tukey's HSD split root elongation per plant into three groups: longest 1 and 5, then 15, and shortest 40 seeds per seed cluster.

PLANT SIZE

To measure the relative success of the plants, plant size was measured using elongation of stems (Section 2.1d above). This measured the tallest plant in each cluster at every time point (as seen in Figure 5-8). At the end of the experiment, the height of the tallest stem in each cluster was also measured (Section 2.1c above).

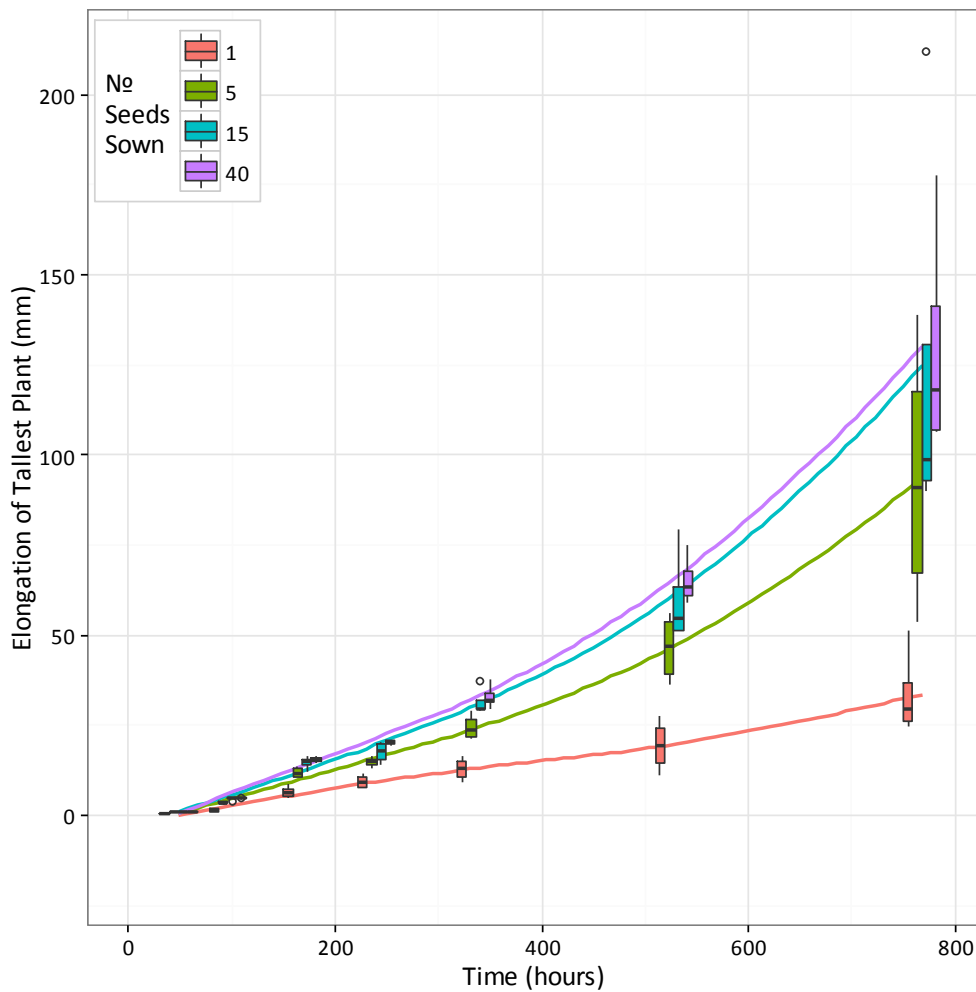


Figure 5-8: Per-treatment elongation of the seedlings over time averaged within the four replicates. The boxes represent the variation in the replicates. The tallest stem per cluster was measured. A simple loess line has been fitted to show the trend.

Elongation over time was tested with a linear mixed-effect model; there was a significant effect on stem elongation from time but not cluster size ($P < 0.001$, P

= 0.59) (Figure 5-8). This is because for all but the last two time points, the elongation is similar and the trend is in the same direction (Figure 5-8).

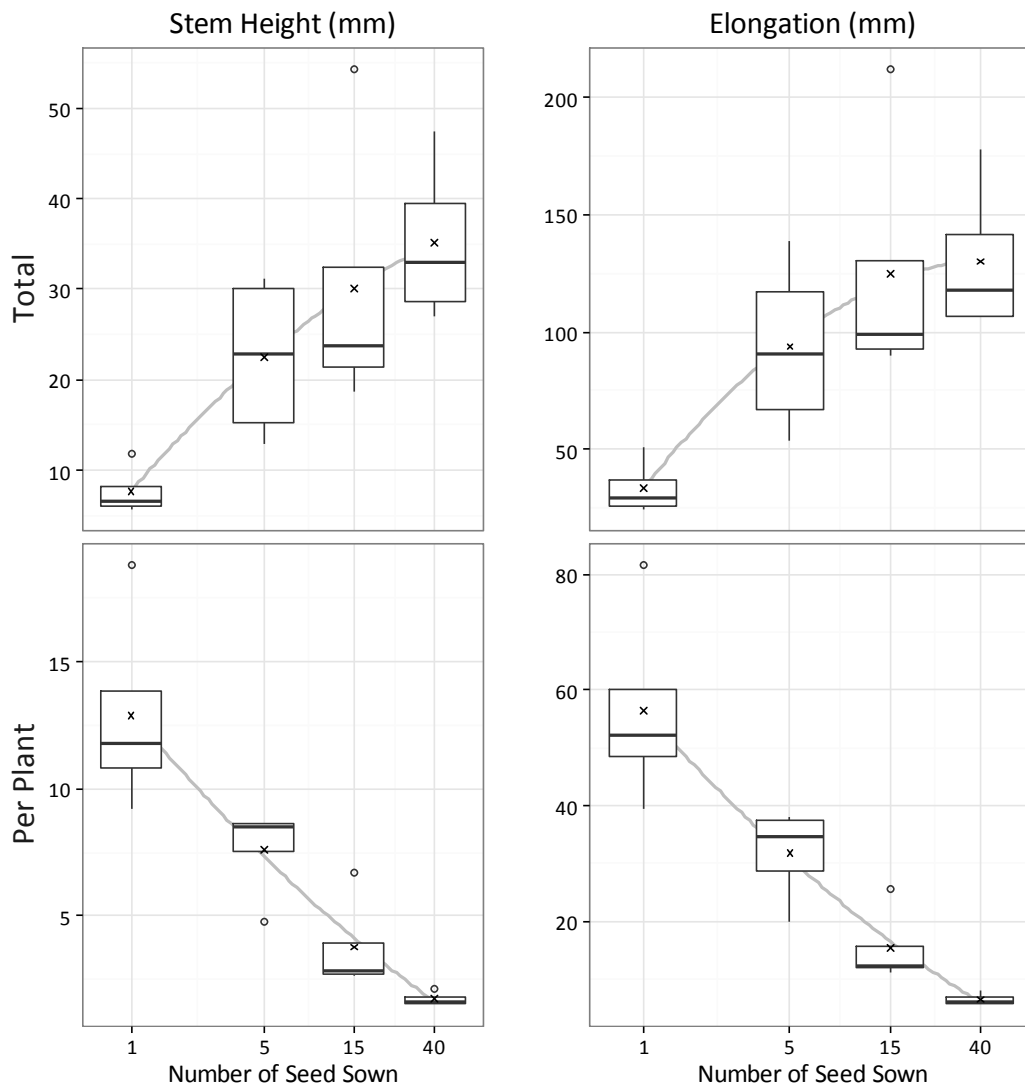


Figure 5-9: Mean stem height (left) and elongation (right) within the four replicates as measured at the end of the ‘Seed Competition Soil Experiment within a Controlled Environment’. The total effect (top) and the effect per plant recorded in the cluster (bottom). The x-axis is on a log to scale and a grey second order polynomial has been added.

For the final measurements at the end of the experiment, both average elongation and average height (Figure 5-9 (top)) were significantly affected by the number of seed per cluster (this was logged for normality and tested with a one-way ANOVA, producing significances of $P < 0.05$ & $P < 0.01$ respectively). Both

had the same groupings from a Tukey’s HSD post hoc test, taller plants were in 5, 15, and 40 seed clusters with shorter plants in 1 and 5 seed clusters (Figure 5-9 (top)). When elongation and height were analysed for their effect per plant (Figure 5-9 (bottom)), they were both more significant than per cluster (logged for normality and tested with a one-way ANOVA, $P < 0.0001$ & $P < 0.0001$). The Tukey’s HSD result for elongation per plant was in three groups: highest for 1 and 5, then 5 and 15, and lowest for 40 seeds per cluster (Figure 5-9 (bottom right)). Height had a slightly different grouping with 1 and 5 highest, then 5 and 15, before 15 and 40 for the lowest (Figure 5-9 (bottom left)).

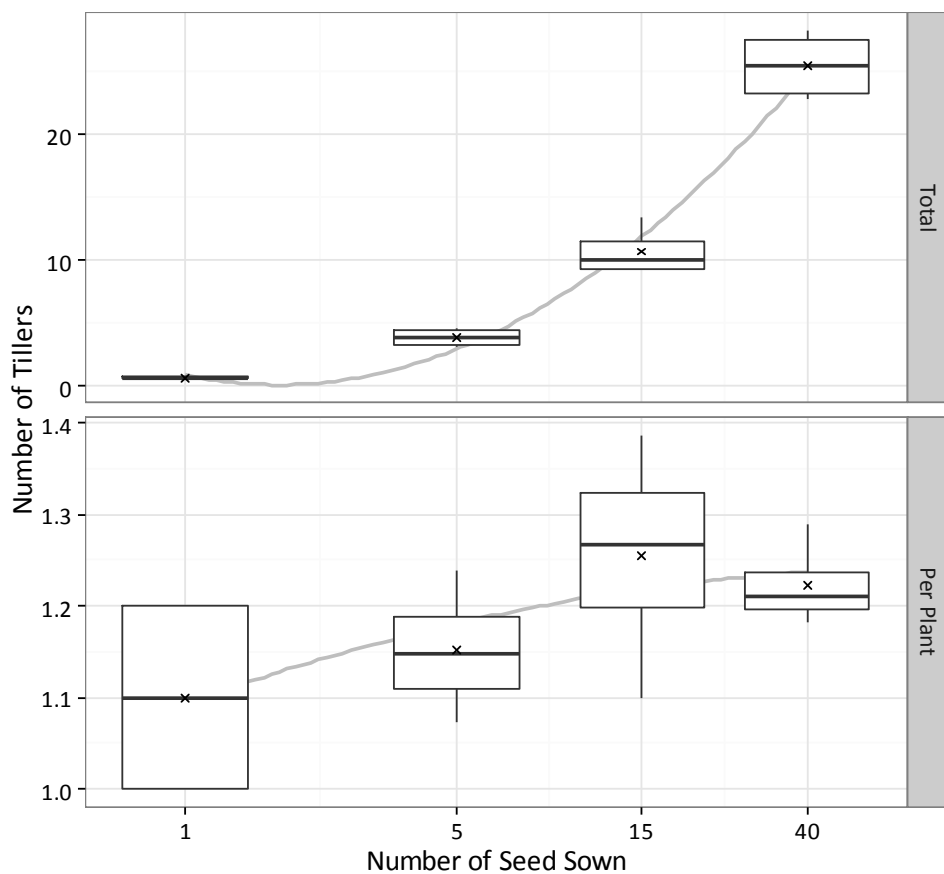


Figure 5-10: Tiller counts at the end of the experiment averaged for all clusters within one of the four replicates. A raw value of tiller number is given (top) as well as tiller number divided by the number of plants in the cluster (bottom). The x-axis is on a \log^{10} scale and a grey second order polynomial has been added.

As another measure of plant size, the number of tillers was used. The average total number of tillers per seedling cluster was significantly affected by number of seeds sown in a one-way ANOVA ($P < 0.0001$). A Tukey's HSD placed the 40 seed clusters in the top group, then 15, and lowest 1 and 5 seed clusters (Figure 5-10 (top)). However, number of tillers per plant was not significantly affected by cluster size when tested with a one-way ANOVA ($P = 0.13$), as seen in Figure 5-10 (bottom). This suggests that plants produced approximately equal numbers of tillers regardless of cluster size.

These stems were then dry weighted; the difference in the dry weights of the stems was significantly dependent on the cluster size (tested using a one-way ANOVA, $P < 0.0001$). A Tukey's HSD produced three groupings; the highest mass was in the 40 seed clusters, then an intermediate group of the 5 and 15 seed clusters, as well as a low mass group of the 1 and 5 seed clusters (Figure 5-11 (top right)). Stem dry weights per plant were not significantly affected by the number of seed sown in the cluster (tested using a one-way ANOVA, $P = 0.1$) (Figure 5-11 (bottom right)).

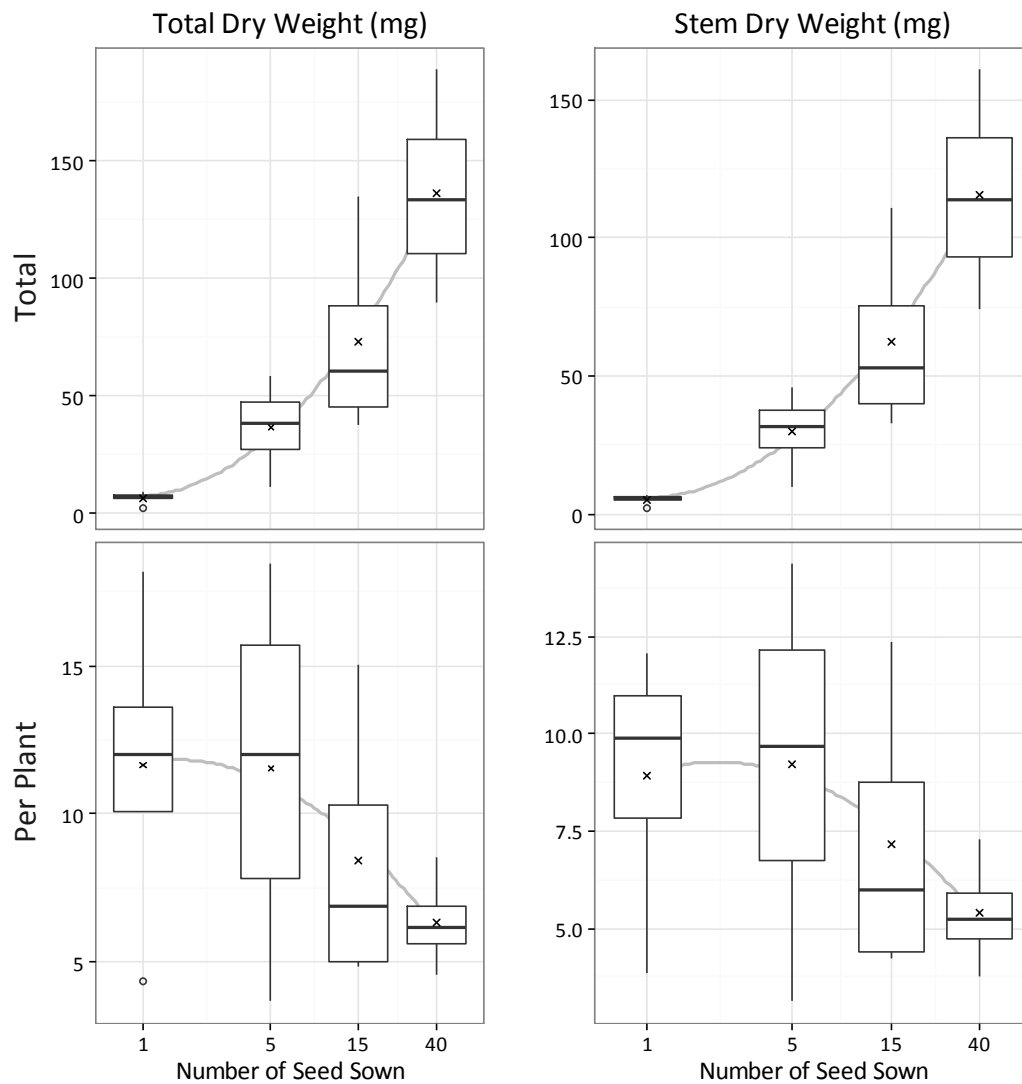


Figure 5-11: Dry weight of stems (right) and total seedling (stem and root) dry weight (left), plotted on a log 10 x-axis, with a grey second order polynomial. Total weight averaged between the clusters in the four replicates (top), and average dry weight divided by the number of plants in the plot (bottom).

The total dry weight including stems and roots was also significantly affected by the number of seeds sown (tested with a one-way ANOVA, $P < 0.0001$). A Tukey's HSD found three significant overall dry weight groups: 15 and 40 (high), 5 and 15 (medium), and 1 and 5 seeds per cluster (low) (Figure 5-11 (top left)). When analysed with a one-way ANOVA, total dry weight per plant was also not significant ($P = 0.09$) (Figure 5-11 (bottom left)).

HEIGHT VS ELONGATION

Finally, the measurements at the end of the ‘Seed Competition Soil Experiment within a Controlled Environment’ were used to examine the relationship between stem height and plant elongation measurements as defined in 2.1c and 2.1d of Chapter 1.6. Figure 5-12 demonstrates a strong correlation of 0.94 between seedling height and elongation.

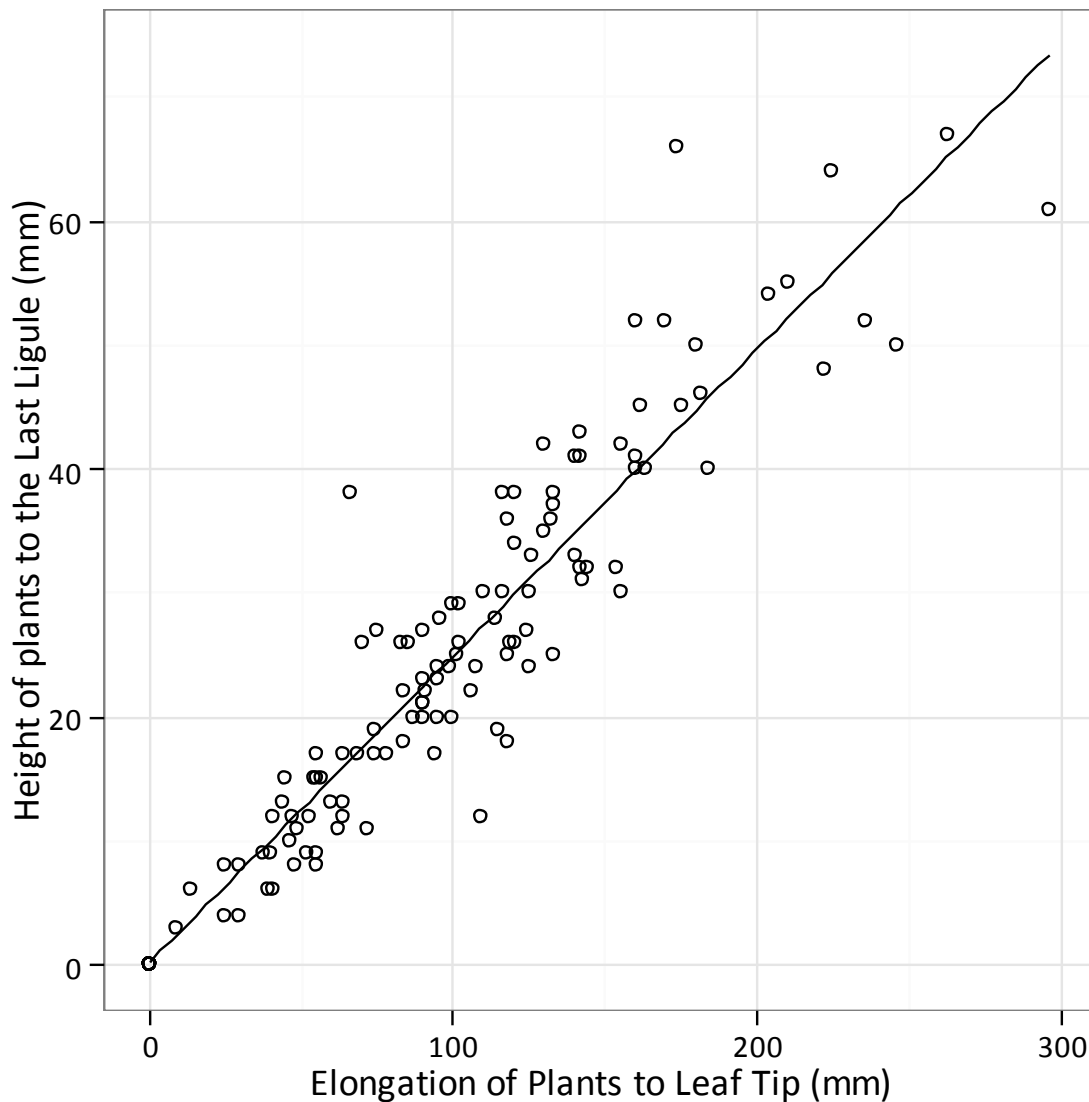


Figure 5-12: Correlation between seedling height and the elongation measurement on 32 day seedlings, for all 128 clusters in the ‘Seed Competition Soil Experiment within a Controlled Environment’.

This correlation should mean that measuring either elongation or height at early stages of growth gives a similar result as in Figure 5-12; however, because the elongation measurements are larger by about a factor of four, it is easier to measure elongation, where an error of a few millimetres is far less of an issue.

5.3C FIELD TRIAL OF CLUSTER SOWING SEED

This trial, of four replicates each with 30 clusters of 5, 15, and 40 seeds (90 cluster plots), was designed to field test the optimal number of seeds to successfully produce a plant (strike rate of 100%), whether there was competition between plants and if this limited growth. At the end of the first year, the plants were removed, measured, and dry weighted to answer these questions.

SURVIVAL

The mean number of plants growing in the clusters at the end of the year appears in Figure 5-13 (top) to increase linearly with the number of seeds in the cluster; when correlated a PPMC coefficient was 0.89. The effect of cluster size on number of plants growing at the end of the year was also tested with a two-way ANOVA using blocking; there was a significant effect ($P < 0.001$). It was then tested with a Tukey's HSD, which grouped the 5 and 15 seed clusters with less stems per cluster, and the 40 seed clusters with more (Figure 5-13 (top)).

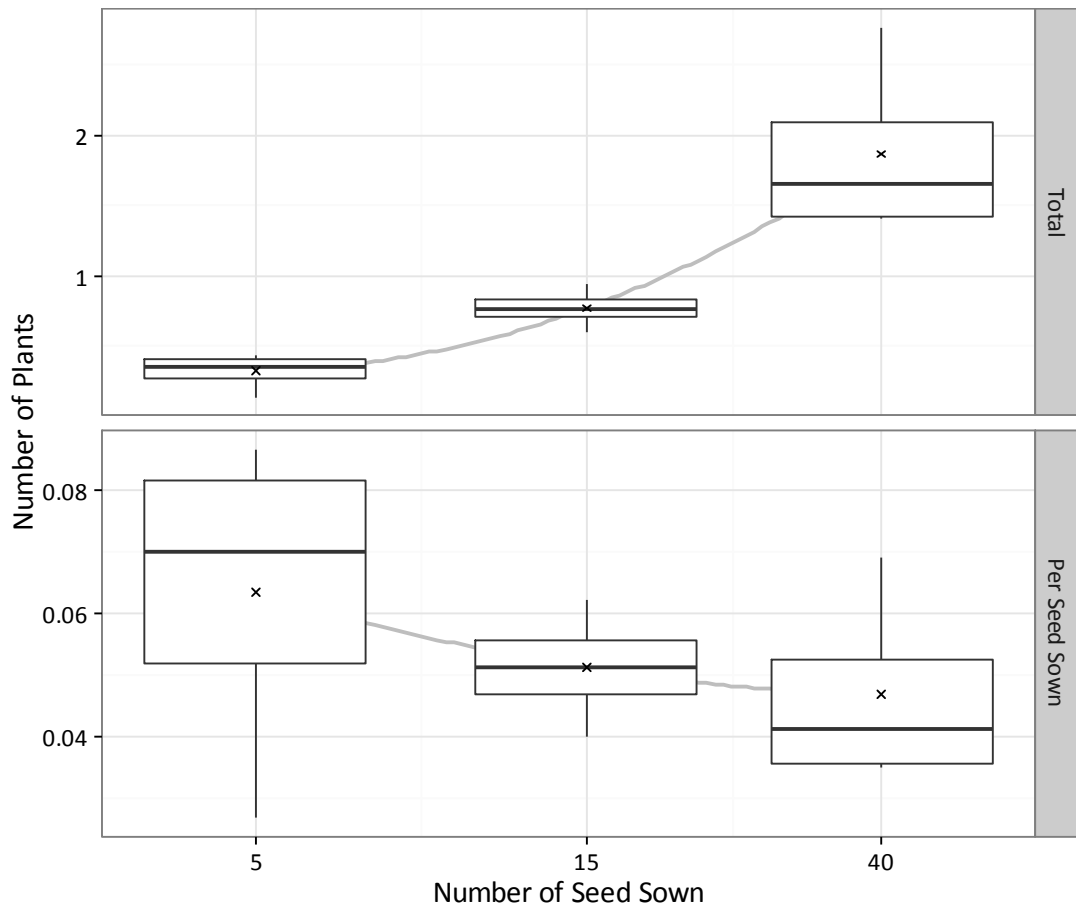


Figure 5-13: Mean number of plants per cluster, at the start of the second season. Top the total mean number, and bottom the mean number per seed sown in the cluster. The x-axis is log 10, with a second order polynomial in grey.

The number of plants per seed sown (Figure 5-13 (bottom)) appears highest at five seeds (~0.06 plants per seed sown) and stable for fifteen to forty seeds (~0.05 plants per seed sown). The PPMC showed a weak negative correlation for the number of plants per seed sown (-0.35). The effect of cluster size on number of plants per seed sown (Figure 5-13 (bottom)) was not significant when tested with a blocked two-way ANOVA ($P = 0.31$). As there is no significant drop in plant survival per seed sown, this indicates that the negative effect of cluster size on the number of plants surviving seen in more controlled conditions did not occur here.

The strike rate clearly improves with number of seeds per cluster (Figure 5-14), with one-way ANOVA producing a significance of $P < 0.001$. However, it is desirable to achieve a strike rate of $\sim 100\%$, where each cluster produces a plant. As there were only four replicates all well below a 100% strike rate, it is impossible to accurately predict the number of seed per cluster required to achieve a $\sim 100\%$ strike rate; however, Figure 5-14 exempts an estimate.

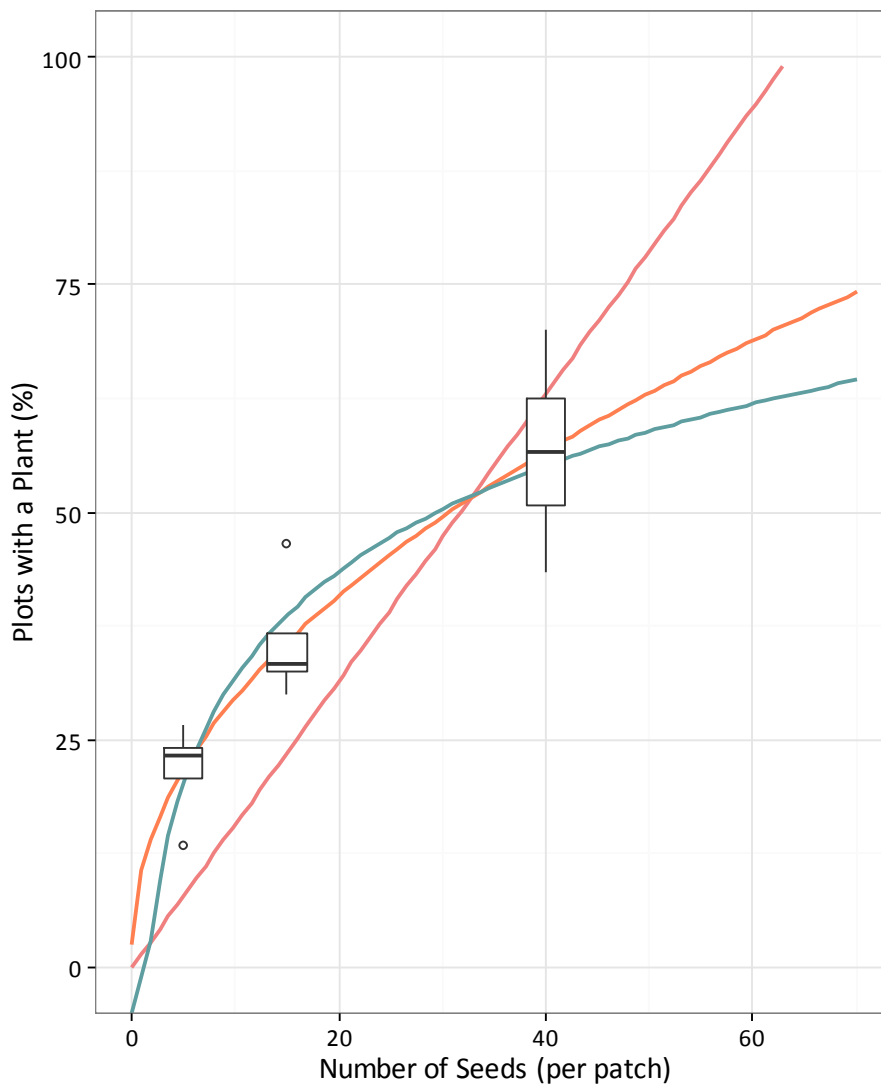


Figure 5-14: Proportion of plots with a plant after 10 months in the field. Three models have been plotted to show the uncertainty of the data (from four replicates): a linear model through the origin (red), a natural log model (blue), and a square root model (orange). These have been extended to 70 seed per cluster, to estimate of chance of a plant per plot.

Three models, fitted through the origin to provide an extra point, were tested to predict the cluster size required. Firstly, if there is a linear relationship between seed planted and strike rate, the linear model can be mapped forwards to produce a figure of ~64 seeds required per plot for the average plot to produce a 100% strike rate (Figure 5-14, maroon). This model has an R^2 of 0.31. Secondly, the natural log model (Figure 5-14, blue) is a better approximation with an R^2 of 0.78; however, 64 seeds would be producing an average strike rate of 63%, and clusters of hundreds of seeds would be needed to approach 100%. Lastly, the square root model (Figure 5-14, orange) fitted the data best with an R^2 of 0.79 and 137 seed required per patch to achieve a ~100% strike rate.

ROOT COMPETITION

The harvested roots at the end of the 'Field Trial of Cluster Sowing Seed' were dry weighted, and the total mean dry weight of roots in each cluster was tested with a blocked one-way ANOVA to determine if the number of seeds sown affected this total (Figure 5-15 (top left)), there was not quite a significant effect ($P = 0.07$). Therefore, there is no significant increase in root mass after one year when more seeds are sown (as long as the cluster survived).

The dry weight of roots per plant (Figure 5-15 (bottom left)) was logged for normality and tested with a blocked one-way ANOVA against number of seeds sown; this was not quite significant ($P = 0.09$), and there was a significant blocking effect ($P < 0.01$) which may obfuscate any true effect. This indicates that individual plants masses in each plot are not significantly lighter when more seed are sown.

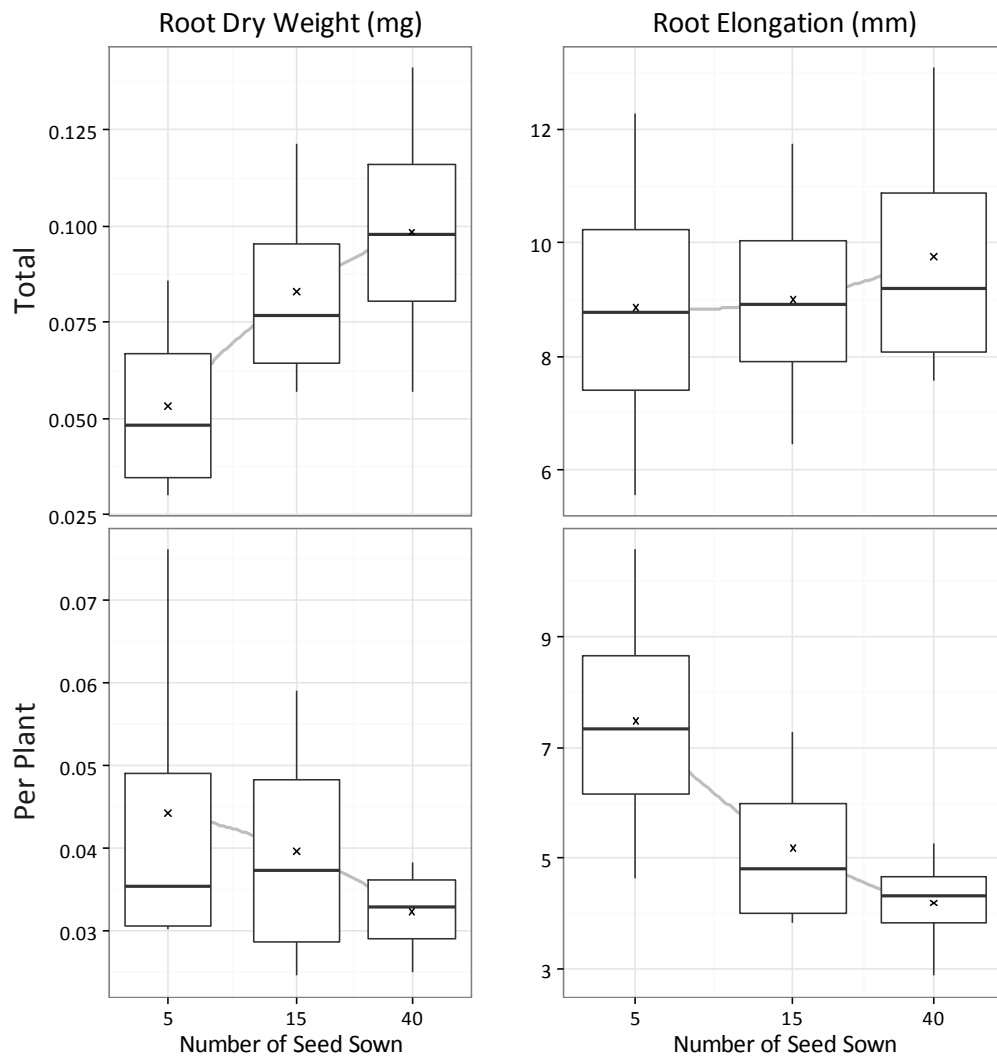


Figure 5-15: Start of second season mean root measurements within the four replicates for the up to 30 surviving clusters. Root dry weight (left) and elongation of the longest root per plot (right), with the mean total (top) and the mean per plant (bottom). Number of seed sown has been log10 scaled and has a grey second order polynomial.

The other root measurement taken was root elongation (see Section 2.1e above). This was used to investigate if the roots were more or less constrained in larger clusters. Figure 5-15 (top right) shows that the difference between mean longest root elongations between number of seeds per cluster is very small. This was confirmed with a blocked one-way ANOVA, which had no significant effect of cluster size on root elongation but had a significant blocking result ($P = 0.35$ & $P < 0.05$).

The difference in mean root elongation per plant counted is more striking (Figure 5-15 (bottom right)), this difference was significant (tested with a blocked one-way ANOVA, $P < 0.05$ & blocking significant at $P < 0.05$). A Tukey's HSD post hoc analysis grouped 5 and 15 seeds together as an upper grouping and 15 and 40 seeds as a lower grouping. Therefore, the longest roots within plots are no longer when more seed is sown in one cluster; the significant, almost linear, decrease in length per plant (Figure 5-15 (bottom right)) shows root elongation is independent of plant number.

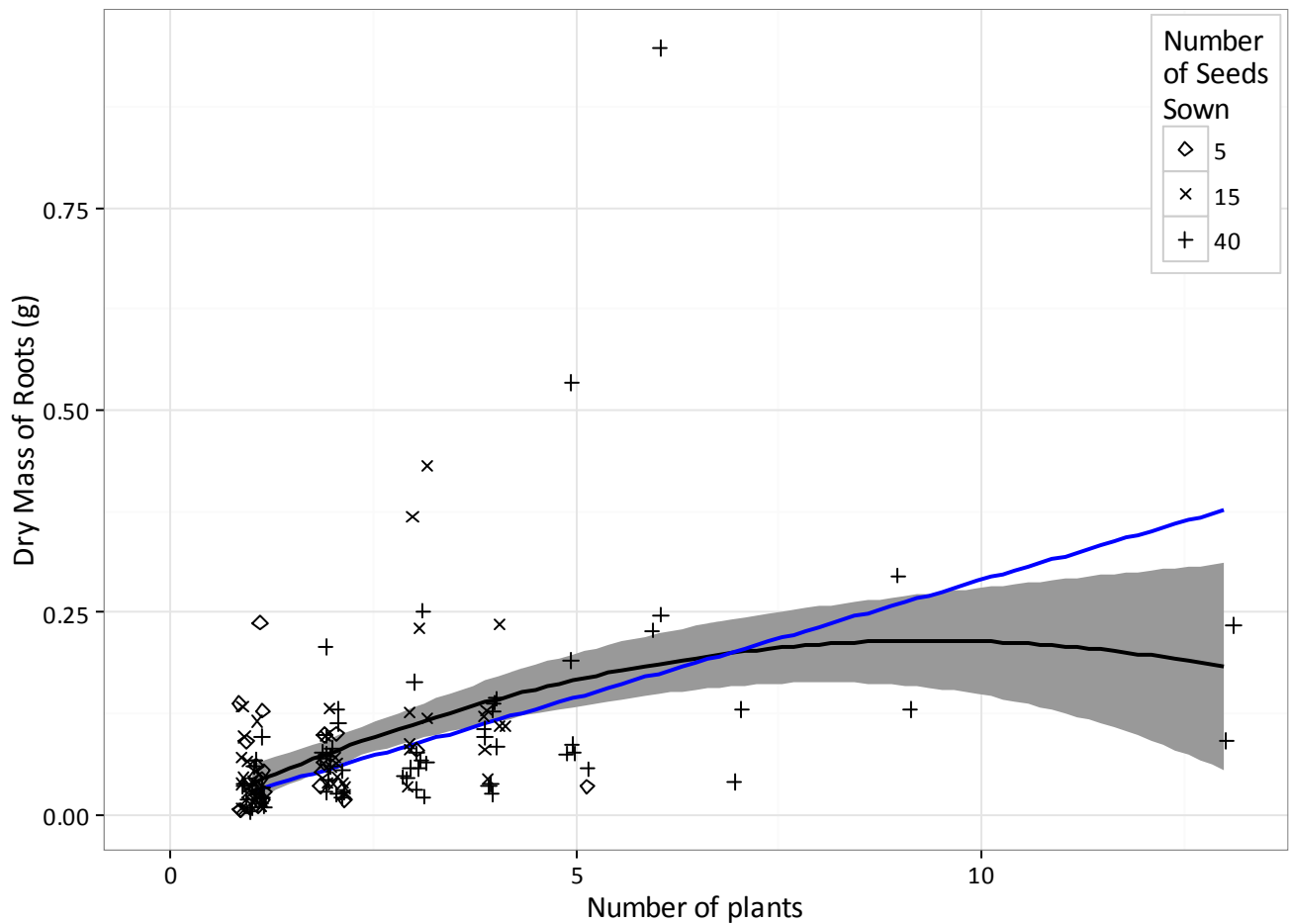


Figure 5-16: The effect of the number of plants on below ground dry weight for all 360 clusters individually. Both measurements were taken at the start of the second year (March), just after the time a mature crop would be harvested. The position of the points on the x-axis has been randomly offset by up to 0.4 to reduce overpotting. A second order polynomial line with 95% confidence interval has been added and a linear model fitted through the origin. Both lines have an R^2 of 0.44. If mass of roots is dependent on number of plants this should follow a clear linear trend.

Finding competition by testing if dry weight was affected by the number of seeds per cluster ignores the relative success of each cluster. Therefore, Figure 5-16 shows the effect of number of plants growing vs. the dry mass of all roots for every cluster; this is similar to what is seen in Figure 5-15 (bottom left), but not averaged within the blocks. The dry weight increases with the number of plants alive per cluster until ~five plants, before levelling off (Figure 5-16). This would be linear if roots were the same regardless of number of plants growing in the

cluster, implying there may be some reduction in the relative dry weight of the roots based on of the number of plants surviving. There are too few plots with over five plants to know if this trend is correct.

PLANT SIZE

The number of tillers, the number of tillers per plant, and a visual assessment of plant health were all used as measures of the above ground size of the plant.

It was observed that clusters with lots of plants tended to have many weak small plants, and one or two large multi-tillered plants. For example, a plot with five plants and ten tillers was much more likely to have a plant with four tillers, two plants with two tillers and three plants with one tiller than to have five plants with two tillers each.

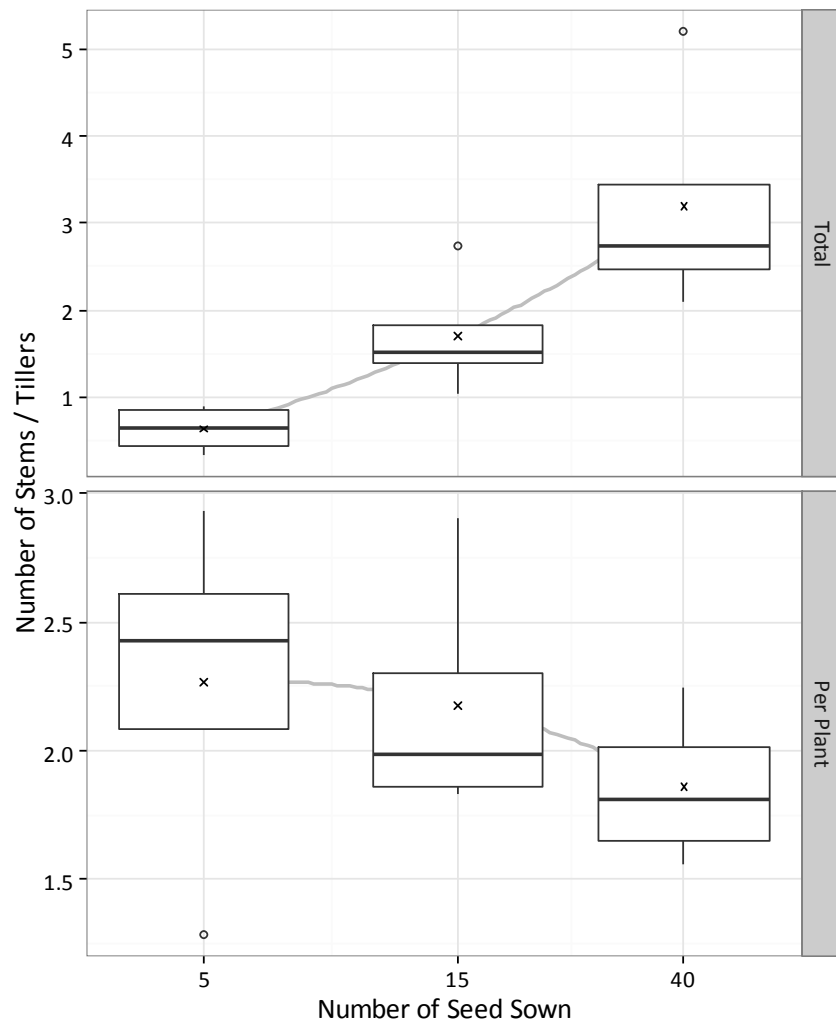


Figure 5-17: Start of second season tiller counts. The boxplot shows the mean total tiller number (top) and the mean tiller number per plant (bottom). Number of seed sown has been log₁₀ scaled and has a grey second order polynomial.

Figure 5-17 (top) gives a strong linear correlation with a PPMC coefficient of 0.79 between stems at the end of the trial and the number of seeds per cluster. The mean number of stems per cluster was significantly affected by cluster size when tested with a blocked one-way ANOVA ($P < 0.01$). A Tukey's HSD on the data produced two groups with the 40 and 15 seed clusters in the higher group and 5 and 15 seeds per cluster in the lower group. This will be primarily due to more seed per cluster producing more plants. There is a slight downward trend seen in Figure 5-17 (bottom) for the number of tillers per plant per seed sown,

which was not significant ($P = 0.13$) (this had a significant blocking factor with $P < 0.05$). Therefore, each plant does not produce significantly less tillers when sown in a larger cluster.

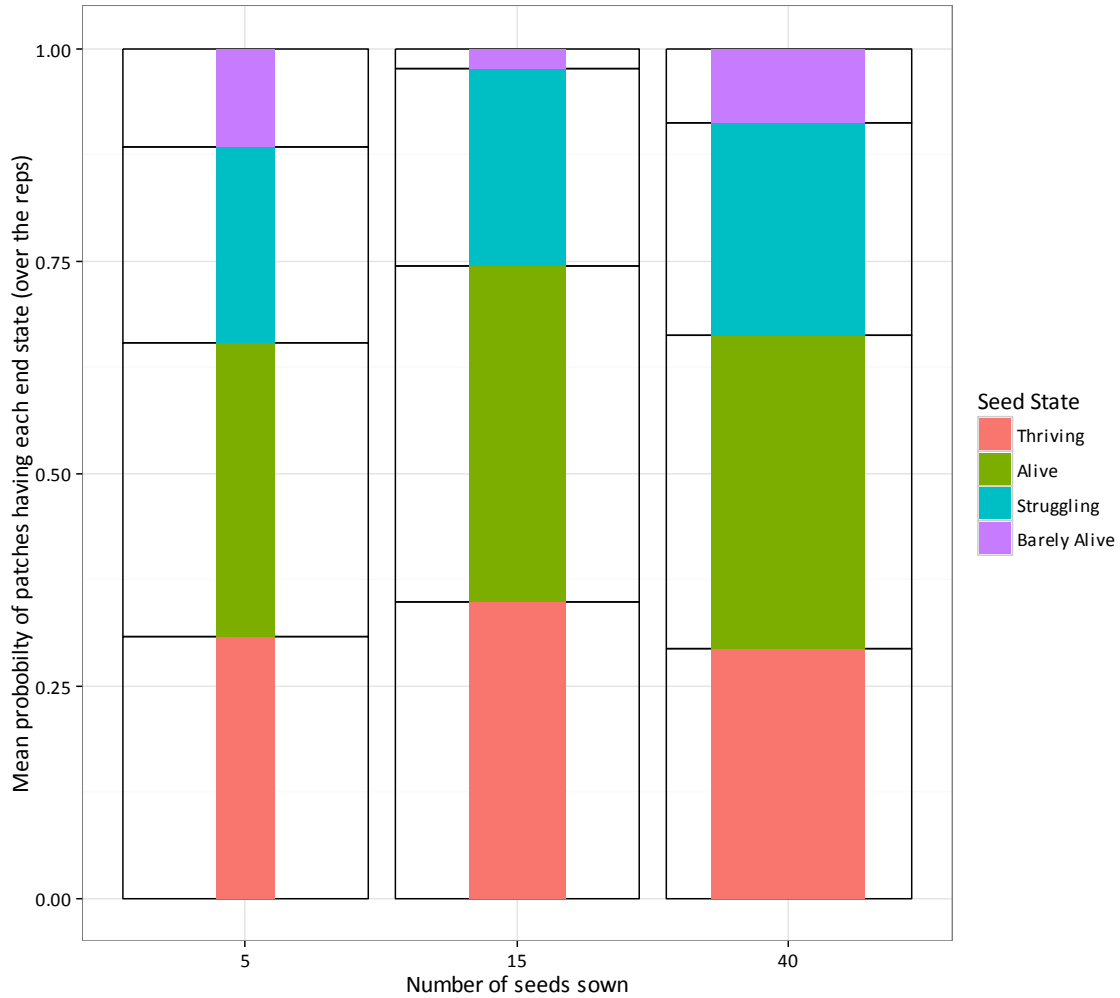


Figure 5-18: The end state of seedlings, at the start of the second season (March). The widths of the coloured blocks represent the proportion of clusters alive of that size (total out of 120).

There were no differences noticeable in Figure 5-18 in the end state of the clusters dependent on how many seeds were sown.

5.4 DISCUSSION

The investigation of allopathy and seed competition within *Miscanthus* has revealed mixed results.

5.4a SEED COMPETITION IN WATER

The seed do appear to compete in the experiments carried out in water, but this may be accounted for by an increased ability of mould to affect successive seeds when they are in close proximity. Pre-sterilising the seed resulted in a more noticeable difference in germination between seed clusters and individual seeds. This could have been caused by the bleached seeds' increased susceptibility to mould; bleaching and softening of the seed coat occurs when the seed is surface sterilised. Once one seed succumbs to mould the other seed in close proximity are less protected. This could explain why clusters performed less well against individual seeds when bleached.

It therefore needs to be determined whether this response is linear with number of seed, or if there is a concentration threshold that has to be met. The broad result of different numbers per compartment indicated strongly that there was a sharp change in germination from concentration of seeds; lower numbers of seed (15 or less) showed normal germination, but larger numbers (35 and 50) showed reduced germination. In the narrower test the 15-plus seed range was expanded upon, this time the effect was more of a gradual decline in germination. The noticeable drop off occurs between 19 and 21 seeds. This implies whatever caused the drop in germination operates over a narrow concentration range. If it were a hormone, ABA would be a likely candidate because it limits germination and has a steep operation curve (see 'Abscisic acid' in Section 4.4a

above). However, there are many other possible allelochemicals (Bais et al., 2003; Siddiqui et al., 2009).

5.4b SEED COMPETITION IN SOIL

The plants appeared to compete in the field, with larger seedlings taking over the plot. However, this was not absolute because at the end of the first year the small seedlings were still living and therefore consuming resources that the larger plants could have utilised. There was less effect from competition in the controlled environment in-soil experiment, but there were many more measurements analysed, giving a broader picture.

GERMINATION AND SURVIVAL

The 'Seed Competition Soil Experiment within a Controlled Environment' had high strike rates; therefore, only single seed clusters had any failure. Single seed clusters performed 41% lower than the 100% germination rate of the seed lot; this is probably due to the reduced water contact from soil and the increased risk of mould. Over the season in the field, the strike rate was dramatically lower: approximately 24% for 5 seeds and 50 to 60% for 40 seeds. If the strike rate does not continue to drop off per seed, approximately 64 seeds would be needed to approach a 100% strike rate. This failure of clusters to succeed in the field even with large numbers of seed is a product of the microclimates around each cluster, the low temperatures, and soil water.

This can be investigated by examining the number of plants growing, which should increase linearly with the number of seeds sown. In both the field and the controlled environment in-soil experiment, there was a strong positive correlation between the number of seeds sown and the number of plants produced. The deviation from this linear change was observed as plants per seed sown. The change in this was not significant in either environment; however, in

the field there was only a decline (from 4~7% in 5 seeds, to 4~5% in 15 & 40 seeds), whereas in the controlled environment in-soil experiment there appeared to be a bump at 5 seeds where germination per seed was higher than in individual seeds. This could be due to the random chance of the seed landing with good hydraulic contact, increasing with seed number but then the inhibitory effects of larger seed numbers restricting germination as seen in Section 5.3a above. This effect in the controlled environment in-soil experiment was significant in the speed of germination, where the low numbers of seed germinated with nearly double the percentage increase over the first 200 hours than the 15 and 40 seed clusters.

ROOT COMPETITION

Root dry weight should increase significantly with number of seeds sown, because more seeds will lead to more plants; however, below ground competition for space could mean all clusters take up about the same space. At the end of the 'Seed Competition Soil Experiment within a Controlled Environment' the below ground dry weight was distributed as expected with more seed having more dry weight; however, at the end of the season in the field trial there was some increase based on cluster size but it was not significant. This indicates the surviving plants losing the effect of number of seeds sown over time. Both of the experiments' decreases in dry weight per plant over the plot sizes were not significant, but the field had a shallower slope.

Size of roots can also be observed through the length of the longest root. This should mostly stay the same regardless of the number of seed sown (possibly with a small increase due to the chance of stronger seeds being present). There is a non-significant increase in the controlled environment in-soil experiment while in the field there is no difference in longest root. This shows that given

time the longest root is independent of the number of seeds sown in the cluster. By dividing the length of the longest stem by the number of plants growing at the end of the test, it should be possible to detect competition effects between plants even if the competing plants are no longer alive. Therefore, this should be flat if there is no competition, yet in both, there is a non-significant decrease with cluster size. This would be the case if one or two of the largest plants outcompeted the others within a cluster, but were slowed down based on the number of others.

It should be remembered that in the field overall below ground biomass did not increase linearly with additional surviving plants per cluster so this effect might not have damaged the cluster productivity in later years. Further investigation would be needed to tell if this is important to yields in the future, and if each plot would eventually end up with one plant.

PLANT SIZE

Above ground plant size, is important as it provides an indication of the success of each cluster. In the field, it would have been preferable to examine the relationship between number of plants and above ground biomass, to determine if there was an effect of cluster size. However, this was not possible due to the post winter condition of the young plants above ground. It is highly probable that there was a big impact on the above ground biomass from smaller root systems per plant. However, it was possible to look at elongation and above ground biomass in the controlled environment in-soil experiment. Elongation was expected to increase a small amount with seeds sown, because the random variation in the seeds would give a better chance of a tallest stem for each additional plant. However, there was a significant proportional increase in height for number of seed sown, and the elongation over time was significant.

This could indicate elongation as competition between seedlings. However, elongation per plant decreased significantly with number of seed sown; which may imply any competition had distanced winners and losers.

Above ground dry weight significantly increased with number of seeds sown, this was as expected as more plants provide more biomass. However, when divided by the number of plants there was a non-significant decrease by number of seeds sown reinforcing the idea of above ground competition.

The controlled environment in-soil experiment and the field can be compared using the number of tillers produced by seed clusters. Both experiments had, as expected, a significant increase in the number of tillers the more seed were in the cluster; however, the mean number of tillers per plant non-significantly increased in the controlled environment in-soil experiment, but non-significantly decreased in the field. This may imply that the early increases could be an effect of competition to outgrow neighbouring plants, but over time in the field, this competition has resulted in fewer tillers per plant because only the successful plants remain. Further investigation would be required to establish if this effect on tillering was a valid conclusion.

The health of individual plants in the field cluster was observed to be more variable in the clusters with more plants, suggesting competition curbing some of the plants. It was expected that this would result in clusters sown with more seed being in worse overall health. However, in the field when assessed, the end state of the clusters did not vary based on the number of seeds sown. This suggests that the larger plants still appeared healthy despite the effects of competition.

Over all more seeds have more chance of producing a plot, but not linearly so; from this experiment, it could be reasonably predicted that sowing between 64

and 120 seeds would produce a plot each time in these conditions using seed with a 100% ideal germination rate. This is because as in the laboratory tests (Section 5.3a) less seeds germinate in soil when there are many seed. There is evidence that the best plants grow more to compete with the weaker plants and will win out over the other plants in the first year. Whether this early period of competition would eventually produce a weaker or a stronger plot requires more long-term investigation.

6 AGRONOMIC MODELLING

6.1 INTRODUCTION

Models of climate and population have pervaded into the everyday language of science and modelling has been used extensively in biology to predict and extrapolate known processes into a wider context. Modelling crop yield is more common than modelling germination and establishment. Modelling generates predictions of germination under different climates and conditions (Dürr et al., 2001) to show how establishment will vary and ultimately demonstrate profitability. Modelling can also be used to predict the effects of land preparations, soil tillage and methods of sowing (Dürr et al., 2001). These models allow for a statistical way of demonstrating the potential of the crop on a global scale with only a few field trials to provide data (Clifton-Brown et al., 2011). Modelling is most commonly used to predict the effect of temperature on a crop; this normally utilises thermal time (Bradford, 1990; Brunel et al., 2009; Gardarin et al., 2010; Shrestha et al., 1999; Trudgill et al., 2000). As a perennial, *Miscanthus*, which has a long establishment period, benefits from modelling, which has been important for crop uptake (Hastings et al., 2009b).

6.1a PRIMING

(See Priming in Section 4.1a above)

6.1b MULCH FILM

Mulch film is a commercial crop product that could prove useful in *Miscanthus* establishment, by providing seeds with additional thermal time. During the establishment phase, film can act in a similar way to a degradable glasshouse; and consequently provide an increased yield (Easson & Fearnough, 2000; Farrell & Gilliland, 2011). It can be applied to fields along rows, appearing similar to a layer of perforated cling film (for details see 2.1f above). It has been claimed that using mulch film increases early soil temperatures by approximately 10°C at the surface, dropping to 5°C at 10 cm deep, thereby halving the time to crop emergence (Farmers Guardian, 2008b). From this report, Maize under film can achieve 20% higher yields as well as producing a more reliable crop, and thus has been economically sound for farmers (Farmers Guardian, 2008b). Mulch film was tested with Maize in China and was found to increase the soil water and the crop harvest mass (Zhou et al., 2009). Also the farming of Maize in Ireland is expanding west and north with the use of film; this could be replicated on marginal sites across the UK, particularly within Scotland (Dr Trevor Gilliland - Head of AFBI plant establishment, via (Farmers Guardian, 2008b)). The increase in dry mass of Maize under film in Ireland was primarily due to the thermal boost mulch film gave the soil (Farrell & Gilliland, 2011). This exemplifies the ability to grow crops outside of normal geographical range, and has particular implications for *Miscanthus*, the aim with which is to target the largest possible geographical area.

6.1c SOWING TIMES

Planting under a range of temperatures and levels of soil moisture can be achieved by planting in different locations; planting at different times of year also introduces the effects of day length and general seasonal climatic patterns.

In existing crops, an early sowing time can maximise yield by extending or moving the growing season. However, early sowings pose not just the obvious risk of young plants dying or seeds failing to germinate in the cold; but also waterlogging of soil in spring stressing young plants. A late sowing can allow an early crop to be harvested before main season planting commences, saving a year of establishment in cost. The crop could also be planted at a warmer time of year when the seedling grows quickly and then senesces late for maximum first year potential, effectively using its second year as the first establishment year. However, in a late sowing the plant may be underdeveloped to cope with winter, or may not senesce in time to prevent frost damage; e.g. Clifton-Brown et al. (2011) postulated that sowings after April 15th would give insufficient time for crop establishment. In addition, late sowings rely on being late enough to allow another crop that requires little time during the year to yield, even on marginal land. This could allow the seed to remain in place then germinate as early as possible the next year making use of all available thermal time.

Even moderately late sowings could be beneficial if sowing straight into grassland, which would be a low carbon, low impact way of sowing (McCalmont et al., 2015). Without ploughing the land, less soil carbon would be released through disturbance of the soil and herbicide use; allowing the crop to more quickly sequester carbon (McCalmont et al., 2015, 2016). With a directed glyphosate line and a pressed groove for the seeds, with the possible addition of mulch film, this could be done after having sheep on the field for spring (Easson & Fearnehough, 2000).

6.1d SOWING METHODS

It has been observed anecdotally that *Miscanthus* seeds may germinate better on firm soil than freshly tilled soil; there are observations of seeds germinating on tyre tracks yet not in plots. This may be due to the size of the seed leading it to be washed down into the soil substructure. It may be relevant that informal observations of *Miscanthus* suggest that germination in the lab, on wet paper or in water, is high but that germination in pots or in the field is much lower. This may be due to a lack of seedling emergence force (Brunel-Muguet et al. 2011) failing to let the emerging hypocotyl reach the surface. As studied in sugar beet by Dürr & Aubertot (2000) average emergence forces within species determine which samples survive in which soils. Testing whether there is a negative impact on germination and establishment based on sowing by sowing method will be important to solve this problem. Previously *Miscanthus* seed was shown to establish better when drilled into the soil than when broadcast over the soil, possibly because the seeds benefit from improved hydraulic contact with the soil (Christian et al., 2005).

6.1e GENETIC VARIATION

There is a wide variation in *Miscanthus* genotypes between and within species in the wild (Deuter, 2000; Songstad et al., 2010, Chapter 7). This variation has been used through breeding mainly to produce bigger *Miscanthus* plants, and to a lesser extent to extend the range of *Miscanthus*. The Aberystwyth University breeding program has many seed accessions that could be characterised by response to germination conditions. Thus far, breeding has not focused on the potential germination range of hybrid seed; however, direct sowing will make this a more important factor, though not as important as biomass and overwintering.

6.1f SOIL WATER AND TEMPERATURE

The main physical environmental triggers for seed germination are water potential (hydrating the seed) and temperature (allowing metabolic processes that also signal germination). The water content of the soil is partly responsible for the water potential, though soil type is also influential. The soil type also influences temperature through density, water content, and albedo. Understanding how *Miscanthus* seed germination changes based on real world soil water and temperature will be vital to direct sowing.

6.1g GEOGRAPHIC RANGE

As a C₄ plant, *Miscanthus* is more suited to tropical than temperate climates; however, it can naturally grow in temperate environments unlike many C₄ plants (Clifton-Brown et al., 2001; Hastings et al., 2009b; Naidu et al., 2003). Because the physical limits on germination are temperature and water, which are genotype dependent, new geographic ranges should be calculable for interspecific hybrids. For example Maize seed has been bred to grow successfully in northern Europe despite also being a C₄ grass (Clifton-Brown et al., 2011).

6.1h COMPUTATIONAL MODELLING

For *Miscanthus* to be direct sown in a range of environments and soils, producing a model to assess its germination and emergence from small-scale trials would improve understanding of crop sowing. Existing crop yield models have been applied to crops of *Miscanthus* (Stričević et al., 2015); custom models of climate and soil have more often been applied to demonstrate the potential yield over time (Hastings et al., 2009a). Modelling could also help optimise the effects of treatments and growing conditions on plug plants, the current (Clifton-Brown et al., 2016) technology for future seed based hybrids. This could be important when optimising on a larger scale with hormones such as in Section 4 above, or

adjusting the temperature required in the greenhouse for uniform emergence. As predicted with priming, a short warm time in the greenhouse could serve to synchronise germination in all the seedlings.

MiscanFor (Hastings et al., 2009a) and before that MiscanMod (Clifton-Brown, Lewandowski, & Jones, 2000) predicted the yield of *Miscanthus* crops. A model of the emergence may make planting by seed drilling predictable.

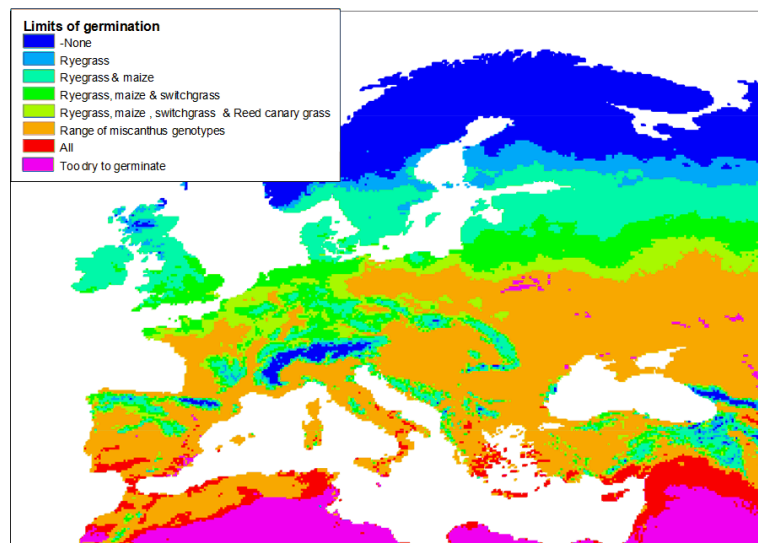


Figure 6-1: Map of seed propagation limits in Europe for *Miscanthus* and other selected grasses (Clifton-Brown et al., 2011).

Modelling factors which influence seed germination such as seedling emergence force (Dürr & Aubertot, 2000) or temperature, result in data which, when applied to a known system, has the capacity to give broad predictions. Figure 2 shows the result of a thermal gradient experiment modelled onto European temperature ranges (Clifton-Brown et al., 2011). As with temperature modelling, other variables rely upon knowledge of the crop agronomy and physiology to create a model that is capable of simulating outcomes in the real world. To do this modelling, basic data about the crop responses to the environment must be obtained (Clifton-Brown et al., 2000). However, through small scale testing

information can be added to the model that will show how a procedure can affect the crop in a wide variety of real world conditions. A good example of a small scale test that can be applied is taking a measure of seedling emergence force to show what an emerging hypocotyl can penetrate; this builds into a wider model of field aggregate size (Dürr & Aubertot, 2000). However, there may be no simple way of expressing how germination changes with time (Garcia-Huidobro et al., 1982) (see Section 2.1a above).

6.2 METHODS

6.2a DIRECT SOWING AGRONOMY TRIAL

The design of this field trial was made in conjunction with Mr Chris Ashman of Aberystwyth University; sowing, harvesting, and measuring of plants at the Aberystwyth site were also done with Mr Ashman. The Blankney site was sown, measured, and harvested by Dr Michal Mos of Blankney Estates Ltd (Blankney, UK) and Terravesta Ltd (Lincoln, UK), with assistance from Mr Ashman.

Simultaneous field trials were conducted in Aberystwyth (West UK) and Blankney (East UK) to compare *Miscanthus* seed establishment by direct sowing while testing two agronomic improvements. The soil surface texture in Aberystwyth is classed as sandy loam whilst in Blankney it is clay loam. Mulch film and priming treatments were used in conjunction with staggering the sowings throughout the growing season to investigate the effect of sowing time and to monitor the treatments under a range of conditions. Sowing late in the year may lead to a shorter establishment period for the crop if the seeds or seedlings overwinter. This may allow commercial growers to sow *Miscanthus* after harvest

lowering the economic impact of crop establishment. The seed type used was SYN55 (see Section 2.2 above).

Similar sites in Aberystwyth and Blankney each used 54 random plots, with two rows of seed (one primed one unprimed) in each plot. The priming was done commercially by Elsoms Seeds to give a high chance of successful priming (see Section 2.1g above). Pairing rows of primed and unprimed seed in the plots allowed mulch film to be used sparingly. Each row was sown with three-hundred seeds to enhance the chance of overcoming the low germination rate of the primed seeds. This was an acceptable density because competition should not be a factor in the first 18 months, which was the major measurement time for the trial.

In both field tests the ground was power harrowed five weeks before the first sowing. Roundup™ was applied 3 weeks before and Grazon® 90 5 days before first sowing. After the experiment began some weeding was attempted by hand because effects of herbicides on *Miscanthus* are still not well enough understood to use with confidence during early plant growth (Anderson et al., 2010; Everman et al., 2011). The seeds were sown into compressed furrows to prevent them being washed down into the sub soil.

Between May and September, seeds were sown every three weeks, under film and exposed with a row of primed and unprimed in each plot (three repeats). The intention of this was supply a better idea of the tolerances of primed seed. This could also determine the best time for planting *Miscanthus* with or without film.

The model (based on SimPIE (Dürr et al., 2003)) was intended to be tested against these results. However, this requires a value for how easily the seeds can emerge from the soil at the sowing depth, given the tilth. The soil on the

field was stony and inconsistent; as a solution, no soil was added above the seeds. Uniform soil could have been used; however, this would not have helped the modelling, and commercial growers and anecdotal evidence is that the seed survival may be improved by sowing into an exposed compressed groove. Meteorological data from both sites was provided by existing weather stations nearby. Soil temperature and soil moisture data was provided by a Campbell CR1000 data logger. Probes (CS616 reflectometers & type T thermocouples) were placed just under the soil on the first sowings, in both film and none film plots. Because there were different climatic conditions each time the mulch film was laid, it would have been helpful to monitor each plot; however, this was not possible so the temperature from four sensors under both film and exposed provided data for the later film plots.



Figure 6-2: A photograph of a plot (with summary information added in the top-left showing plot and treatments). A series of images such as this were taken of every plot in Aberystwyth in the first year to record weed cover, seedling successes, and plot health.

Seedling emergence for each plot was counted approximately every week, labelled photographs (Figure 6-2) were also taken to provide more information about possible fluctuations in seedling emergence and provide a record of weed growth. The emergence of seed at the Aberystwyth site was analysed with a generalized linear model using a negative binomial distribution (from the MASS package (Venables & Ripley, 2002)). This was tested against Poisson and zero inflated models with a Vuong's test (Zeileis, Kleiber, & Jackman, 2008). The

effect of each factor was checked by running the model with and without it included, and comparing the likelihood ratios using with an ANOVA. In Blankney the same set of tests were tried but a zero inflated Poisson was used and the likelihood ratios were checked with a nested likelihood test (Bilder & Loughin, 2014, p. 249) (from the 'lmtest' package (Zeileis & Hothorn, 2002)). This may be because a zero inflated model treats the zeros as a separate nested model within its structure.

End of year plot measurements were taken when the plants began to senesce (November 2013); these consisted of counting the number of stems and estimating the number of plants in the row. Then the tallest stem in each 50 mm section was selected for senescence, leaf number, height (to the last ligule 2.1c), and stem thickness measurements. This gave detailed data on up to 20 stems per plot (as seen in Figure 3-14a). Regular senescence scores were collected after the end of year counts, these were done per row by eye using a 1-10 scale (from zero for no senescence to ten for full senescence), to chart the senescence occurring. The same measurements were taken at the end of the second year (October 2014), without the senescence scores, because the plants senesce normally in the second year.

Both of these data sets were analysed by using three-way ANOVAs on height, thickness, leaf number, and senescence. The three factors used in the ANOVA were film, priming, and sowing number. Each was analysed with interactions first, if the interactions were not close to significance they were removed from the model and the model was run again. The two models were then compared and if not significantly different, the simpler model was used, unless its residuals deviated significantly from normality. Tillering was analysed in the same way as emergence, with a generalized linear model using a Poisson or negative binomial distribution depending on the result of a Vuong's test. To determine

the effects of each treatment the model was run without the treatment, an ANOVA of the likelihood ratios was used for significance (Pinheiro & Bates, 2000).

Dry weights were collected at the start of the third year, an effective harvest time for *Miscanthus* (Clifton-Brown & Lewandowski, 2002). Plots were harvested with a hedge trimmer and kept in sacks at 45°C for 3-5 days until their weight was unchanged, dry weights were then taken. The Blankney site harvest data was lost so biomass index was used as a substitute; this is an informal breeder's measure for non-destructively assessing biomass and consists of number of stems multiplied by height. Both of these data sets with zero values removed were analysed using a three way ANOVA without interactions (if there were no significant interactions). If sowing was a significant factor, a Tukey's HSD was used to highlight significant groupings.

6.2b MULTI GENOTYPE DIRECT SOWING TRIAL

The design of this field trial was made in conjunction with Mr Chris Ashman of Aberystwyth University. Sowing, harvesting, and measuring of plants at the Aberystwyth site were also done with Mr Ashman.

This experiment was similar to the 'Direct Sowing Agronomy Trial' above, but focused on the differences between *Miscanthus* genotypes for direct sowing in the UK. This experiment was a smaller, concurrent direct sowing field trial using five seed crosses and primed seed was carried out in Aberystwyth (West UK), with all plots replicated four times, both under mulch film and without film. This trial consisted of six seed types: primed seed (see Section 2.1g above), four crosses from Texas crossing blocks (SYN55, SYN56, SYN58, & SYN16), and SYN17 which was crossed under glass in Aberystwyth. The SYN55 is widely used in this study, as is the primed SYN55; the other crosses were chosen to

have parents from a range of climates. Comparing direct sown seed in Aberystwyth with and without film should give an idea of how seeds/seedlings with different temperature requirements respond. Also sowing the different crosses under film will investigate the effect of the thermal boost film should give. Seed were sown in compressed open grooves made with a v-shaped length of wood. This trial also used 300 seed per row to ensure a measurable difference in germination/ emergence. Sowing took place on the 30th of May 2013.

Field plots were scored for seedling emergence every week for 4 weeks, then biweekly. The emergence total was recorded for film-covered seeds; however, this was less accurate for the reasons explained in Section 6.2a. These plots were also photographed regularly to give a better understanding of the ground, weeds, and plant growth patterns.

Overall emergence was tested with a generalized linear model using a negative binomial distribution. Unlike Section 6.2a above, emergence was otherwise scored using the 40-day count, the counts were synchronised from sowing for all replicates, and counts were not made until day 33. Therefore, day 40 allowed plenty of time to pass without the tillering complication indicated to occur at approximately 45 days (Section 6.2a). The emergence data was then logged for normality and analysed with a two-way ANOVA. This was done to allow for a Tukey's HSD post hoc test, the results of which could be compared to Tukey's HSD results for final year wet weight, to give a general idea of to what extent the groupings were maintained once the plants were grown.

Plots were harvested using a forage harvester at the start of the third year. Larger plots had fresh weights taken, and then a subsample (~600 g) was used to calculate moisture content, and work out DW. These samples were lost so fresh weight data was used to analyse plot success. This data was analysed

without failed plots (zeros) using a two-way ANOVA and a Tukey's HSD; the effect of film was further tested with zeros included using a Kruskal-Wallis rank sum.

6.2c SOWING METHODS EXPERIMENT

An experiment was used to test the sowing method used in the field. Drilling *M. sinensis* seeds has previously been shown to be more successful than broadcast sowings as the seeds have better soil contact (Christian et al., 2005); but observations indicated seed sown beneath the soil had very low emergence. Seed sown in the two trials above (Sections 6.2a & 6.3b), were sown in open grooves and had relatively low germination rates.

This experiment used 330 mm lines of 100 seed, to approximate the same sowing density as 6.2a and 6.2b above (300 seeds per meter), each tray having four lines of seed sown using four different sowing styles randomly ordered. This experiment used the same cabinet and monitoring setup as the 'Seed Competition Soil Experiment within a Controlled Environment' experiment (Section 5.2b above) but to 31 days rather than 32. The film was removed at 22 days. This was done using SYN55, which should grow faster than the MX300 seed in Section 5.3b, resulting in an early harvest. For this test, soil from the field (experiments 6.2a & 6.2b) was used after being sieved and autoclaved. For one random line of seed in each tray, the soil was compacted to make a groove as done in the field experiments; for the three other comparison sowing styles, a soil-covered groove was used, and a surface sowing and finally a soil covered surface sowing. The soil was wet by adding water to the troughs the seed trays stood in, the grooves were made, and the seed was applied by shaking from an envelope as performed in the field. The trays were then covered with the same mulch film used in the field. This was removed and reapplied when taking

measurements. At decreasing intervals (2, 4, 7, 10, 14, 22, 31 days) the seeds' emergence/tillering and the elongation of the seedling were measured. When necessary and when the seed were measured the water in the reservoir troughs was topped up, to keep this experiment none water limited. At 31 days, the plants were dug up and the soil was washed off. Above ground elongation, plant number, tiller number, and the elongation of each tiller was recorded because the Seed Competition Soil Experiment within a Controlled Environment experiment (Section 5.2b above) showed that elongation was valid for small seedlings and was more variable than last ligule. The above and below ground plant material for each row was washed and separated, then dried (70°C for 48 – 72 h) and weighed, to obtain above and below ground dry masses.

The emergence and elongation data used one treatment with four levels, and was analysed with a Friedman's test over time (this used the mean value at each time point for each treatment). This was broken down using one time point with two Kruskal-Wallis rank sums for groove and soil covering or a two-way ANOVA depending on normality. The final measurements of above and below ground dry weight and root length treated the sowing method as two pairs of treatments: groove sowing vs. atop ground and under soil vs. on the surface. A two-way ANOVA was used for the analysis; if the results did not fit a normal distribution a transformation was used.

6.2d SOIL WATER CONTENT

For comparisons of germination between the laboratory model and field to be better assessed, 100 MX300 seeds were placed in Petri dishes containing around 85 g of 3 mm sieved, autoclaved and dried Aberystwyth soil (sandy loam). This was repeated 24 times; the dishes were then numbered randomly and the mass of each was recorded and the mass of soil in each calculated.

Then each of the 24 dishes had SDW (sterile distilled water) added to 1 of 6 soil water percentages (4, 8, 12, 16, 20, 24), these were chosen to represent the soil moisture range as recorded by reflectometers during sowings in Aberystwyth in 2013. The dishes were then placed in a random grid into a germination cabinet at 25°C and 60% RH [max] for 15 days.

Germination was then counted at 5, 7, 12, and 15 days. Each time the germination was counted, the dishes were weighed and the amount of water lost was calculated; SDW was added to replace it, this did not account for the mass of the growing seedlings, which should have been low compared to the dish and the soil. By monitoring the water loss over differing periods, it could be observed whether opening the dishes to count the germination lost most of the water, or if it was lost from venting while in the cabinet. At the end of the experiment, the soil with seedlings was dried and a mass was recorded to determine the errors accumulated over the course of the experiment.

Any effect soil water had on germination was checked with a Friedman test using time of count as the blocking factor. To calculate the minimum water for germination, Ψ would be required (Bradford, 1990); however, because this is difficult to measure in field sites seed germination to hydrotime was not used in the analysis. Instead an approximate linear model was made between proportions of seeds germinated and soil W/V, in order to calculate a minimum. This will only truly reflect a minimum for this soil, so may misinform the model. However, because the main model evaluations are done in the same soil this should not be a problem in this study. External to this study the model may need more refinement or a parameter for soil type, as Ψ is difficult to collect.

6.2e THERMAL GRADIENT FOR SEED GERMINATION

To provide detailed information on *Miscanthus* seed germination and allow for a calculation of base temperature, the same primed and control *Miscanthus* seed as sown in the UK field trials (Section 6.2a) and the genotypes from the 'Multi Genotype Direct Sowing Trial' along with eight promising hybrids; were assessed on a thermal gradient plate (Grant Instruments Ltd. Cambridge, UK). At each temperature, 60 seeds were placed on wet blue roll for the primed and control seed batches. Seeds were germinated under constant fluorescent light at a PAR of $\sim 70 \mu\text{mol m}^{-2} \text{s}^{-1}$ as measured by Clifton-Brown et al. (2011); this was combined with a constant temperature to avoid diurnal effects.

The bar was monitored by a 'Campbell CR10PW' data logger, which was used to confirm the consistency of the temperatures throughout the experiment. A glass thermometer and a multi-meter (CA5233 Chauvin Arnoux) thermocouple were used to calibrate and assess the temperatures at the beginning and the end of the test; the temperatures varied by 1°C or less within each set of cells. This was particularly necessary to check because there was a 1-2°C variation in the thermal gradient plate thermocouples at the same temperature; this may have been due to corrosion from extended use in a wet environment. During the experiment the thermal gradient plate was kept as wet as possible while avoiding floating the seeds (done using the wick system adding tap water every 48 hours).

SEEDS

The thermal gradient plate could test up to fourteen seed lots; the genotypes chosen for characterisation were picked based on diversity and using genotypes that could inform other experiments. Therefore, the main SYN55 and MX300 seed lots were used on the thermal gradient. Then seed lots SYN56,

SYN58, SYN16, and SYN17 were used to mirror the seed in the multi genotype experiment (Section 6.2b above). Newer seed were also used to inform future development of *Miscanthus* breeding: GNT1, GNT2, GNT3, GNT4, GNT5, GNT22, and GNT36. Primed seed (Section 2.1g) were included to determine the effect of priming in a controlled environment. The seeds were not surface sterilised to provide the model with more accurate parameterisation of germination.

TEMPERATURE RANGE

The thermal gradient plate was set to create a temperature gradient to approximate the range of field temperatures seen in the field trials in 2013 from the two UK field sites. Diurnal fluctuations were not used because the field sowings had been conducted throughout the year so there was no consistent day length. The field temperatures fluctuated a lot from -5°C to 42°C ; however, these extremes were not maintained for long so the thermal gradient temperatures were derived from running 6-hour averages on the data. From this the temperatures 4.6°C and 33.3°C were reached. These were simplified to give regular spaces of 2 degrees per cell on the thermal gradient (5 to 31°C).

COUNTING

Counts were done manually every day at the same time and visibly dead (very mouldy/squashy) seed was removed at approximately 150-hour intervals to stop mould spreading to other seeds and highly squashy seeds disintegrating beyond recognition. Germination of seeds was determined by eye when the radical had visibly emerged (Bewley, 1997a; Ellis et al., 1985a). The experiment was ended once no seed had germinated for 3 days. The end procedure was similar to Clifton-Brown et al. (2011); all-remaining seeds after the test was ended were given a temperature boost to 30°C to determine viability. Unlike Clifton-Brown et al. (2011) this was done only for 3 days after the germination had stopped.

However, as soon as the higher temperature germination started dropping the tweezer method (Borza, Westerman, & Liebman, 2007) was used to determine firmness. The temperature boost and the tweezer method were done to help establish which remaining seed were firm and/or viable; any firm seeds that did not germinate may have been in a deep state of dormancy. At the end of the test all seeds were placed into three categories according to their condition at the end of the test: Germinated, Mouldy or Viable.

RELIABILITY & ANALYSIS

The thermal gradient plate has been shown to be a reliable non water-limited way of assessing the effect of temperature on germination (Clifton-Brown et al., 2011). A snapshot of germination at seven days was used, chosen as a standard germination comparison time, this tested which seed lot, and which temperature performed best on the thermal gradient. This was done with both poisson and negative binomial generalized linear models (Venables & Ripley, 2002).

Using the range of temperatures and rates of germination on the thermal gradient plate the base temperature for the seeds were interpolated with information from Dr Ruth Sanderson at Aberystwyth University based on the work in Clifton-Brown et al. (2011). This was done by fitting a logistic curve to the proportion of seeds germinated along an axis of thermal time with a default base temperature (0°C); from this curve, the point of inflation (T_{50}) could be extracted. $1/T_{50}$ gives the rate of germination for each cell on the thermal gradient plate; a linear regression could then be done to find the intercept from all the rates ($1/T_{50}$) for each seed lot. Outliers outside $4/n$ of Cook's distance were removed (Bollen & Jackman, 1985, p. 268) and a robust regression from the 'MASS' R package was used (Venables & Ripley, 2002). However, this method removed too many points, and did not remove some high values that seemed to be lifting the

robust models; therefore, the data was trimmed to remove growth rates above 0.05. The intercept of this was then used as an approximation for the seed lots' base temperature because it is the temperature where the germination rate reaches zero (Gummerson, 1986). Confidence intervals were bootstrapped to this intercept using estimated standard error (this used the 'boot' package (Davison & Hinkley, 1997)).

6.2f THERMAL GRADIENT FOR SEEDLING STEM ELONGATION

An experiment was conducted to parameterise the effect of temperature on seedling stem elongation. Both SYN55 and MX300 seed were used because those are most compliant with the other experiments. Seed were not sterilised, in line with the first thermal gradient plate experiment. 400 SYN55 and 230 MX300 seed were germinated in the dark at 25°C on wet blue roll, to produce 196 germinated seeds. After 72 hours, the germinated seeds were moved to the thermal gradient plate (Grant Instruments Ltd. Cambridge, UK) with lighting added by Clifton-Brown et al. (2011). The thermal gradient plate was covered with glass panels to produce a consistent environment for the seedlings. There they were placed in a pre-determined random arrangement of rows across the temperature gradient, seven for each genotype. Each temperature interval had two germinated seed placed in it (for redundancy); these seed were measured as they were placed for stem/leaf elongation. The same temperature range was used as 'Thermal Gradient for Seed Germination' experiment (Section 6.2e above), 5 to 31°C in fourteen 2°C increments, but to reduce risk of stem breakage the seed were measured at decreasing intervals rather than daily (0, 1, 2, 4, 7, 11, 16, 22, 29, 37, 46 Days). The same lighting arrangement was used as in the 'Thermal Gradient for Seed Germination'. The water on the thermal gradient plate's wick system was regularly topped up. The seeds' health was

scored at the end using the same system as the germination tests used (Appendix K, without fluorescence imaging).

The difference between the seed lots' elongation rates was tested with a three-way ANOVA. For a selection of the central temperatures, t-tests or Kruskal-Wallis rank sums were done to check if there was a difference between the seed lots for the difference between the final elongations of the stems.

The results were analysed to find the base elongation temperature. This was done by modelling the elongation rate against temperature. The rate could be calculated by dividing the final elongation change by the final time elongation; however, it was decided to be more reliable to calculate the elongation rate per hour between each time point and the next then average these results. This method requires less reliance on accuracy in the final (most difficult) elongation measurement and may account better for the rate of elongation varying with time. The base elongation temperatures were calculated for each seed lot from the germination rate using a linear model; the outliers, where Cook's distance was less than $4/n$, were removed (Bollen & Jackman, 1985, p. 268).

A three way ANOVA was also done on the calculated elongation rate (logged for normality). This was used to test the relative significance and interactions of genotype time and temperature.

6.2g COMPUTATIONAL MODELLING OF GERMINATION

A *Miscanthus* model was created based on the SimPIE model (SIMulated Plant Emergence) (Dürr et al., 2001, 2003), this model by Dürr et al., was originally written in the C programming language and produced a mathematical seedbed in soil with stones positioned in the bed to simulate obstacles. The model was run on a daily loop of soil temperatures to determine the thermal time accumulated by each seed, and then its growth up around clods and through the soil surface

(Dürr et al., 2001, 2003). The model has been used for many crop and weed plants from mustard to weed beet (Brunel-Muguet et al., 2011; Brunel et al., 2009; Constantin et al., 2015; Dorsainvil et al., 2005; Sester et al., 2007).

A version of this model for *Miscanthus* was first attempted in Simile (Muetzelfeldt & Massheder, 2003), as it is user friendly and based on C++. However, as the model grew in complexity it was built in R and later improved and integrated with Python to allow an object-oriented approach. R Shiny was used as a graphical UI that sent inputs to the Python program. Data from Python was passed back to R for analysis and graphing. In this system, R saves user-selected data from Shiny into CSV files and passes these to a Python program, which runs the model. The Python program can run independently; however, when doing so it must be configured manually through input CSV files.

The model operates by producing a seedbed of clods and seed (Figure 6-3); the clods are added in size categories as in Dürr et al. (2001), normally starting with the largest clods and working towards the smallest. This makes them easier to place, because the smallest clods would limit the range of larger clods. Size of clod was assigned by placing clods in sets which represent sieve sizes, in line with Dürr & Aubertot (2000). For example, a clod in the 5 mm set would not pass through a 5 mm sieve, and would be the right length to pass through the sieve size above. Each clod is given as L, h and l dimensions, though different to Dürr & Aubertot (2000), L, the longest axis, represents the sieve size above.

Dürr et al. (2001) makes it clear that using ellipsoids is better: "This [ellipsoids] is more realistic than a spherical shape, and influences the hypocotyl length and the probability for seedlings to encounter a clod". Although ellipsoids are used for this model, it was excessively complicated to determine the intersec-

tions of ellipsoids in Python, especially because *Miscanthus* is not sown below the soil. Therefore, to calculate when clods overlapped, they were simplified into spheres. The radii of the spheres as calculated in equation (7) could then be used to determine easily if clods would overlap.

$$r = \frac{\left(\frac{L + h + l}{3}\right)}{2} \quad (7)$$

Clods can still be rotated through the primary (L) axis, so that seedlings encountering clods experience varying levels of difficulty in circumventing them. This also allows for full implementation of ellipsoids if needed in the future. The clods are then placed and rotated randomly to create variation in the seedbed, and their positions relative to other clods checked to prevent overlaps.

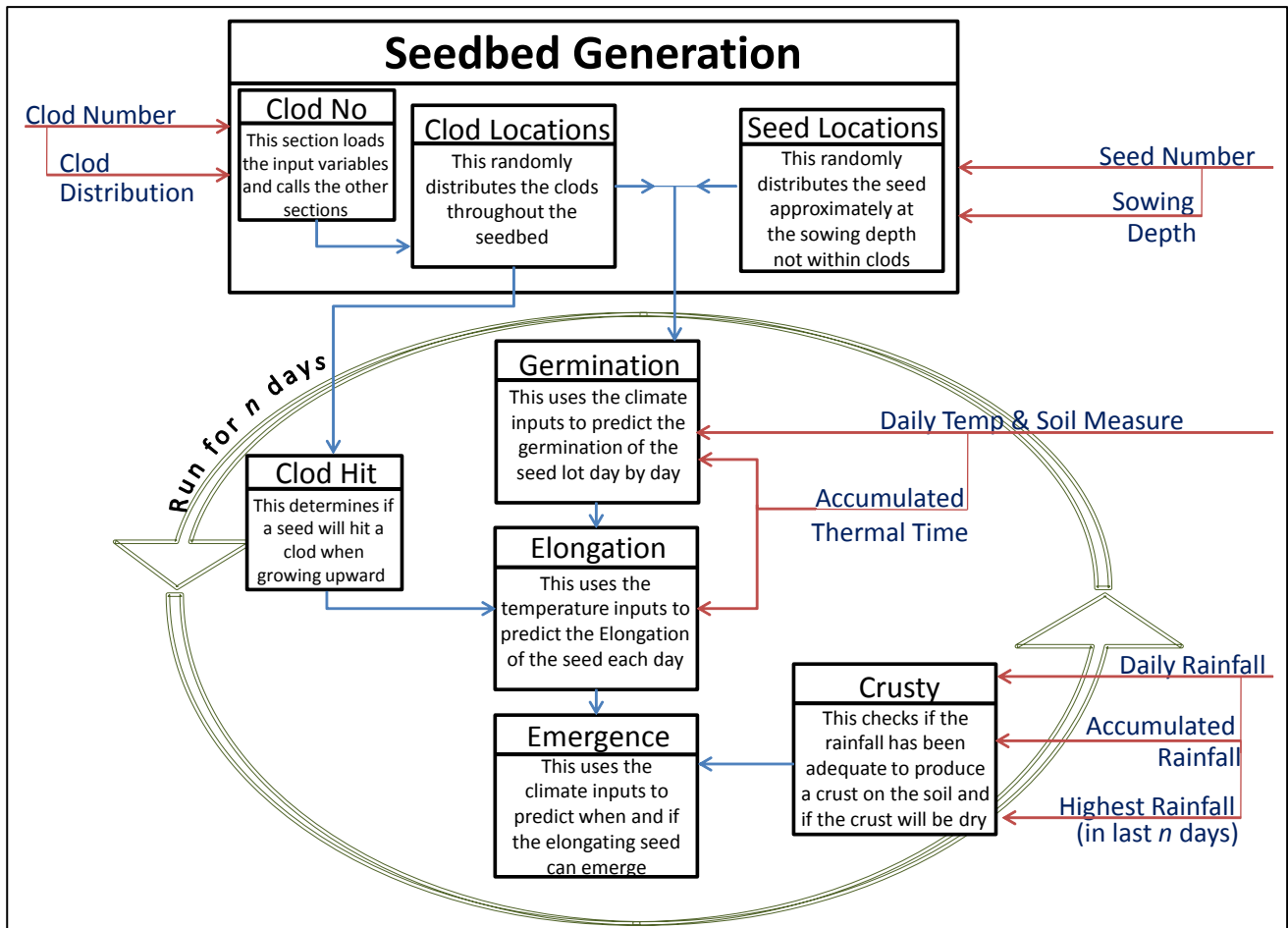


Figure 6-3: A basic diagram of each section of the model and what it does [for more information see Appendix P]. Within the section that is run each day, each seed is checked.

To allow for different soil types, the placement of a clod on the z-axis can be given a probability of being fully or partially above the soil surface. For example, a recently ploughed field may have more surface clods. Seeds are then added randomly to the seedbed at the sowing depth, and the seeds check their position so no two seeds occupy the same square millimetre and that the seeds were not placed in a clod. The seeds placement can be controlled on the y-axis, to provide one or two rows of seed to represent a field sowing better.

After the seed is placed, its thermal time requirement is selected proportionately from a user input distribution of thermal times by proportion of seeds germinated. The same thing is done with soil water volume required to germinate. This is

different to how the SimPIE model used water, as an on/off requirement using water potential (Brunel-Muguet et al., 2011). All this is done on day zero (Figure 6-3), then the seedbed advances by one day at a time. Then for each day, rainfall data allows a sub model to calculate if the soil surface has a crust and if that crust is wet or dry. This sub model works as outlined by Dürr et al. (2001) as based on earlier work (Dürr & Boiffin, 1995; Tamet et al., 1996).

Then each seed is advanced one day: First, the model has a seed death/loss factor that has been added to account for the seed being lost into the sub soil, so each un-germinated seed has a random chance of dying. This is to account for situations where the seed sit for a long time in the soil before conditions become ideal, and when they do, the seed does not seem to germinate and emerge. Then if the seed is alive, the thermal time it has gathered since sowing is calculated; if this is more than the number selected for that individual seed the water level is tested and must be equal or above the selected seed's requirement, for it to be marked as germinated [Appendix P]. If not it will wait until the next day. If the seed does not become lost in the soil it will pass the thermal time and retry the water the next day, this will continue until lost or germinated. Once the seed is germinated, the lost in the soil factor will no longer apply to it; however, a separate death factor is used for if the soil becomes dry enough to damage the seedling. *Miscanthus* seedlings seem to go through a secondary thinning process, which is only seen in soil based sowings, probably due to lack of water. Therefore, this model includes a chance of young seedlings dying if the soil is dry, if the soil is drier than a seedling's base soil moisture for germination it has a 1 in 5 chance of dying, which is re-calculated each day. However, each day it lives the probability of dying (p) is reduced (8), where N_d is number of days alive and p_y is the probability of dying yesterday.

$$p = \frac{1}{5} - \left| \frac{N_d}{10} + p_y \right| \quad (8)$$

In Dürr et al. (2001), after germination the early growth of the seedling is calculated by a weibull function; however, *Miscanthus* early growth better fitted a second order polynomial, this was determined from elongation data on the thermal gradient plate, where a second order polynomial fitted with an R^2 of 0.92 (Figure 6-4). Each time the seedling grows the position of the new tip is calculated, if this tip is inside a clod, the seedling may be stopped. Firstly, if the clod is under a set clod minimum size the seed can proceed unimpeded, if not the seed may be stopped from advancing. If the seed hits near the centre of the clod and the clod's first angle (incline along the longest axis) (L) is near to flat, the clod is determined to be impassable and the seed will wait 5 days and then die. However, if the clod is passable the seed will attempt to circumvent the clod each day for up to five days, during this time it does not grow taller. After five days, the seed will die if it has not passed the clod. In the original model the seed's ability to pass the clod was determined by random, either following the line of the ellipsoid or dying (Dürr et al., 2001).

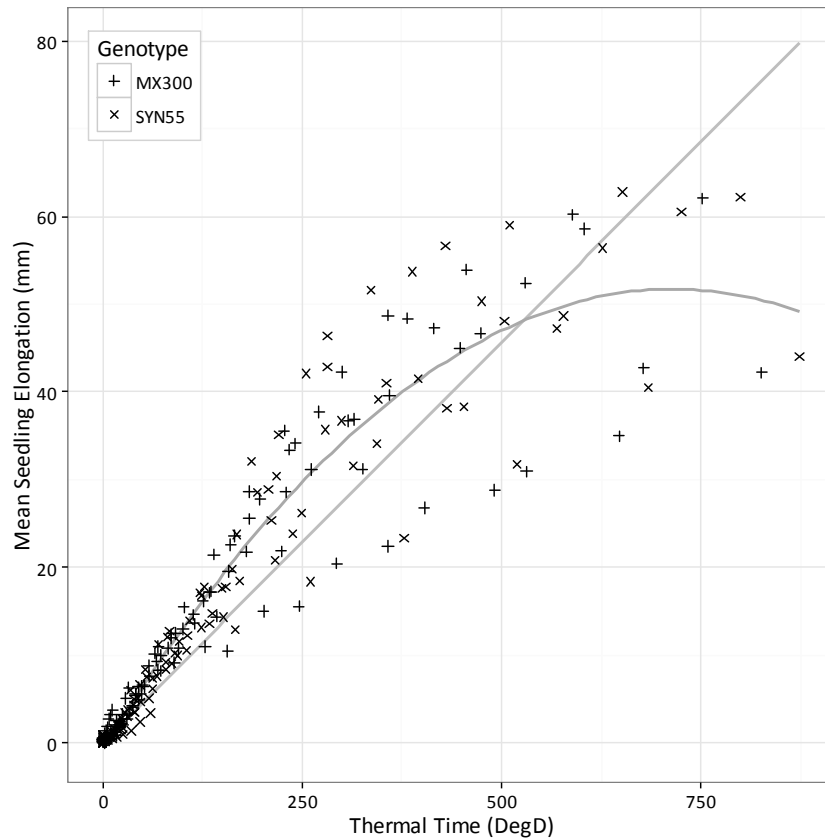


Figure 6-4: Parameterisation of early growth model, using data from the ‘Thermal Gradient for Seedling Stem Elongation’ experiment (section 6.3f).

When the seed reaches the soil surface, a sub model, the same as Dürr et al. (2001), determines if it can emerge. This is based on rainfall, and previous state of the soil crust. As with the clods, if the seedling is stuck under the soil crust for five days it is counted as dead. The model for growth after emergence of true leaves in the SimPIE model was not used, because there is no clear distinction in growth patterns for *Miscanthus* (which is a monocot). This was therefore based on the extra elongation seeds achieved on the ‘Thermal Gradient for Seedling Stem Elongation’, which at each time point was linear per degree-day (Figure 6-5). A linear model of growth was estimated from this (grey line in Figure 6-5); this was used for seedlings after they achieved the maximum growth (~5 cm) using the early growth model.

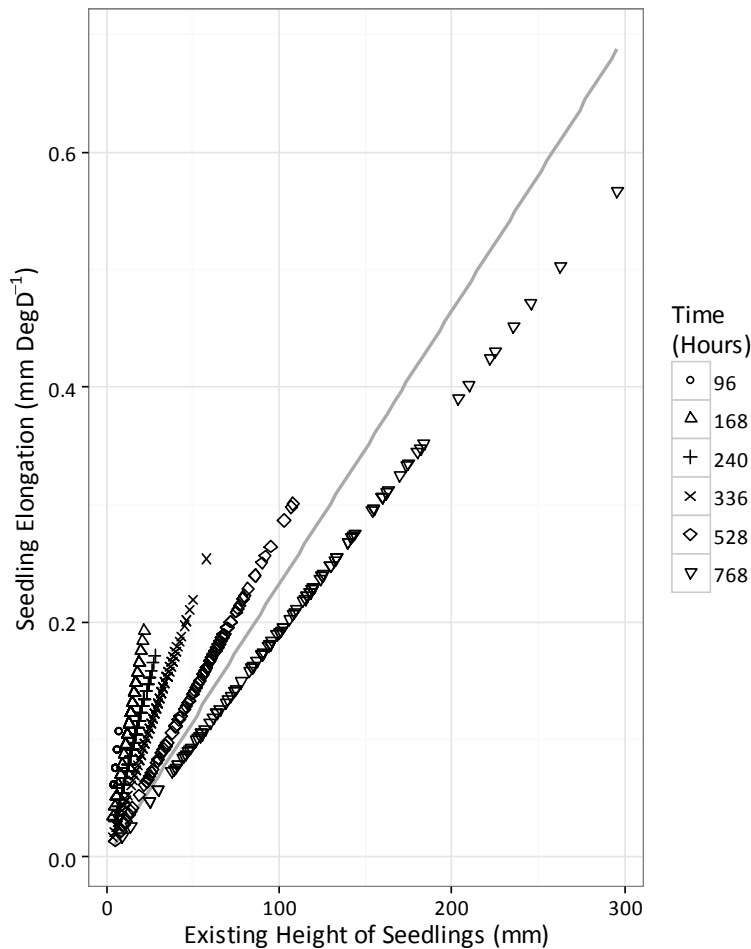


Figure 6-5: An estimation of the linear equation needed for late stage growth, based on thermal time (data from the 'Thermal Gradient for Seedling Stem Elongation' experiment (section 6.3f)).

The model was tested without clods because the only testing with soil above the seed was with sieved soil in a controlled environment, so clods nor surface crusting have been fully tested. However, they are still implemented to make the model more expandable.

The model was trained and compared each time on sets of data from different experiments. It can be compared to three types of growing environment: Firstly, the Petri dish with blue germination paper, which is closest to the thermal gradient plate from which the model's germination values are based; here seed loss can be turned off; water, clods, and crusty surfaces are also not an issue. The modelling was repeated six times to give the same replication as the com-

parison experiment. Germination was compared with a Friedman Rank Sum using time as the blocking factor, to the results of six of the controls from results in Physical & Chemical Germination Factors (Section 4.4), using both a specific (25°C), and all temperature range model. Wilcoxon tests were also used to compare the model to the real experiments for final germination and elongation.

Secondly, there is the controlled environment where water is not an issue but other aspects work similar to the field. This was then compared to the section ‘Sowing Methods Experiment’ (Section 6.3c), because this tested sowing beneath the soil. ‘Seed Competition Soil Experiment within a Controlled Environment’ (Section 5.2b above) was not used because the effect of seed competition is not included in the model, and its post emergence growth was used as a comparison during model development. To compare emergence and elongation across all times, likelihood ratios were compared between generalized linear models (Zeileis & Hothorn, 2002) produced with and without modelling (real or modelled data) as a factor. Final 31-day emergence and elongation were compared using Wilcoxon tests.

Thirdly, there is the field environment. This has the most hurdles to overcome, although, the model still does not have to account for stones or a crusty surface because the seeds were sown on the top of the soil in a groove. This was compared to both the ‘Direct Sowing Agronomy Trial’ (Section 6.3a) and ‘Multi Genotype Direct Sowing Trial’ (Section 6.3b), which allowed for comparison of a selection of seed lots. The modelling of both trials was tested as with the ‘Seed Competition Soil Experiment within a Controlled Environment’, by comparing generalized linear models with and without if the data was real as a factor. The emergence of seedlings were also compared at the closest time to 45-days, this

seemed the best end time for a comparison because after 45-days tillering affected the accuracy of field counts.

Across all levels, an R^2 was tested between mean predicted final results for each type; 40 or 45 days in the field, 31 days in the controlled environment, and 11 days in the lab.

6.3 RESULTS

6.3a DIRECT SOWING AGRONOMY TRIAL

The results for the 'Direct Sowing Agronomy Trial' are detailed below. Results were collected with Mr Ashman of Aberystwyth University and Dr Michal Mos of Blankney Estates Ltd (Blankney, UK) and Terravesta Ltd (Lincoln, UK).

This experiment tested the effect of priming mulch film and sowing time in two locations. Both the Aberystwyth and Blankney sites were analysed separately when testing the effects of treatments on metrics of success.

ENVIRONMENTAL EFFECT

Blankney was the warmer location during the trial, on average 3.7°C warmer over the first five sowings. The highest difference for a sowing recorded was 4.08°C warmer in Blankney when sowing 4 was sown (Figure 6-6). In the 180 days shown in Figure 6-6, on only three days did the Aberystwyth site achieved a higher mean temperature.

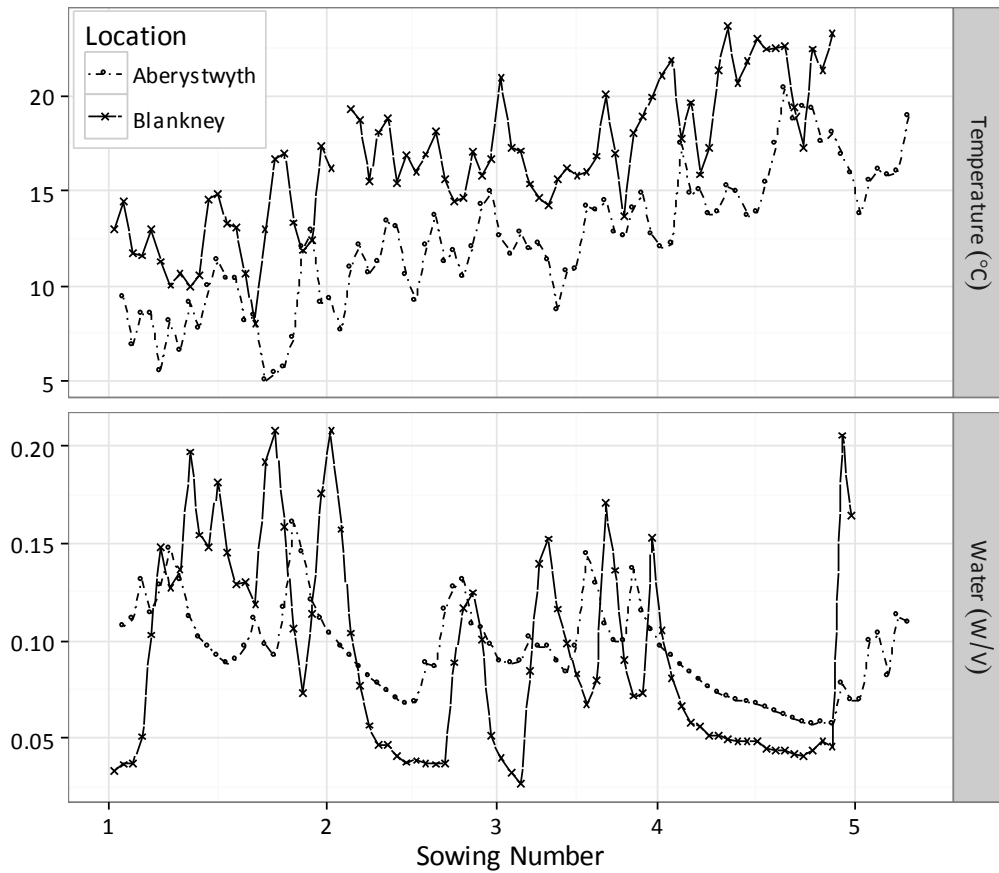


Figure 6-6: Mean daily temperature and soil water in the two locations. The sowings are shown on the x-axis, and the dotted line is Aberystwyth. These are averaged for both sensors not under film.

The soil moisture varied widely, particularly in Blankney (Figure 6-6), where the soil had high peaks with low troughs, dropping consistently below Aberystwyth in June as temperatures rose above a mean of 15°C. In the 76 days between the 10th of May and the 24th of July uncovered Blankney plots received ~344°Days more thermal time than uncovered plots in Aberystwyth. The temperature at the Aberystwyth site never peaked as high as the Blankney site, but the stonier top soil at Aberystwyth maintained more consistent soil moistures throughout the year (Figure 6-6). The Blankney site also had lower rainfall during the year (84 mm less during March to September), particularly July to September, which was during sowings 4 to 7 [metrological data in Appendix O].

Mulch film was observed to degrade fastest for the July-August sowings (in about 4 weeks); also, the 17th of October sowing did not have the film degrade until the following spring.

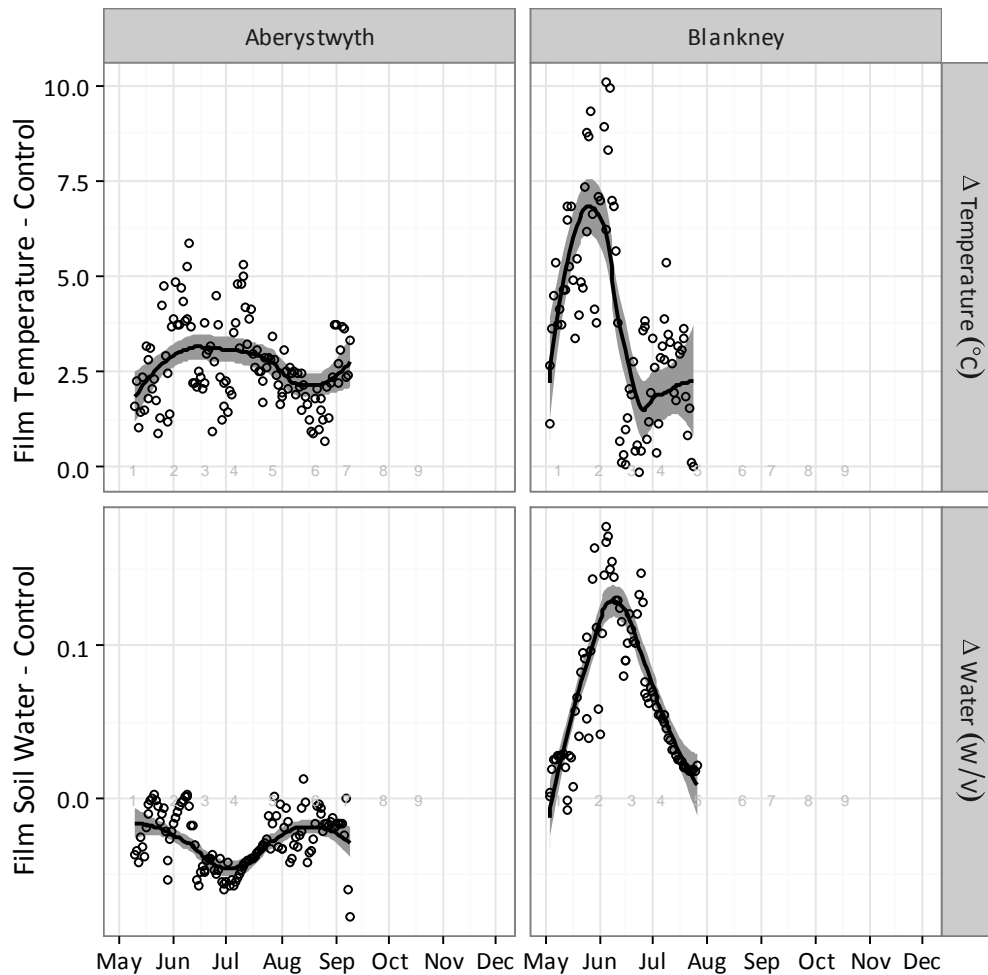


Figure 6-7: Change in soil moisture and temperature under film in both locations. Sowing times are visible numbers at the bottom of the graphs. A loess line has been applied to each set in black with a 95% confidence interval in grey.

Film was monitored under the first sowing; producing a positive effect over the first two months, after which the effect collapses (Figure 6-7). The net positive effect film had on temperature was much more pronounced in Blankney than Aberystwyth due to the higher temperatures in that location (Figure 6-7). During the first two months, the film had a $2.74 \pm 0.46^{\circ}\text{C}$ (SE) daily mean boost

in Aberystwyth and a $4.17 \pm 0.58^{\circ}\text{C}$ (SE) in Blankney. The effect of the film on soil water is more complicated, causing a drop in soil moisture in Aberystwyth and a spike in Blankney (Figure 6-7).

SCORING EMERGENCE

The later emergence scores were impaired by tillering, visible in the increase in emergence (Figure 6-8) after 50 days; new tillers were probably being counted as separate plants. When emergence is referred to in this experiment, it is taken as the mean emergence between 15 and 43 days – this is the flat section, visible in Figure 6-8 after most seeds have emerged yet before tillering. This averaging has been done to account for the inaccuracies in field counting, as well as to not include plots before most of the seed that is going to germinate has emerged, or after counting is failing due to tillering of seedlings.

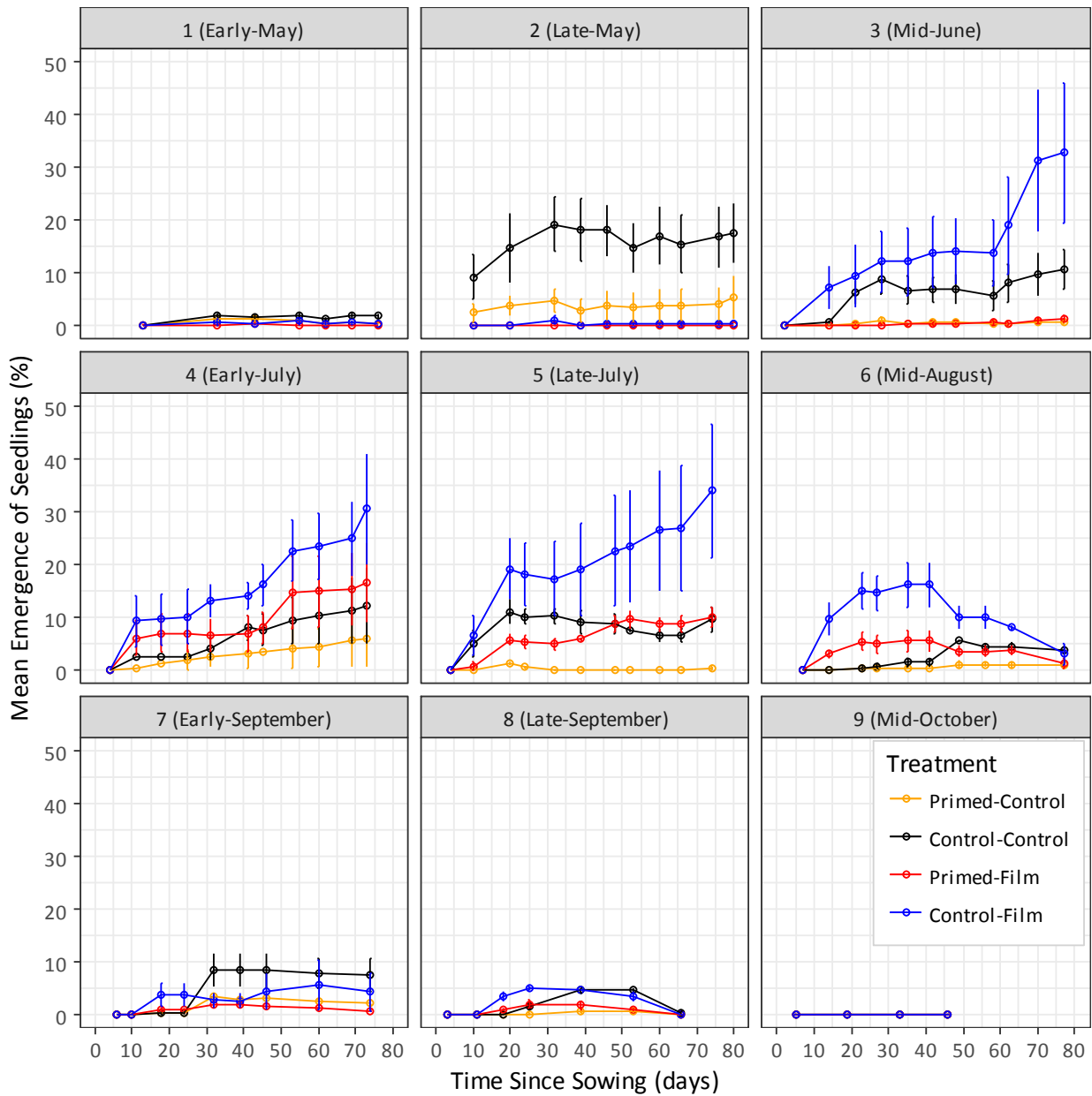


Figure 6-8: Emergence counts for seed sown in Aberystwyth, over the first 80 days after each sowing at all nine sowings. Lines are coloured by treatment combinations and have standard error lines added to show the variation in the three replicates.

Comparing the likelihood ratios from generalized linear models for the emergence resulted in sowing and priming having a significant impact on the model, being $P < 0.0001$ and $P < 0.0001$ respectively. This was not the case for mulch film with a significance of $P = 0.14$.

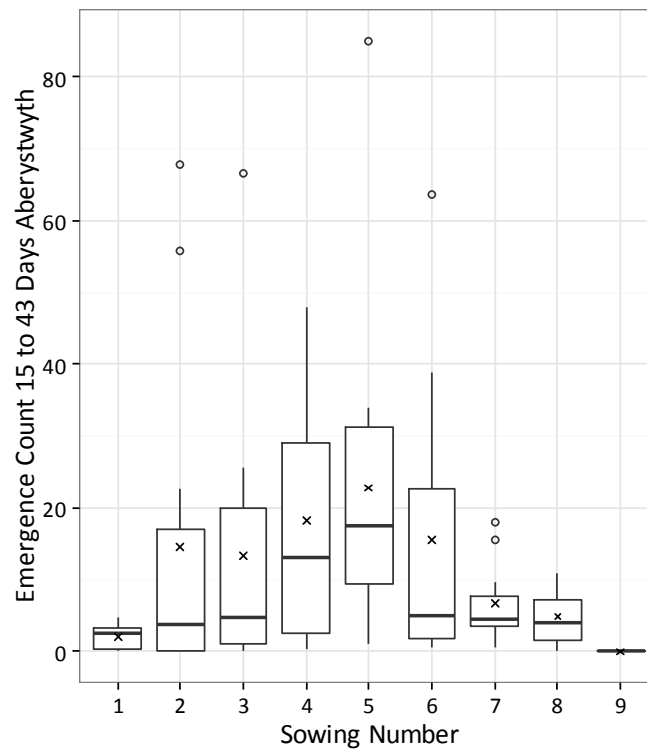


Figure 6-9: The mean number of seed emerged in the Aberystwyth site, as counted 15 to 43 days after each sowing out of 300 seed sown. The boxes show the variation in the twelve plots: Three replicates of each set of film and control, with primed and control for each.

The sowings show a clear trend over the year with an emergence increase at sowing 2 (late-May) (Figure 6-9); this was when the soil temperature was regularly averaging above 10°C. This effect would not show single sowings as distinct if analysed with a post hoc test. The emergence peaked at sowing 5 (late-July) (Figure 6-9) in Aberystwyth, this was the warmest month (mean average daily temperature 15.85°C).

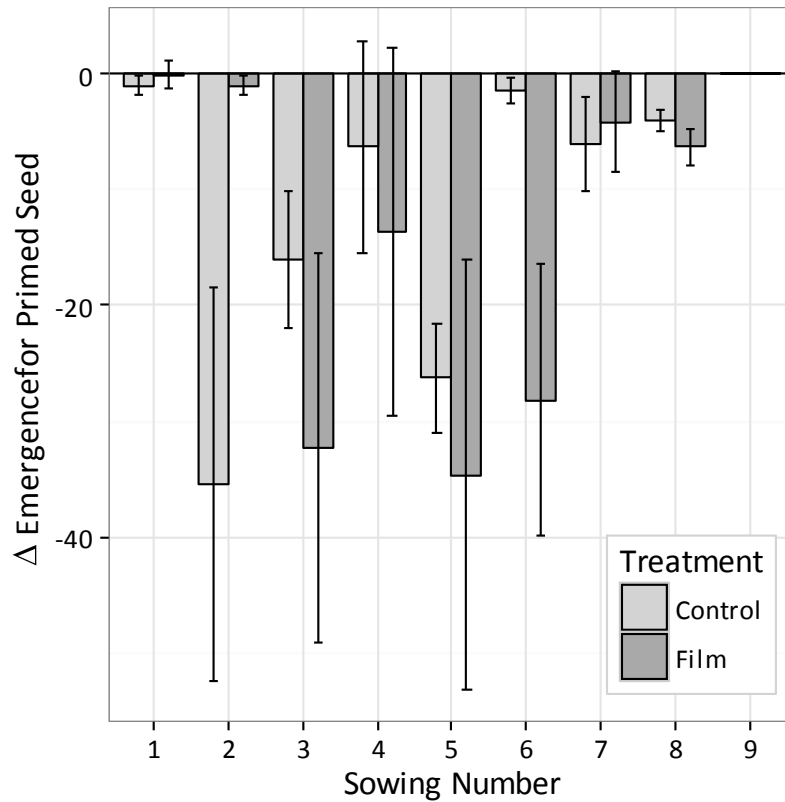


Figure 6-10: The change in seedling emergence due to priming in Aberystwyth, for emerged seed counted between 15 and 43 days. The number of emerged seed each primed plot had the number of elongated seed in each corresponding unprimed seed plot subtracted from it. Standard error bars have been added to show variation in the three replicates.

Figure 6-10 shows primed seed had negative effects in all sowings and treatments in Aberystwyth; however, these effects were variable, but are most noticeable in the sowings where more seedlings emerged. However, a drop in the percentage of emergence after priming was performed was already known (Section 2.1g above).

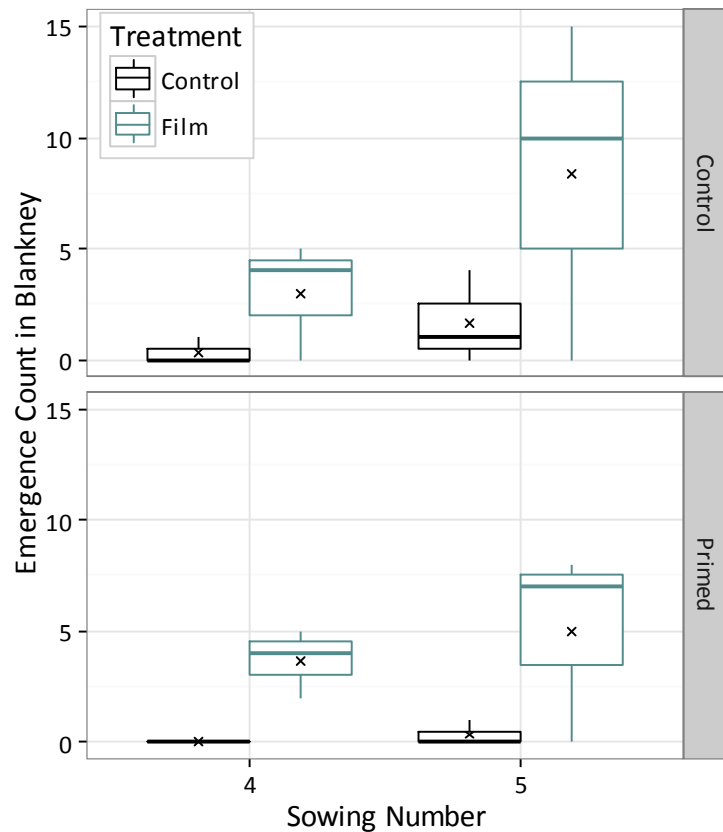


Figure 6-11: Emergence counted in Blankney for sowings 4 (early-July) and 5 (Late-July) out of 300 seeds sown. The boxes show the variation between the three replicates; means have been added as an x.

Blankney lacked clear emergence data because emergence was only counted once. Only sowings 4 and 5 had emergence scores recorded between 15 and 43 days. Therefore, only these two sowings were analysed, with a generalized linear model using a zero inflated Poisson distribution. Likelihood ratios showed most significance for film ($P < 0.0001$), then sowing ($P < 0.001$), with priming only just significant on $P < 0.05$. More seedlings emerged in the later July sowing and the film treated plots; however, less of the primed seedlings emerged (Figure 6-11).

END OF FIRST YEAR

In both locations, end of first year measurements were carried out in the last week of November and the first Week of December. The measurements will be covered in the order of total tillers, height, stem thickness, number of leaves, and senescence. Aberystwyth results will be given first in each case.

The comparison of likelihood ratios between negative binomial models for the number of tillers in the Aberystwyth sowing showed a significant effect for priming ($P < 0.0001$) and sowing time ($P < 0.0001$). Film did not have a significant effect on the model ($P = 0.81$). In all Aberystwyth sowings, control seed produced more tillers than primed seed by the end of the first year (Figure 6-12 (top)). Film plots did produce more tillers in Aberystwyth for sowings 3 to 6 but film produced negative effects in sowings 1 and 2.

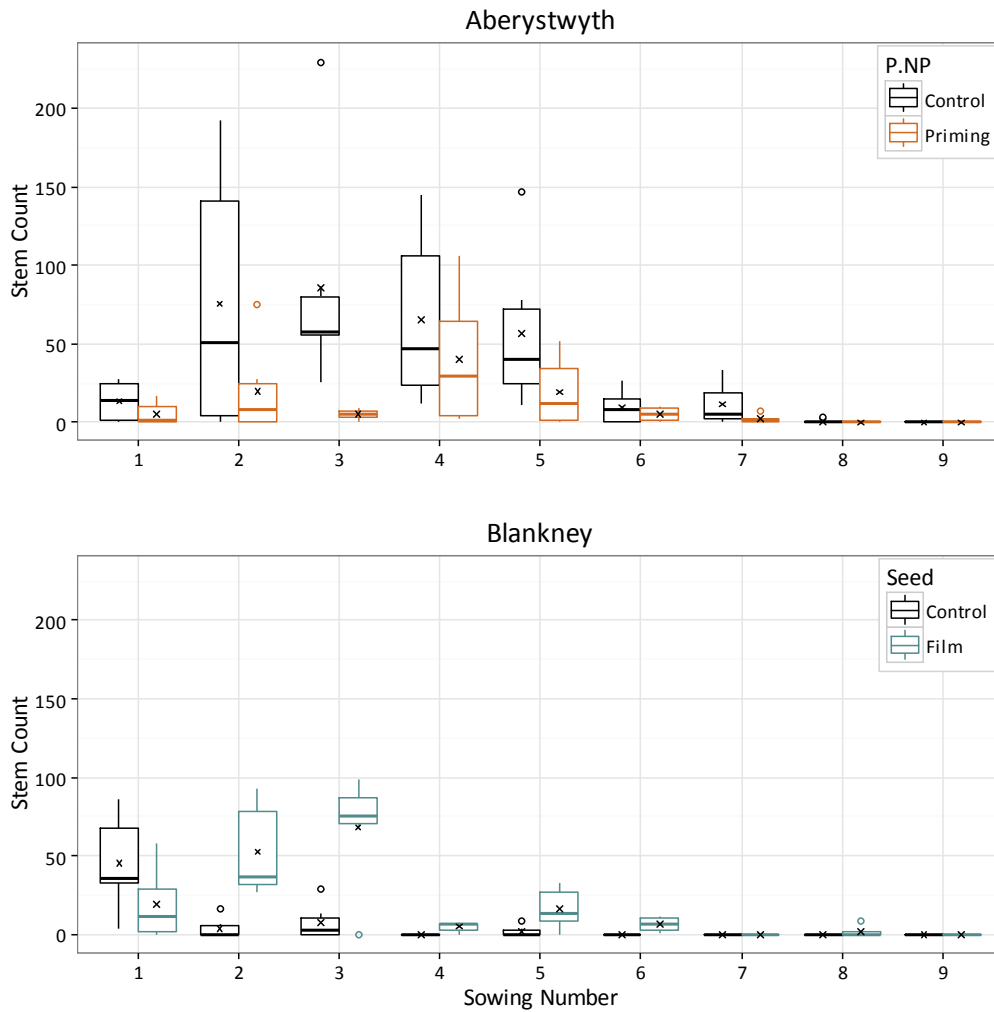


Figure 6-12: Significant factors for first year tillering in Aberystwyth and Blankney. Boxes show the variation in the non-significant factors and the three replicates; means are represented with an x.

In Blankney, comparison of likelihood ratios used the same negative binomial distribution; this found that mulch film and sowing had a significant effect on the model (both $P < 0.0001$), while priming did not ($P = 0.4$). Mulch film in Blankney generated the largest improvement in tillering in sowing 3 (mid-June), and noticeable improvements in sowings 2 to 6 (Figure 6-12 (bottom)). However, film had a negative effect on sowing 1 (early-May); when tested with a Kruskal-Wallis rank sum this effect was not significant ($P = 0.08$).

Both locations had no tillering in sowing 9 (mid-October); therefore, there was no first year information on sowing 9.

Height for sowings in Aberystwyth was tested with a three way ANOVA, without interactions. From this there were significant effects on height from mulch film ($P < 0.01$) and sowing ($P < 0.0001$), but not from priming ($P = 0.68$). A Tukey's HSD placed sowings 1 to 5 in the high means group, with 6 to 8 in a low group and sowing 5 and 6 in an intermediate group. Sowings in or before July (high group) had mean heights more than 60 mm, while sowings sown after July had heights of less than 30 mm (Figure 6-13 (top)). Plots covered with mulch film, had stem heights on average 23 ± 11.7 mm (SE) longer.

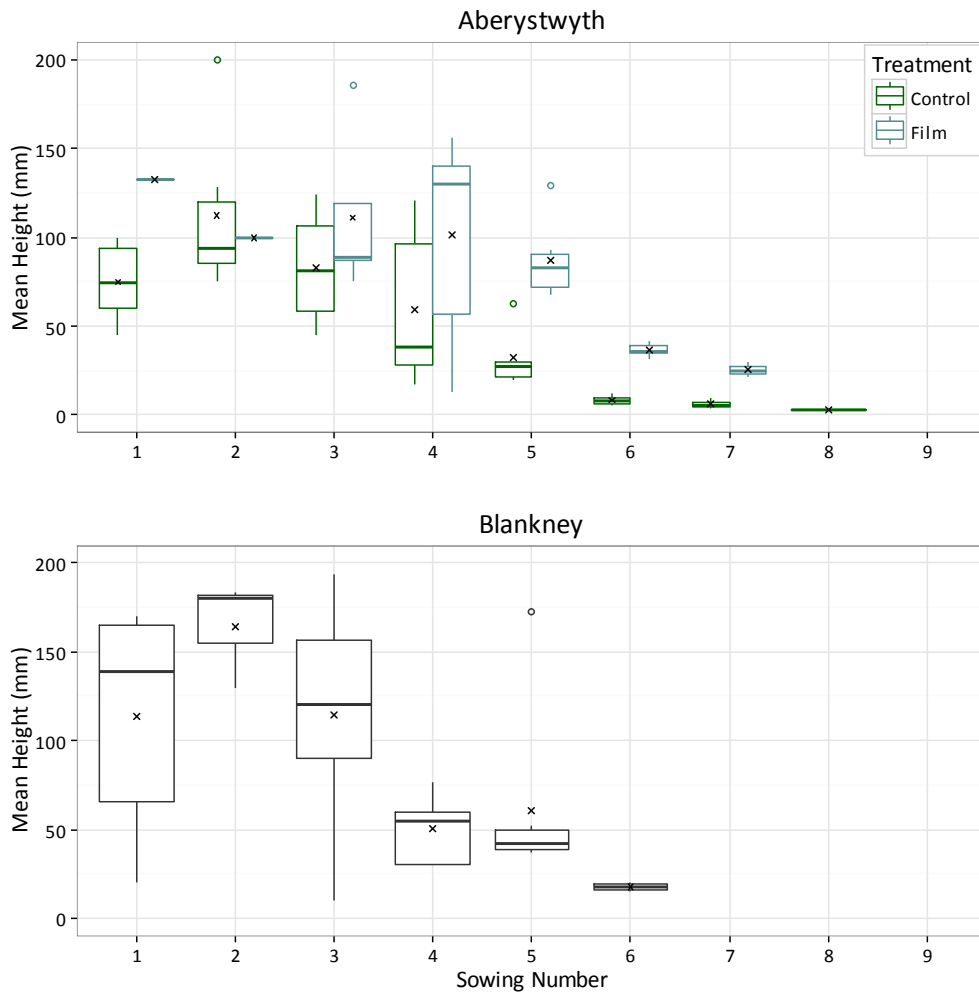


Figure 6-13: Significant effects on height in both locations, using data from the end of the first year. Boxes show the variation in the non-significant factors and the three replicates; means are represented with an x.

The Blankney data was sine transformed for normality, from this there was only a significant effect from sowing ($P < 0.0001$), and there was no significant effect from mulch film ($P = 0.27$) or priming ($P = 0.34$). A Tukey's HSD showed an upper grouping of sowings 1 to 3 (May/June) with heights more than 160 mm and a lower grouping of sowings 4 to 6 (July/August) with heights equal to or less than 60 mm (Figure 6-13). Sowings in Blankney after sowing 6 (September onwards) failed to have any measurable height.

Both mean thickness of the stems measured, and mean number of leaves for those stems, behaved collinearly as a measure of plant success and were therefore analysed together.

When tested with a three way ANOVA, Aberystwyth stem thickness measurements were only significant for sowing number ($P < 0.0001$), not mulch film ($P = 0.83$) or priming ($P = 0.15$). There were no significant interactions so a model without interaction was used. A Tukey's HSD produced two groupings: Sowings 1 (early-May) through 5 (late-July) had mean thicknesses between 3.2 and 4.3 mm, while the lower grouping of sowings 4 (early-July) to 8 (late-September) had thicknesses between 1 and 3.2 mm. Sowing 1 to 3 had very similar heights, with height decreasing from 4 onwards (Figure 6-14). Mean number of leaves per stem counted was also only significant for sowing when tested with a three way ANOVA, with sowing having a significance of $P < 0.0001$, film of $P = 0.16$, and priming of $P = 0.36$. There were no significant interactions. However, the Tukey's HSD was more complex (seven groupings) because the number of leaves followed a seasonal curve up to sowing 2 (late-May) and decreased from there (Figure 6-14 (bottom-left)).

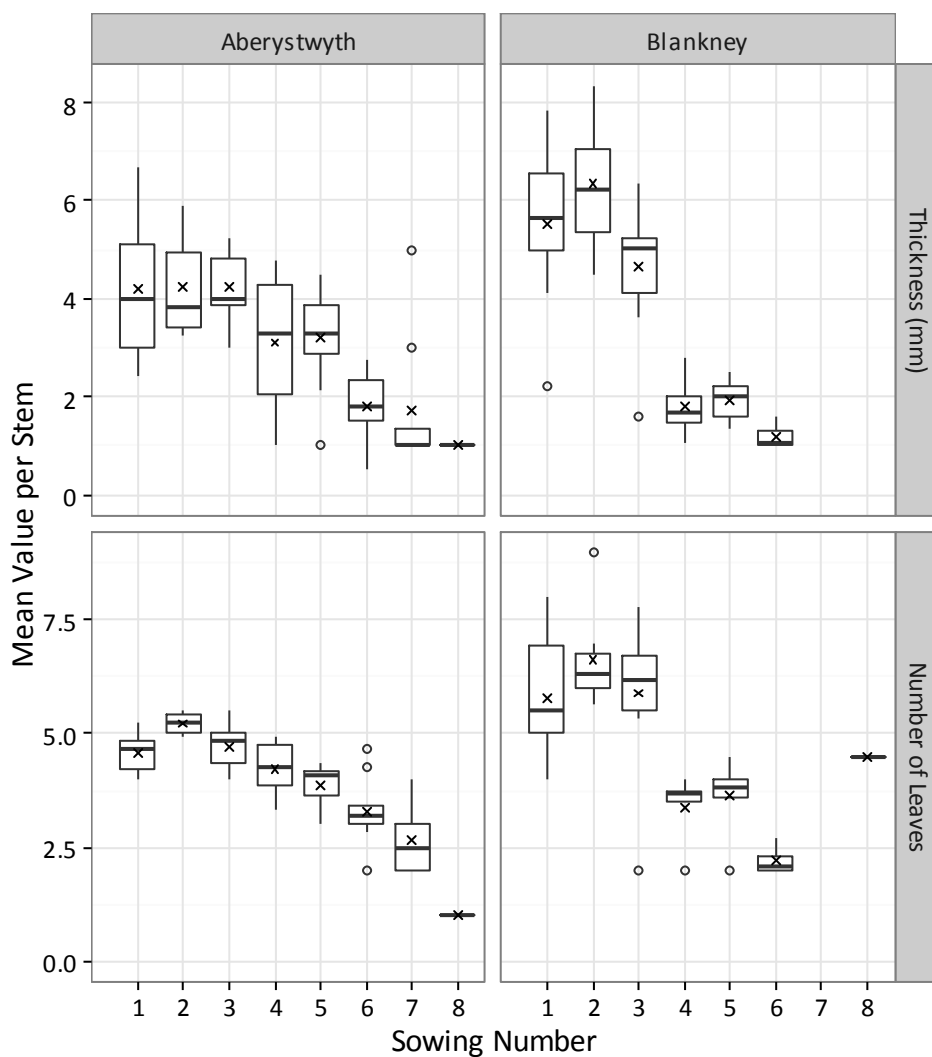


Figure 6-14: The end of first year measurements for stem thickness and number of leaves, in Aberystwyth and Blankney. The boxes show the variation in the twelve plots; film and control, as well as primed and control with three replicates of each. Mean values added as an x.

Blankney end of first year stem thickness was tested with a three way ANOVA, done without interactions as a prior test had shown them to be insignificant. This test resulted only in significance for sowing number ($P < 0.0001$), and not film ($P = 0.15$) or priming ($P = 0.95$). A Tukey's HSD placed the sowings into three groups: 1 and 2 (May) in the thickest stemmed group (mean > 5.5 mm), 1 and 3 in the next group, and 4, 5 and 6 (July to August) in the thinnest group (mean < 2 mm) (Figure 6-14). The mean number of leaves per stem measured followed the same pattern, where there was only a significant effect from sowing

number ($P < 0.0001$), not film or priming ($P = 0.32$, & $P = 0.99$), when tested with a three way ANOVA (interactions were not significant but were maintained to keep normality). A Tukey's HSD produced two groups; sowings 1 to 3 and 8 were in the leafiest group (≥ 4.5 leaves per stem), 5 to 6 and 8 in the low leaf group – sowing 8 (late-September) was an outlier with only one successful plot (Figure 6-14).

Figure 6-14 shows the first three Blankney sowings (May to June) performed better in stem thickness and number of leaves than in Aberystwyth; however, Blankney sowings dropped in both rapidly at sowing 4 (early-July).

The senescence scores in Aberystwyth were analysed with a three way ANOVA with interactions. There was no significant interaction for priming and a close interaction for mulch film ($P = 0.73$). Therefore, this was re-modelled testing only for an interaction from film, these models were not significantly different ($P = 0.96$) so the simpler model was used. This found a significant effect on senescence from sowing ($P < 0.0001$) and a significant interaction between sowing and film ($P < 0.05$), though there was no significance of priming ($P = 0.47$) and film ($P = 0.12$).

Film placed at the start and at the end of the year increased the senescence score in Aberystwyth, yet film application on sowings 2 to 5 (late-May to late-July) reduced senescence score; overall plants tended to score higher when sown later (Figure 6-15).

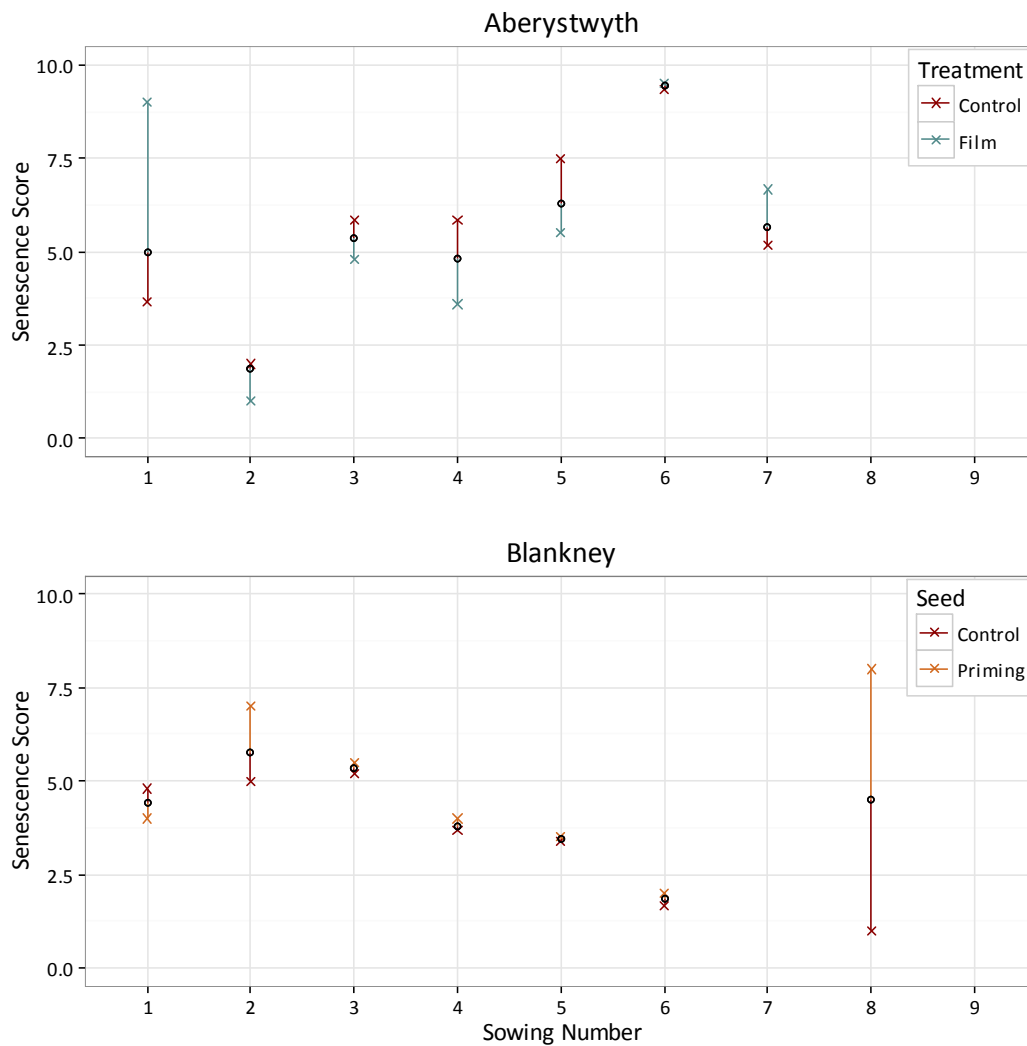


Figure 6-15: Effect of sowing on senescence and interactions in both Aberystwyth and Blankney. The black dots are the mean senescence score for each sowing; the effect of the interacting factor is shown as a line from the sowing mean to the means for both levels of that factor's sowings.

In Blankney the senescence scores followed a similar pattern when analysed with a three way ANOVA, except priming produced the close interaction ($P = 0.92$), and film was dropped as an interaction factor. The models were not significantly different ($P = 0.97$), so the simpler model was used. This ANOVA found sowing to be significant for senescence ($P < 0.0001$), and there was a significant interaction between priming and sowing ($P < 0.05$). Again, film and

priming on their own did not have a significant effect on senescence ($P = 0.48$, $P = 0.2$).

Figure 6-15 (bottom) shows earlier sowings in Blankney tend to have higher senescence with priming having notable increases in senescence in sowings 2 (late-May) and 8 (late-September).

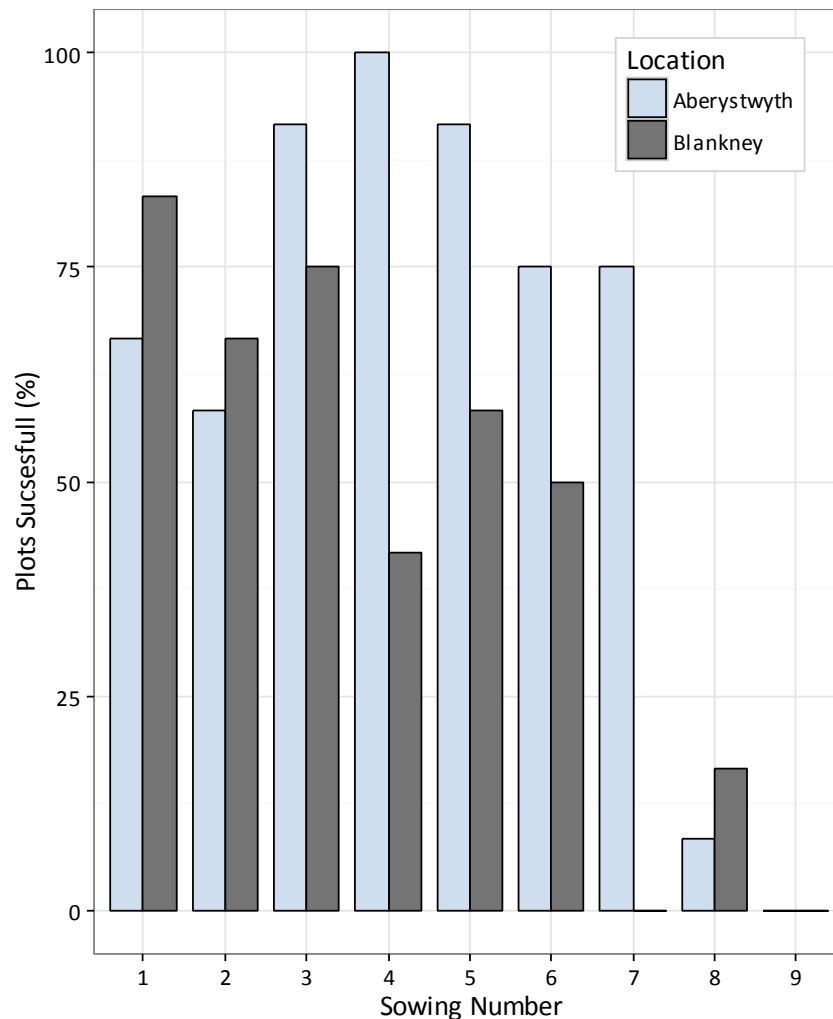


Figure 6-16: The percentage of plots that had any plants growing at the end of the first year, by location and sowing number.

Comparing counts made at the end of the first year in Aberystwyth and Blankney shows Blankney produced less consistent plots with a failure rate of 56.5% to 37% in the westerly location (Figure 6-16). This was exacerbated due to

Blankney's hotter drier weather later in the year (Figure 6-7) [metrological data in Appendix O]. Over the growing season, Blankney only reserved 75% of the precipitation the Aberystwyth site did.

SECOND YEAR COMPARISON

Blankney's warmer climate was more pronounced on the second year results. No plots that had no emergence in the first year showed any emergence in the second year in Aberystwyth, whilst in Blankney second year emergence only happened in sowings 7 & 9 (Early-Sept & Mid-Oct) with sowing 9 achieving a height of 16 cm but few plants.

Aberystwyth follows the same pattern as in the first year, with sowings and priming having a significant effect on the negative binomial generalized linear model ($P < 0.0001$ & $P < 0.0001$), while film did not ($P = 0.8$). Tiller counts had increased in earlier sown Aberystwyth site sowings; plots without film at sowing showed no change or less tillers from sowing 5 (late-July) onwards, whereas plots with film decreased at sowing 6 (mid-August) (Figure 6-17).

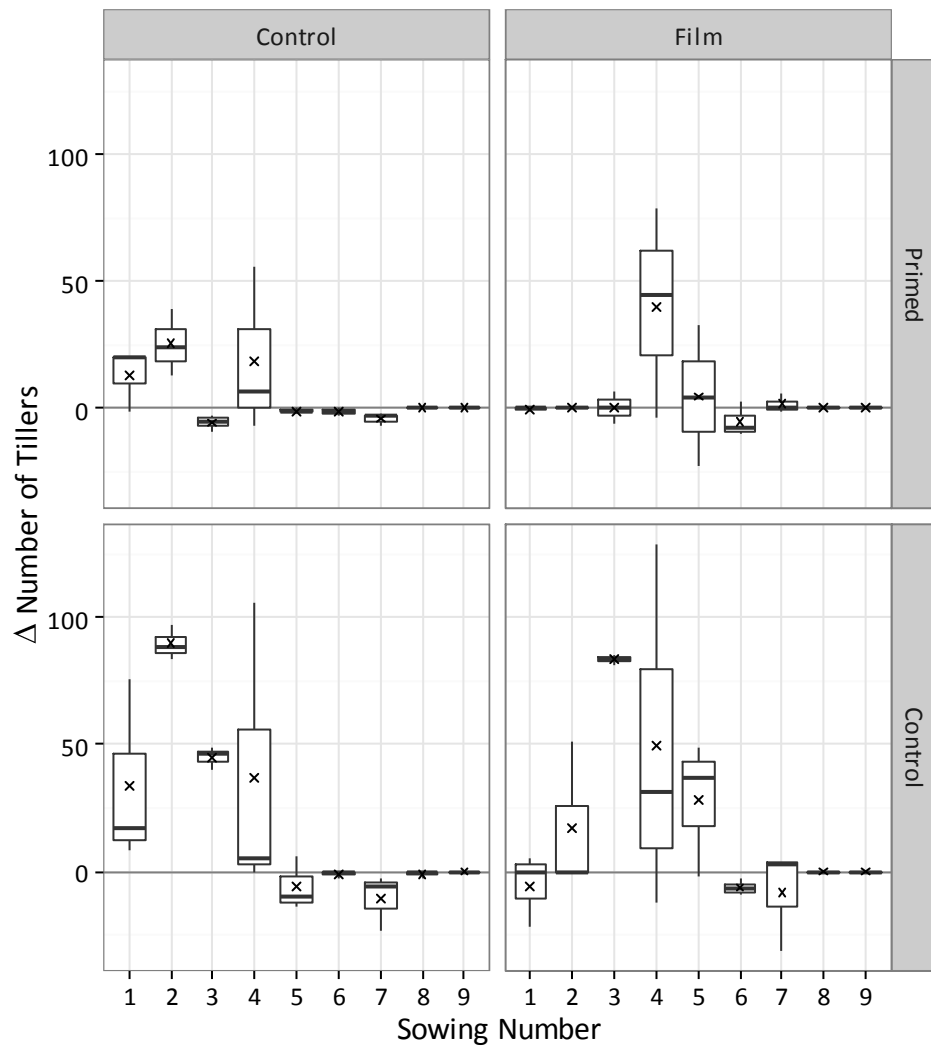


Figure 6-17: Change in tiller number in Aberystwyth field site from the first to the second year, separated by mulch film and priming treatments. Boxes represent the variation between the three replicates.

Figure 6-17 also shows smaller changes in the primed seed sets over the control. In Aberystwyth, the second to fourth (mid-June & early-July) sowings have the most improved tiller numbers in the second year (Figure 6-17). In all the sowings with mean positive effects in Aberystwyth using un-primed seed, the primed seed had smaller increases.

Testing the likelihoods between negative binomial generalized linear models of these changes showed all factors, film, sowing, and priming to be significant ($P < 0.0001$, for all three).

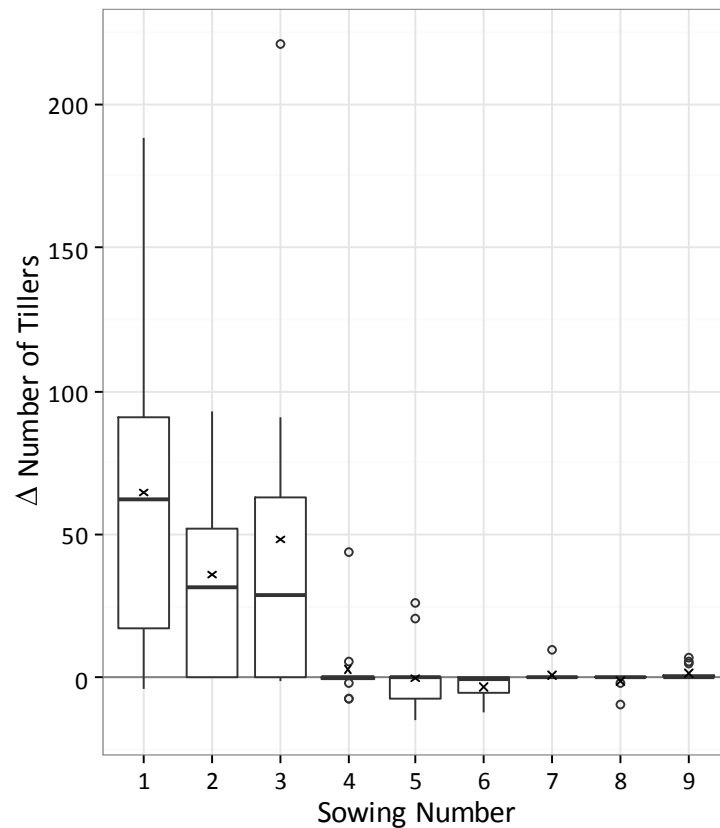


Figure 6-18: Change in tiller numbers at the Blankney site between the first and second year. Grouped into sowings with an x representing the mean change in each sowing. The boxes show the variation in the twelve plots; film and control, as well as primed and control with three replicates of each.

In Blankney, the second year had significant effects on tillering from priming ($P < 0.0001$), sowing ($P < 0.0001$), and film ($P < 0.01$) when likelihood ratios were tested from negative binomial generalized linear models. The change between the first and second year was only significant for sowing ($P < 0.0001$), and not mulch film or priming (both $P = 0.06$). Sowings 1, 2 and 3 (May to June) in Blankney show large (30+ tiller) improvements in the second year (Figure 6-18), while many plants in later sowings failed to over winter.

Table 6–1: An analysis of end of second year height, thickness, and number of leaves in Aberystwyth and Blankney. Second year values and a comparison of change since first year were used; both analysed with three way ANOVAs followed for a sowing significance with a Tukey's HSD (written in order of mean).

	Second year	Sowing			Mulch Film		Priming	
		Post Hoc Tukey's HSD			P	Highest Mean	P	Highest Mean
Aberystwyth	Height	2 3 4 1 5 6 7	a ab ab abc bc bc c	< 0.001		0.51		0.8
	Thickness	1 3 4 5 2 6 7	a ab ab ab bc b c	< 0.0001	Control	< 0.05		0.136
	Leaves	2 3 4 1 5 6 7	a a a a a ab b	< 0.001		0.303		0.94 # ^
	Δ Height	2 3 4 1 6 5 7	a ab ab ab ab b b	< 0.01		0.83		0.445
	Δ Thickness	1 3 6 4 5 2 7	a ab ab ab ab b b	< 0.01		0.302		0.674
	Δ Leaves			0.18		0.456		0.691 #
	Second year							
Blankney	Height	1 3 2 4 5 6 7	a ab ab bc c c c	< 0.0001		0.854		0.916
	Thickness	1 3 6 4 5 2 7	a ab ab bc ab b b	< 0.001		0.405	Control	< 0.05
	Leaves	1 2 3 4 5 7 9	a a a ab ab ab b	< 0.001		0.783		0.622
	Δ Height	1 3 2 4 5	a ab bc bc c	< 0.001		0.985		0.943
	Δ Thickness	4 5 1 3 2	a ab bc b b	< 0.05		0.903		0.09
	Δ Leaves			0.174		0.795		0.768

The three way ANOVA included an interaction test because an interaction was close to significance.

^ Priming had a significant interaction with sowing number (P < 0.05)

The averaged per stem second year measurements, and the comparisons of these to first year measurements, are analysed in Table 6–1 and demonstrate

only two points of significance for film and priming. Firstly, un-filmed plots were significantly thicker in Aberystwyth. Secondly, unprimed seed were significantly thicker in Blankney. Both of these significances were not the case in the first year.

Sowing number is significant for all tests in Table 6-1 except change in leaf number between the first and second years. Stem Height and thickness as well as the changes in them between years, are highest for the earlier sowings and lowest for the later sowings (this can also be seen in Figure 6-19). Figure 6-19 also shows some Blankney plots grew in the second year while no Aberystwyth plots did; however, this was inconsistent because sowing eight produced plants in the first year but none in the second.

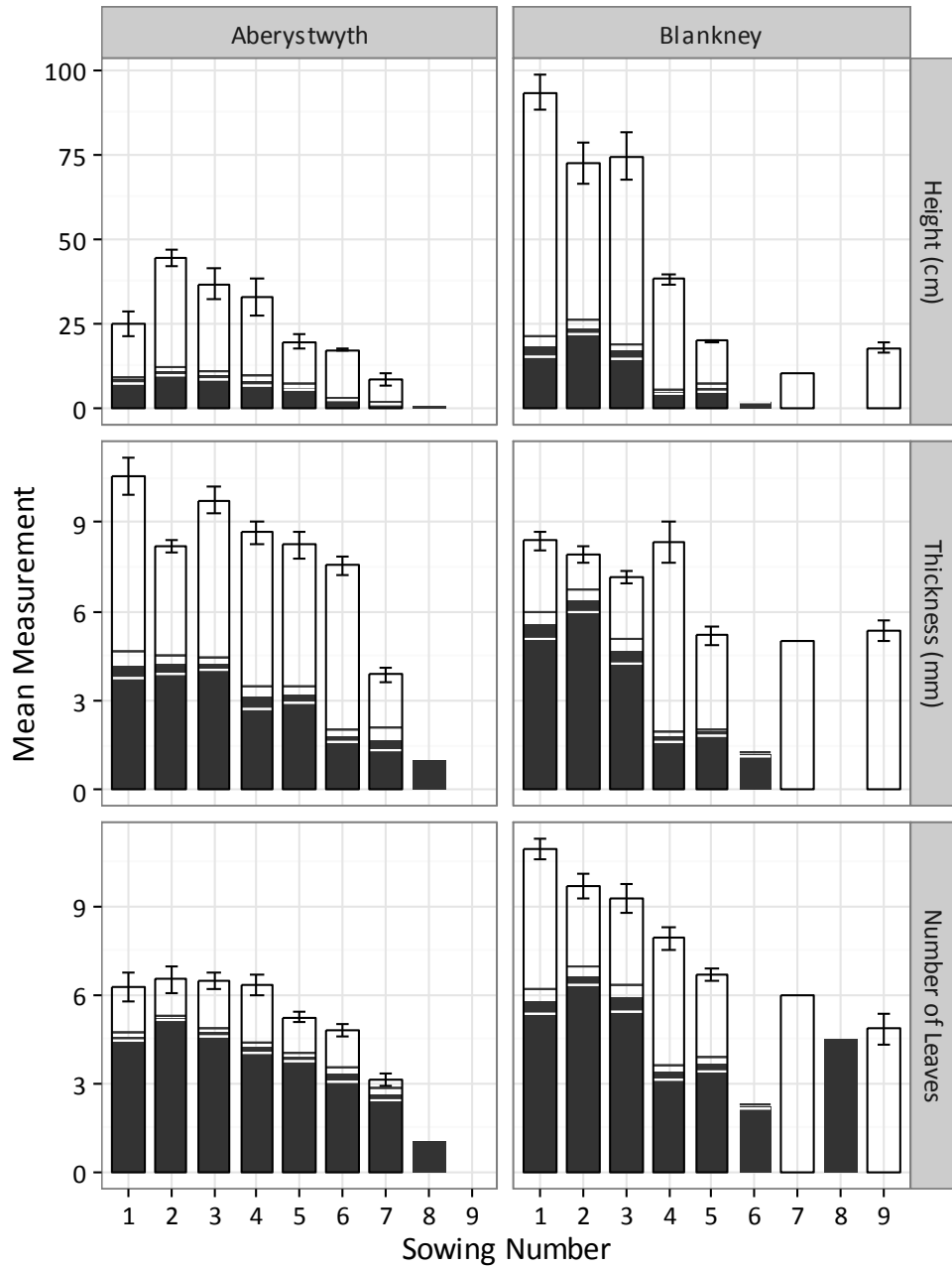


Figure 6-19: Measurements per stem for each plot, for the metrics taken (means of up to 20 measurements per plot of stem height, thickness, & number of leaves). First year recordings are in dark grey with standard errors as stripes. Second year readings are in white with standard error bars. Both show the variation in the twelve plots; film and control, as well as primed and control with three replicates of each.

The plots that improved the most were primarily the plots that had the best start in year one. Plots in Blankney can be seen in Figure 6-19 to improve in year two faster more than the plots in year one due to the warmer climate.

Table 6-1 also adds a significant interaction between priming and sowing number in Aberystwyth for mean leaf count per stem. This interaction is due to primed seed being leafier in the first sowing (mean, +2 leaves), but less leafy in sowings 2 and 3 (mean, -0.5 to -1 leaf), these two sowings were the peak for leaves per stem; all other sowings had small effects. Therefore, the model is predicting as total leaves increase effect of primed seed decreases (Figure 6-20).

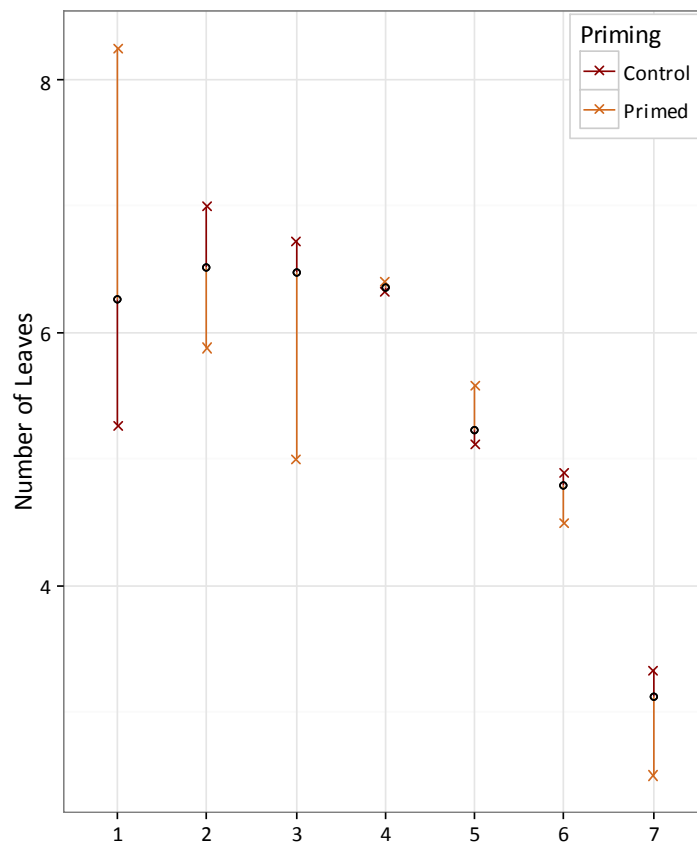


Figure 6-20: Mean number of leaves in Aberystwyth as measured at the end of the second year (black), with the control and primed seed means shown with lines to demonstrate the interaction.

FINAL OUTCOMES

In Aberystwyth the dry weights without zeros from plots harvested at the start of 2015, were significant for sowing ($P < 0.05$) and close to significance for priming ($P = 0.07$), but not for film ($P = 0.76$); there were no significant interactions. There were no significant groupings in the sowings based on Tukey's HSD. Primed seed lines produced only 53.5% of the dry weight not primed seed lines produced (Figure 6-21).

To test the final survivorship a binomial generalized linear model was compared with and without each factor; the mulch film did not have a significant effect on survivability ($P = 1$, both had exactly 18 out of 54 plots harvestable), yet priming and sowing number did ($P < 0.01$ & $P < 0.0001$). The sowing that was most likely to be harvestable was sowing 3 (mid-June) in control seed then sowing 2 and 4 in primed seed (Figure 6-21). All treatments in the Aberystwyth site had dry mass at or near zero if sown in or after August; however, control seed still had a high (66.7%) chance of successful harvest in sowing 5 (late-July) (Figure 6-21).

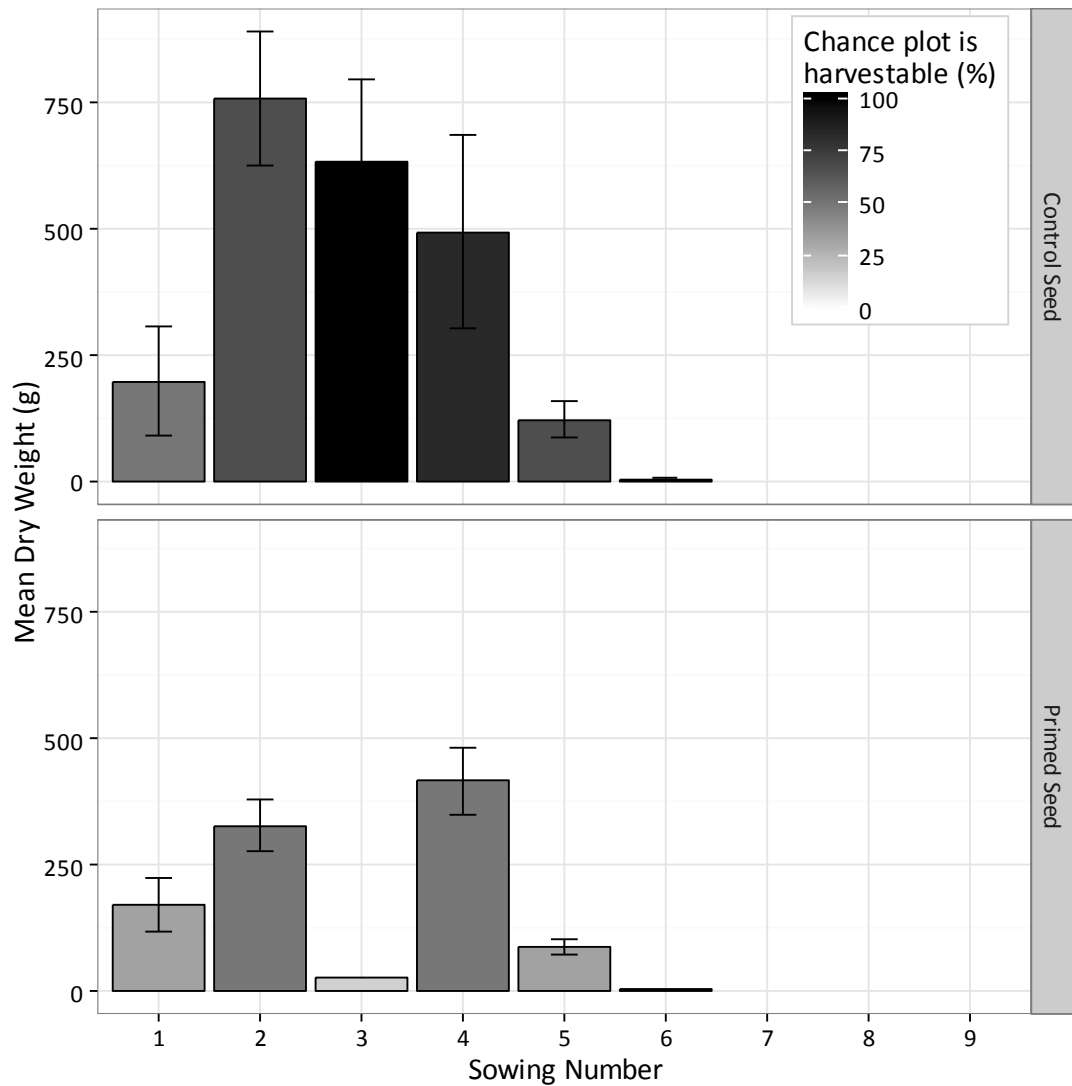


Figure 6-21: The mean dry weights for harvests at the end of the second year in Aberystwyth with standard error bars that show the variation in the six plots; film and control with three replicates of each. The bars are coloured by the percentage chance plots in this set of six were harvestable.

The moisture content was $(\log + 1)^4$ transformed for normality and found to be significant for film ($P < 0.05$). The difference in means was $50.2 \pm 5.2\%$ (SD) moisture in none film plots while filmed plots had a moisture content of $53.5 \pm 3.4\%$ (SD) at harvest. Sowing time and priming did not have a significant effect on moisture content ($P = 0.53$ & $P = 0.3$).

In Blankney the biomass index in the second year followed the same pattern as Aberystwyth dry weight without zeros, with sowing significant ($P < 0.05$) but priming and film not ($P = 0.13$ & $P = 0.76$). In Blankney, sowing 1 (early-May) had the highest biomass index and sowing 3 (mid-June) the second highest, although the Tukey's HSD found no significant groupings.

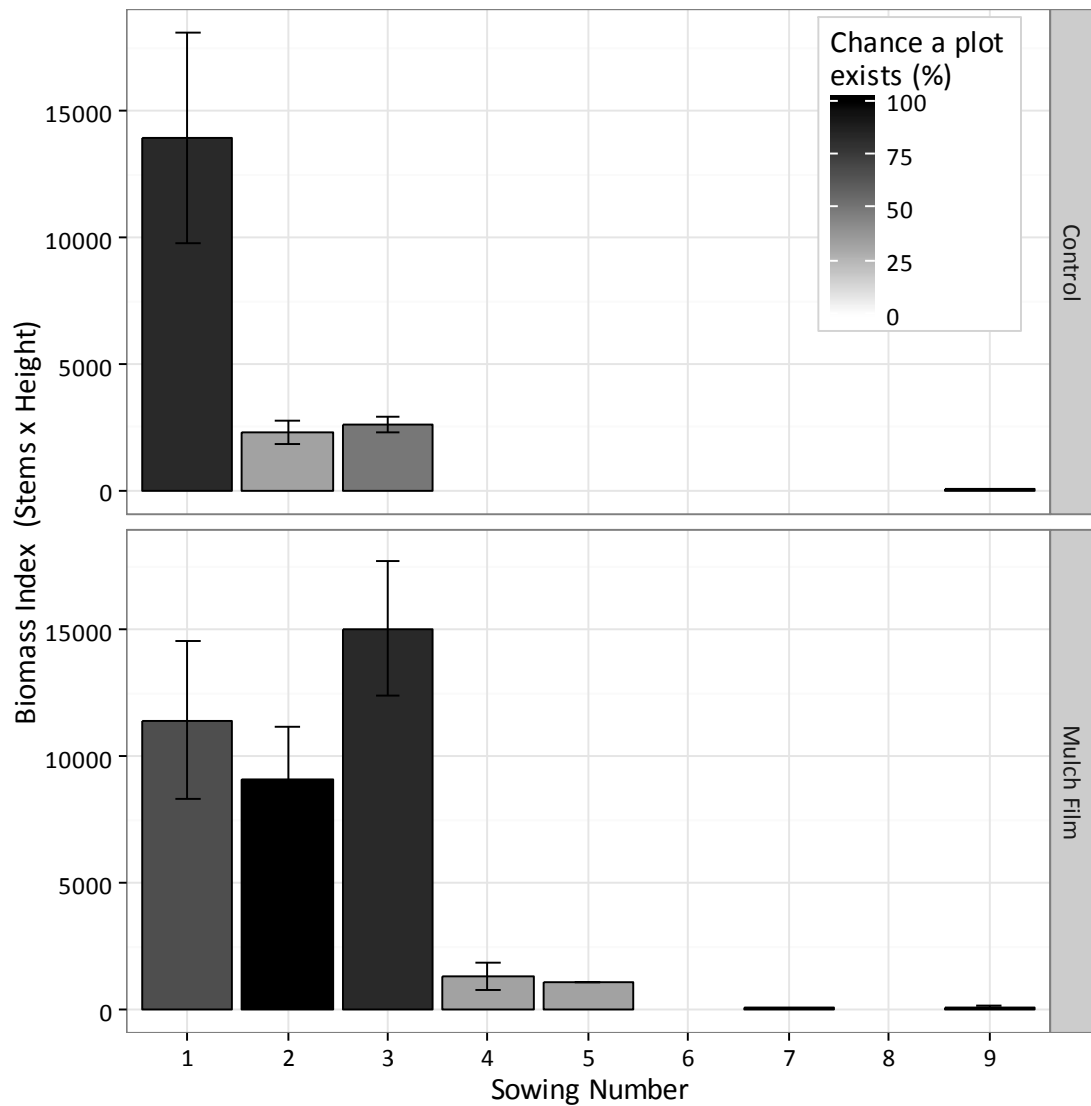


Figure 6-22: Blankney biomass index, with standard error bars that show the variation in the six plots; primed and control with three replicates of each. Bars are coloured by the chance a plot exists at the end of the second year.

When the chances of a plot having plants in at the end of the second year were tested with a logistic regression, and generalized linear models with and without each factor had likelihoods compared, there was no significance of priming ($P = 0.4$) but there was for film ($P < 0.01$) and sowing ($P < 0.0001$). Overall, the Blankney plots that started with film had a 33% higher chance of being alive at the end of the second year than plots did on average (Figure 6-22). Sowing 9 (mid-October) had a 25% overall chance of being alive. Figure 6-22 shows there is a wider range of successful planting times when sowing under film.

6.3b MULTI GENOTYPE DIRECT SOWING TRIAL

The results for the Aberystwyth ‘Multi Genotype Direct Sowing Trial’ are detailed below; these were collected with Mr Ashman of Aberystwyth University.

This experiment tested five *Miscanthus* genotypes and primed *Miscanthus* SYN55 seed in a direct sowing site in Aberystwyth. This aimed to test the relative success of the seed lots and the effect of film under which all plots were replicated. This trial was sown next to the ‘Direct Sowing Agronomy Trial’ at the same time as the second sowing (late-May). This trial was not individually monitored for environmental data but temperature and rainfall data from the metrological station nearby was collected [shown in Appendix O]. There was only ~40 mm of precipitation in June but there was adequate soil moisture as seen in Figure 6-6 above, due to the ~70 mm of precipitation in May.

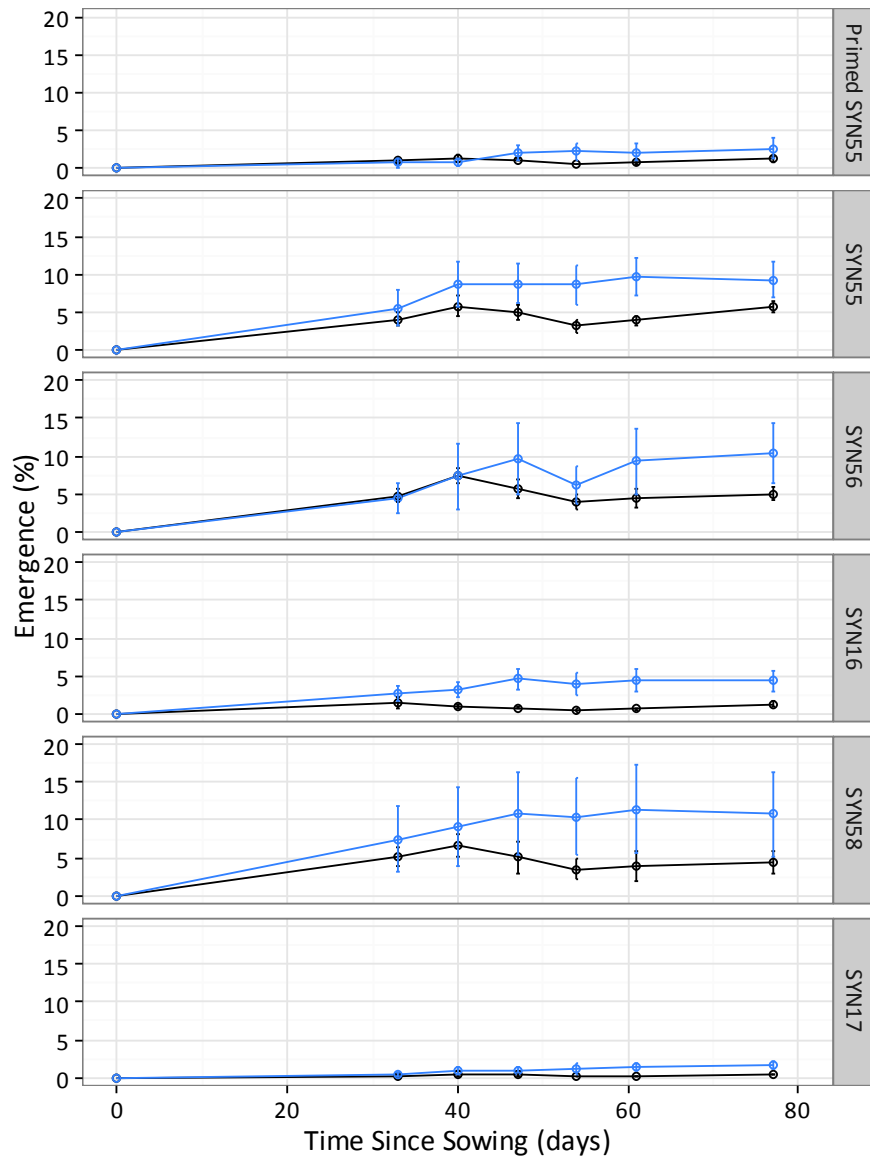


Figure 6-23: Seedling emergence as counted in spring/summer 2013; film (blue) and control (black), with all six seed sets (labelled). Counting was carried out weekly for five weeks after thirty-three days, then on an occasional basis. Standard error bars have been added to represent the variation in the eight plots four replicates with film and four without.

Mean emergence in all seed lots at 40 days was less than 10%; differences between mulch film and no film were significant over time ($P < 0.0001$) with film notably higher in most seed lots (Figure 6-23). Extra emergence occurred after 60 days; this is not shown extensively in Figure 6-23, which cuts off at 80 days, because this is mostly a counting error due to tillering. The generalized linear model likelihood ratios also found a significant difference between the geno-

types. These effects were investigated further at 40 days from sowing to discount the massive effect of time.

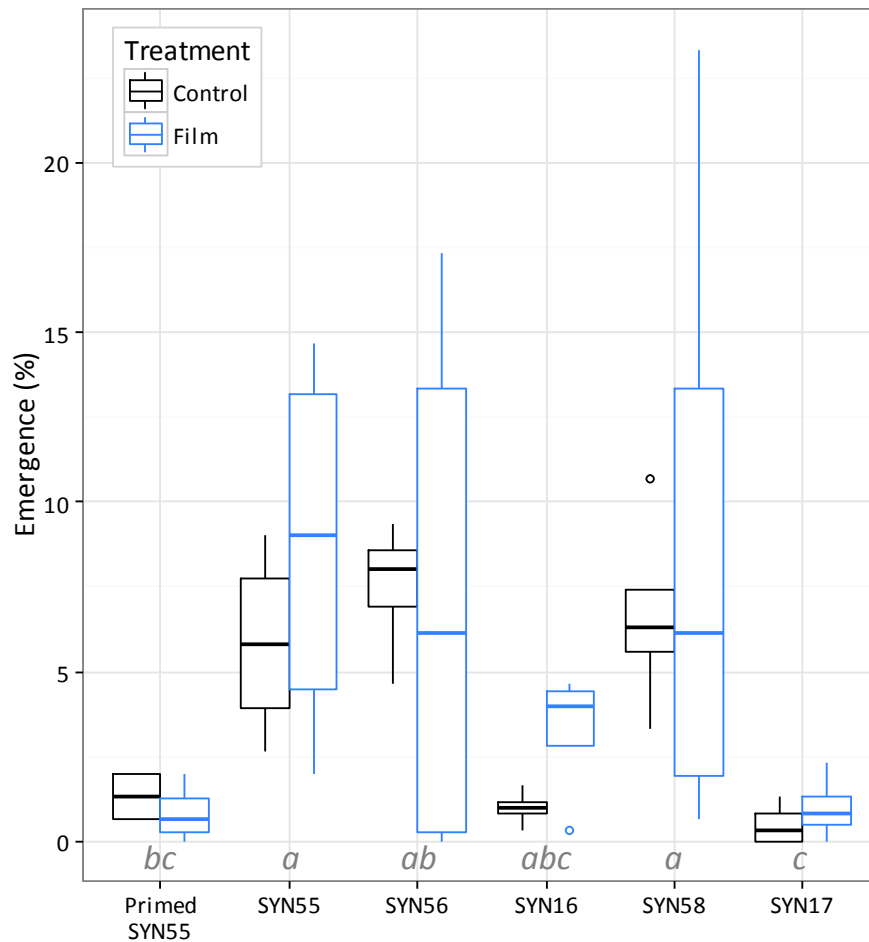


Figure 6-24: Boxplot of emergence of seed at 40 days after sowing. Boxes represent the variation between the four replicates. Separated into film and control treatments, primed seed is on the far left. The seed categories from a Tukey's HSD are shown along the bottom for each seed lot / genotype.

At 40 days after sowing the seed should have mostly emerged and produced a visible sign, so this time point was analysed with a two way ANOVA. This showed a significant effect of seed lot ($P < 0.001$) but no overall significance of film ($P = 0.85$) and no significant interaction with film ($P = 0.4$). The complex effect of film can be seen in Figure 6-24, where film's effect varies dependent on genotype. Using a Tukey HSD post hoc analysis, primed and unprimed SYN55

significantly differed ($P < 0.05$) with more emergence for primed seed (Figure 6-24). The Tukey's HSD with just the genotypes put SYN56 and SYN16 in the same group, and SYN16 with SYN17; all except SYN17 had overlapping groupings (Figure 6-24).

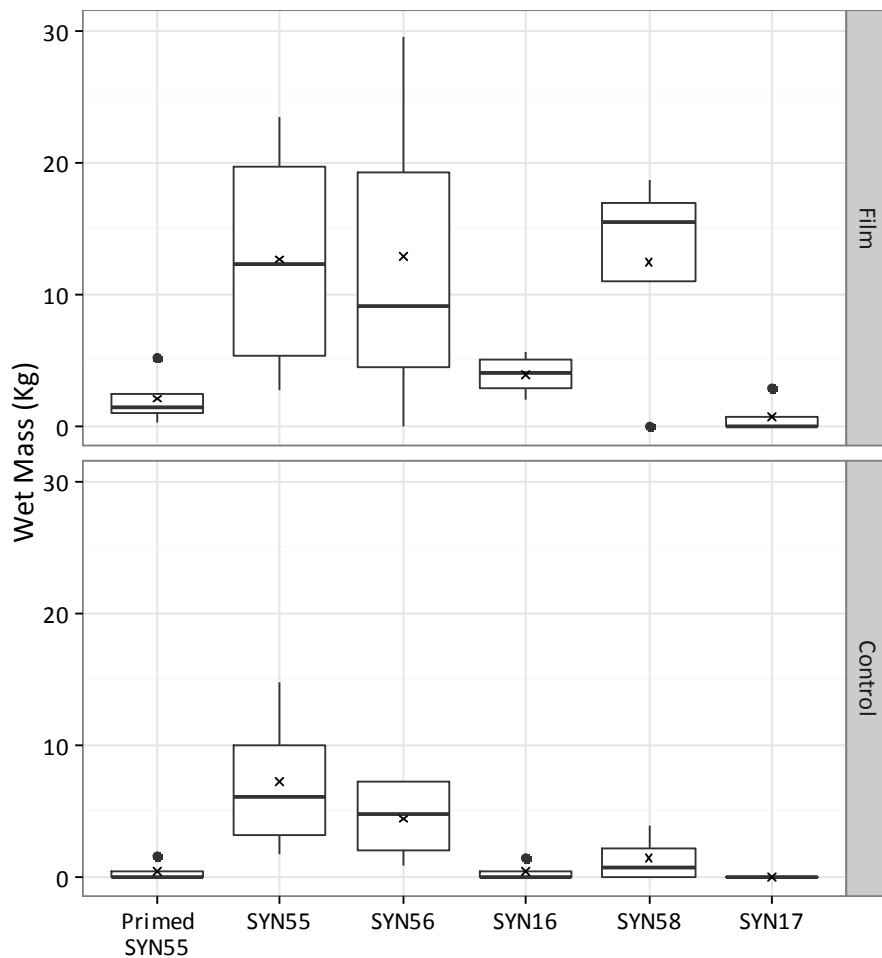


Figure 6-25: Boxplot of total wet biomass generated by each seed lot, film, and control. Mass taken at the end second year in a standard spring (March 2015) harvest time. Boxes represent the variation in the four replicates.

When harvest wet mass was analysed with a two-way ANOVA with seed lot and film as factors, both had a significant effect on wet mass, being $P < 0.05$ for seed lot and $P < 0.01$ for film (Figure 6-25). This occurred even when failed plots were removed from the analysis. The genotypes followed a similar distribution under

film, with SYN56 being the only one to change order. SYN56 and SYN58 performed disproportionately well under film. SYN16 and SYN17 did not thrive in either circumstance, despite being cold suited *M. sinensis* synthetic crosses. A Tukey's HSD produced two groupings, one with all the seed lots and a lower set of SYN16, SYN17 and primed seed.

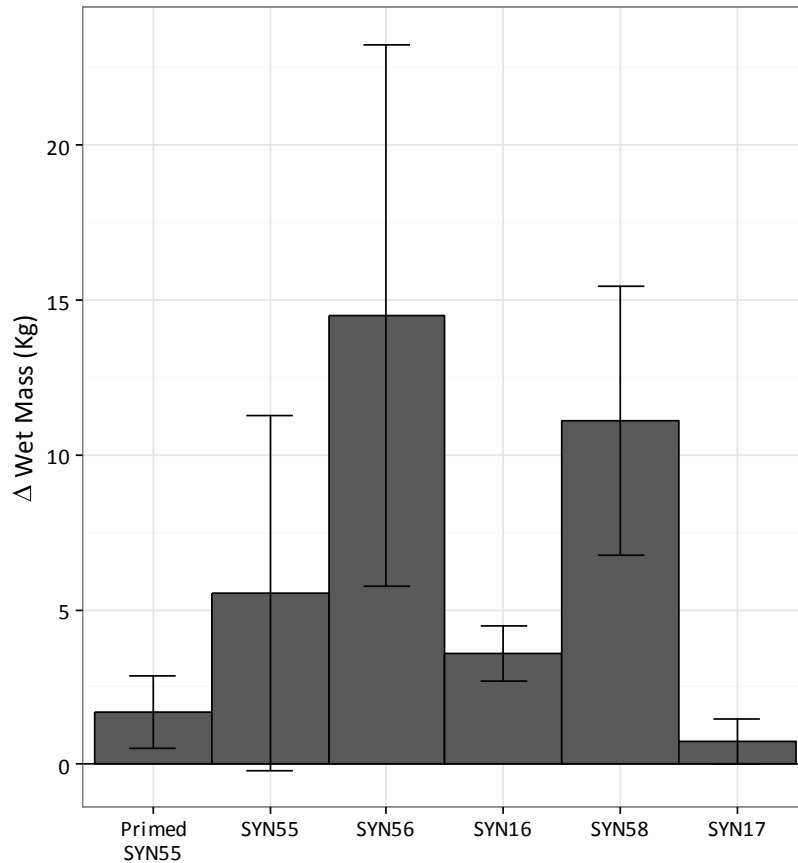


Figure 6-26: Change in end of wet weight between film covered and control plots as harvested at the start of the third year. Standard error shown for the variation in the four replicates.

The film always improved the mean wet harvest weight as seen in Figure 6-26 above and detailed in Table 6–2 below. For untransformed fresh weights with failed plots included, there was still a significant result for the overall difference in film vs. control when analysed with a Kruskal-Wallis rank sum ($P < 0.01$).

Table 6–2: The improvement of the six seed lots' wet weight with the application of film at sowing, measured at the end of the second year at harvest time (March 2015). The standard error to the right is between the four replicates. A percentage increase of film on control is also shown on the right.

Seed lot	Mean film Effect (Kg)	±SE	Mean film Improvement (%)
Primed SYN55	1.69	.58	441
SYN55	5.53	2.86	77
SYN56	14.51	4.38	332
SYN16	3.61	.45	1,053
SYN58	11.12	2.17	830
SYN17	.73	.37	
Mean	6.2	3.6	547

6.3c SOWING METHODS EXPERIMENT

This experiment tested the effect of several different in-soil sowing methods. All sowing methods had less than 40% of seeds emerge (see Figure 6-27), less than the laboratory tested germination rate of ~58% for SYN55 in Section 6.3e below, but not as low as germination in the real field sowings such as those in 6.3a and 6.3b above. Using the mean over time emergence data, a Friedman rank sum found a significance of sowing method with time as a random factor ($P < 0.001$) (Figure 6-27).

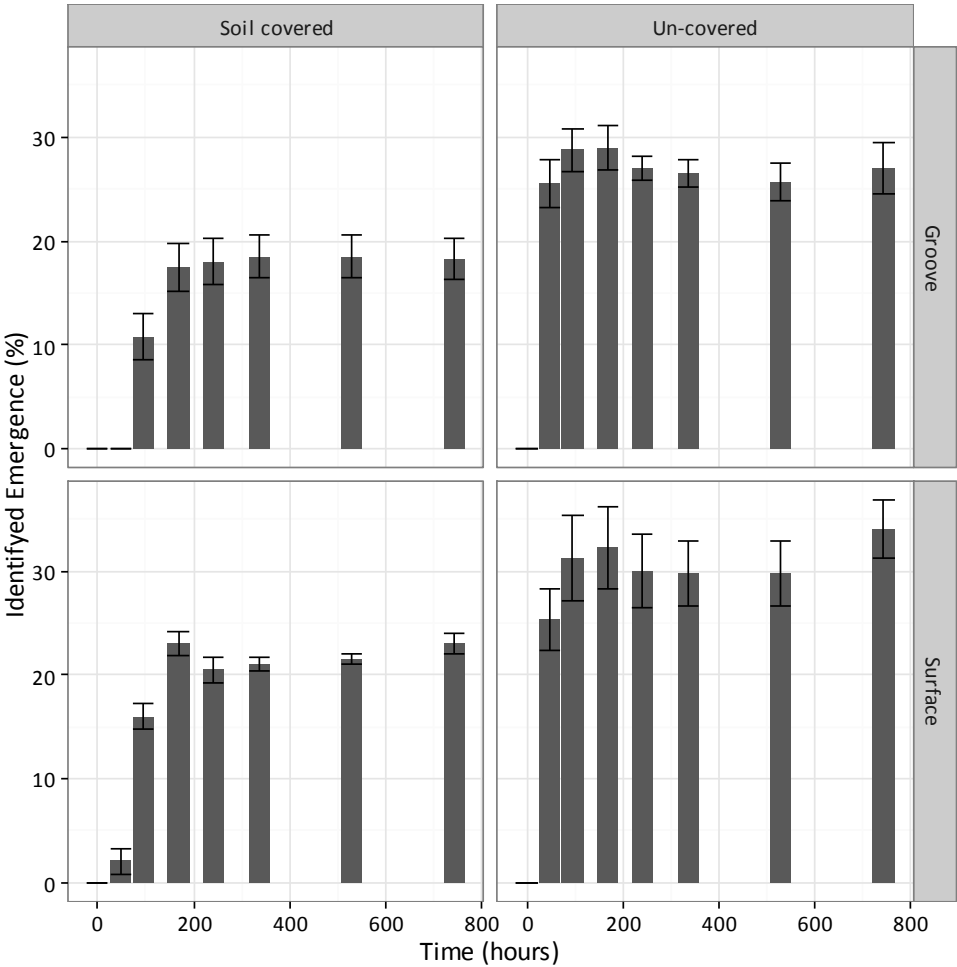


Figure 6-27: Percentage of emerged seed identified over the experiment, standard error bars shown in dark grey depict the variation in the four replicates. The SYN55 seeds were germinating in a constant light at environment 25°C.

Figure 6-27 shows emergence counts remained stable after 96 hours, therefore, emergence at 96 hours was tested using two Kruskal-Wallis rank sums; these found the effect of the soil covering was significant ($P < 0.001$) yet not the effect of the groove ($P = 0.46$). As seen in Figure 6-27 the soil covered seed performed worse than the uncovered seed.

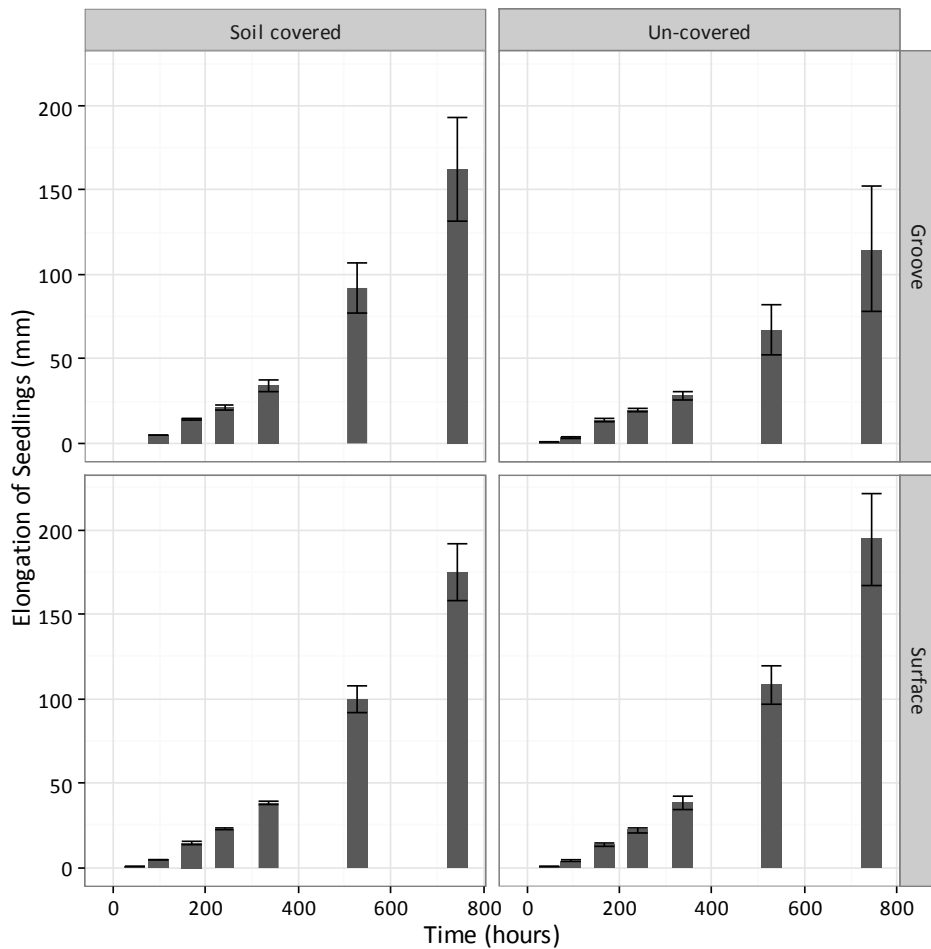


Figure 6-28: Elongation of seedlings averaged for each rep, standard error bars, of differences between replicates, shown in dark grey illustrate the variation in the four replicates. Seedlings were grown in constant light at 25°C.

Using time as a random effect, the Friedman's test found a significant effect of treatment on stem elongation ($P < 0.05$). The final elongation time of 744 hours (the right most bar in Figure 6-28) was chosen to analyse further using a two-way ANOVA (data logged for normality), this found no significance of groove or soil covering ($P = 0.08$ & $P = 0.42$).

Root elongation at the end of the test gave a similar result when logged and tested with a two-way ANOVA; it was not significant for either groove or soil covering ($P = 0.06$ & $P = 0.66$).

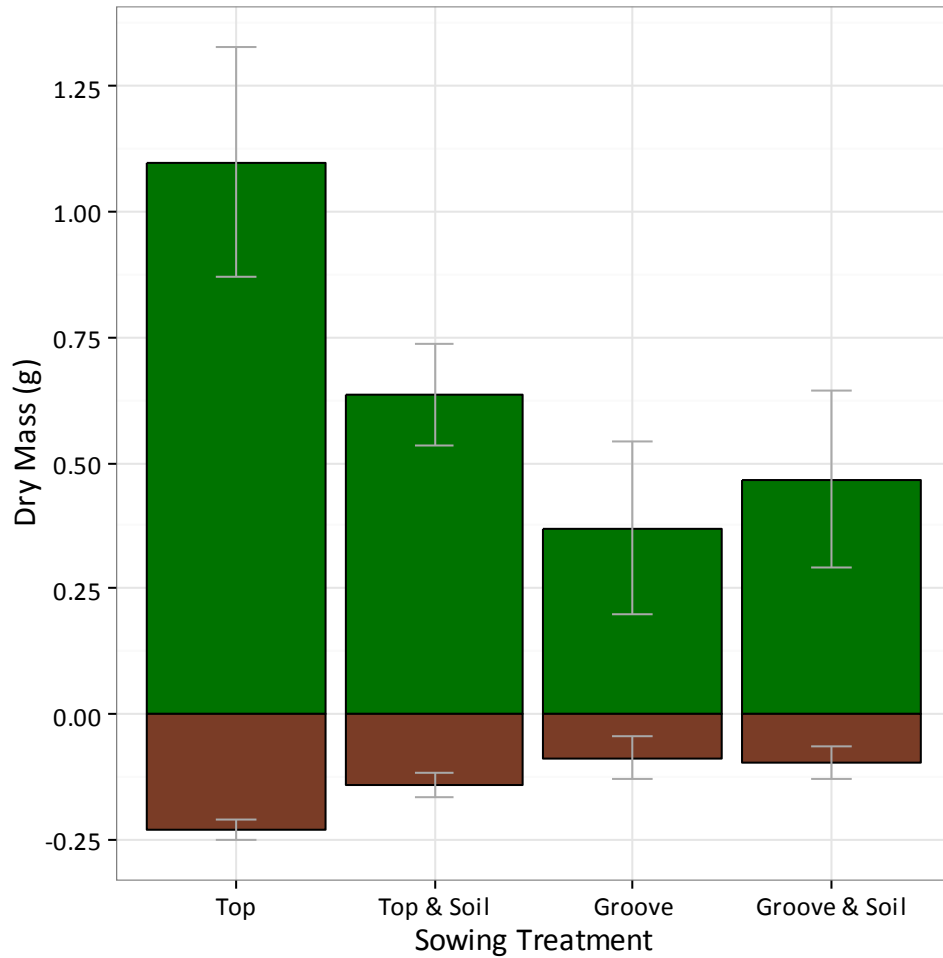


Figure 6-29: Above (green) and below ground (brown) dry biomass at the end of the experiment (31 days). The standard error between replicates is shown in the grey error bars illustrative of the variation in the four replicates. Below ground biomass has been inverted to give a view of overall biomass.

The above ground biomass, below ground biomass and total biomass were all logged to produce a normal distribution and tested with two-way ANOVA's against grooves and soil covering. All three showed a significant effect of groove (all $P < 0.05$) (see Figure 6-29). However, none of the dry masses had a significant effect from soil covering (above ground $P = 0.73$, below ground $P = 0.26$ & total $P = 0.72$).

6.3d SOIL WATER CONTENT

It was important to determine the effect of soil moisture on germination, and from this to determine a minimum level of soil moisture required. Figure 6-30 shows the pattern of germination changing from a shallow line to a more typical s-shaped germination curve as soil moisture increases. The seed only approaches 50% germination for the 0.24 water W/V; 100% was achieved at the same temperature on the thermal gradient plate (Section 6.3e). Over all the germination times, there was a significant effect of water W/V (Friedman's rank sum, $P < 0.01$).

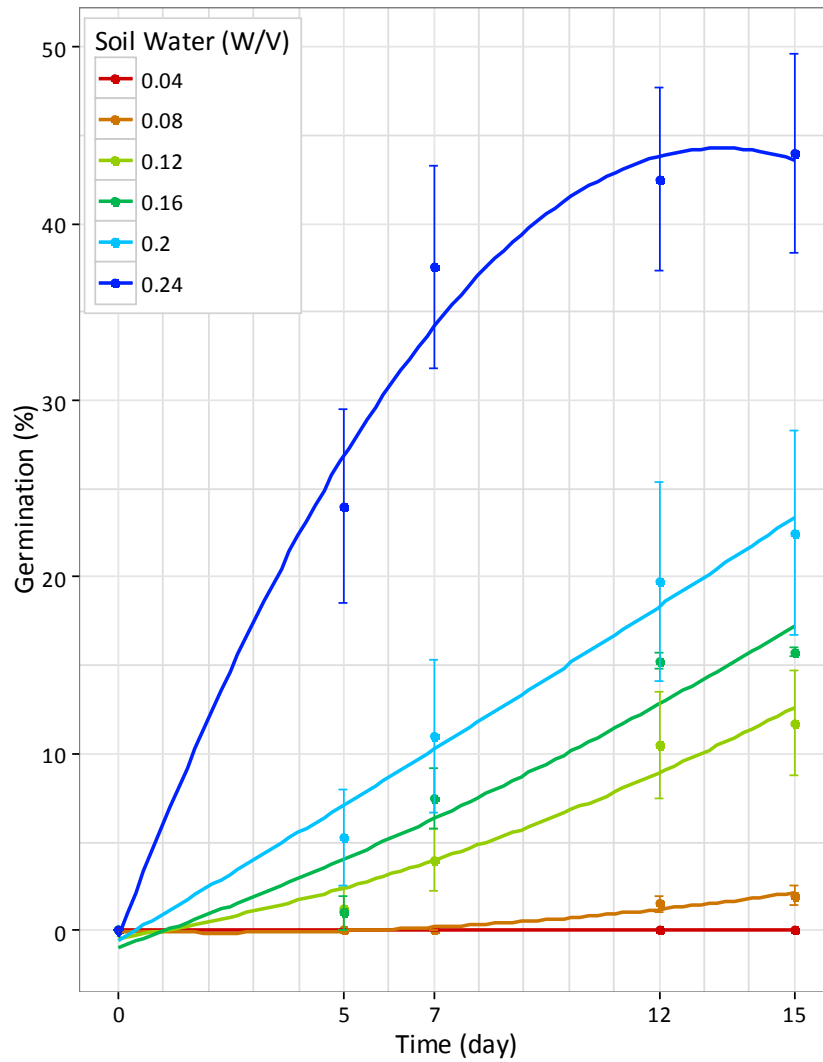


Figure 6-30: The percentage of germination measured at 25°C, over a range of soil moisture levels. Second order polynomials have been added as lines with standard error bars on each point to depict the variation in the four replicates.

The minimum soil water content required for germination was 0.062 W/V. This was calculated from the intercept of a linear model of the fifteen-day germination count against water concentration (Figure 6-31). The model was highly significant ($P < 0.0001$), and the R^2 for the fit was 0.77.

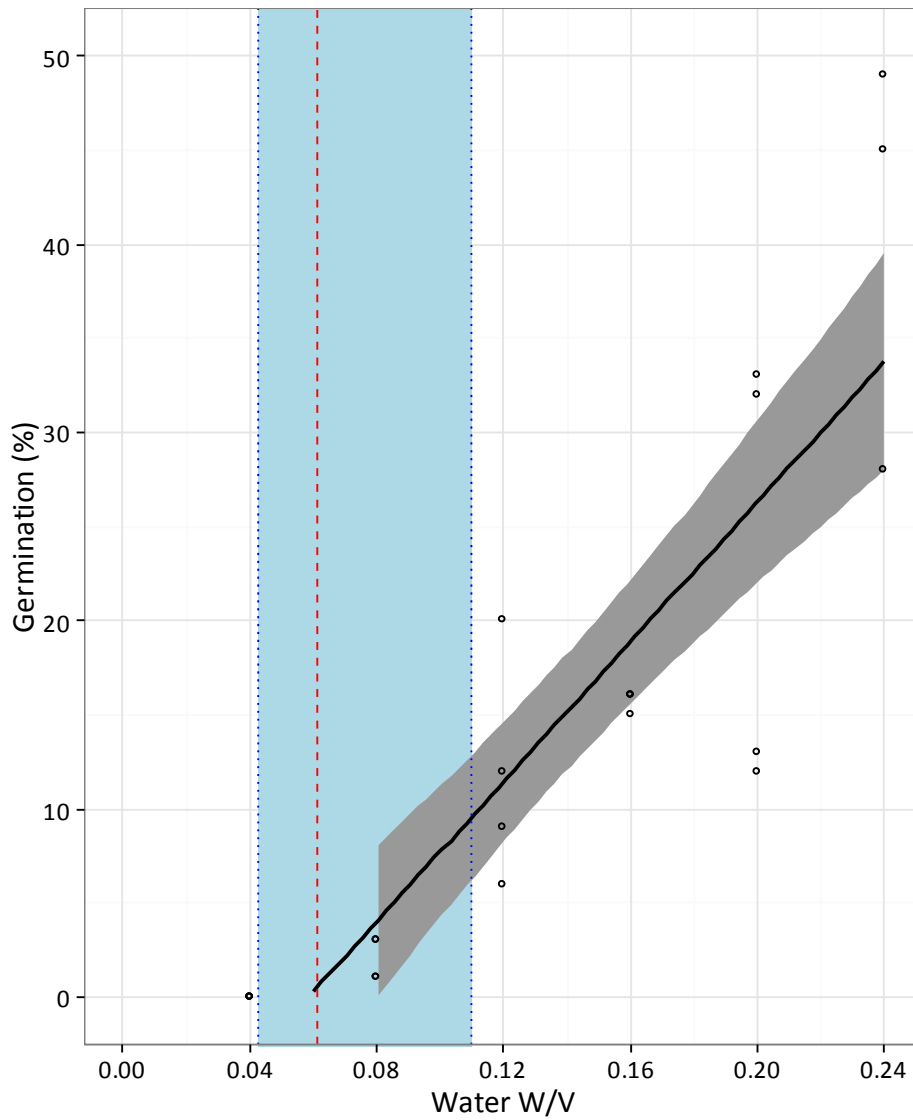


Figure 6-31: A graph of the germination (at 15 days) by soil moisture, with a linear model added to calculate base germination and with a 95% confidence interval. The blue zone shows the range of field water W/V recorded in Aberystwyth sowings 1-7 with and without film (section 6.3a), and the red line is the intercept of the model.

About 0.5% of water was lost per day from the dish (Figure 6-32 & Table 6-3). Therefore, because the water top-ups were every 2-3 days there was some variability. During this time, the water percentage could have been lower than the target level.

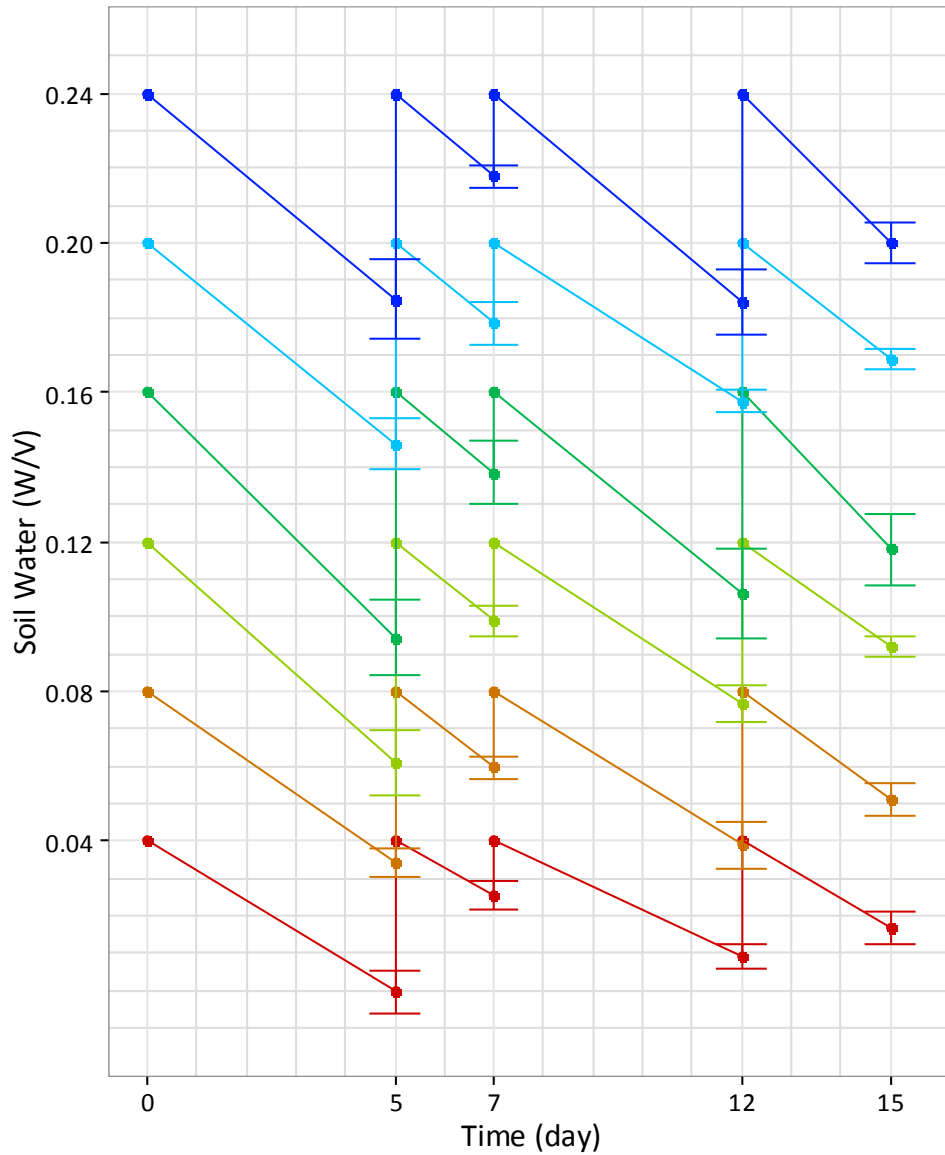


Figure 6-32: The mean soil water measured for each set of dishes at each time, dishes were topped up with the calculated amount of water at each time. The discrepancies between the four replicates are represented with standard error bars.

As most dishes contained around 100 g of soil, the average amount of water added at the start was close to the W/V percentage (see Table 6–3).

Table 6–3: Mean water loss per day in μL and the percentage water, with standard errors for the four replicates over the fifteen days. Information is given for each water percentage 4 to 24%.

Proportion of Water per Dish	Water Quantity (W/V)					
	4%	8%	12%	16%	20%	24%
Loss per Day W/V (%)	0.34±0.15	0.43±0.15	0.51±0.22	0.59±0.26	0.50±0.19	0.54±0.21
Loss per Day (mL)	0.29±0.13	0.36±0.13	0.53±0.23	0.54±0.23	0.53±0.20	0.53±0.17
Mean Starting Water (mL)	3.4	6.7	12.5	14.9	21.8	24.8

6.3e THERMAL GRADIENT FOR SEED GERMINATION

A thermal gradient plate was used to parameterise the germination of fourteen seed-based hybrids at a wide range of temperatures. The temperature probes placed at approximately 5, 18, and 31°C recorded stable temperatures (Figure 6-33) and checks at the start and end of the test confirmed the temperature range [for details see Appendix R].

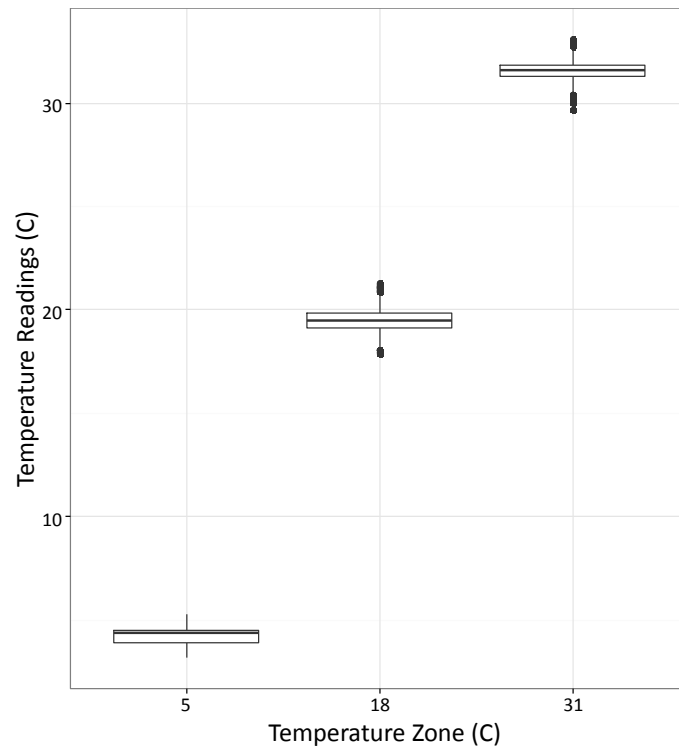


Figure 6-33: Temperature variation across the thermal gradient plate for three sensors at 5, 18, 31°C temperature and across all 40 days of the experiment. Mean temperatures were logged every thirty minutes. The standard deviations are 5°C ± 0.15, 18°C ± 0.46, and 31°C ± 0.36. [For the calibration of the thermal gradient plate, see Appendix Q]

Figure 6-34, visually confirms that ~25°C appears to be the best temperature for germination in most seed lots. The generalized linear models both agreed on a best seed lot of GNT2 and an optimal temperature of 25°C. At seven days, there were also noticeable differences between the minimum germination temperature and the maximum germination percentage (Figure 6-34).

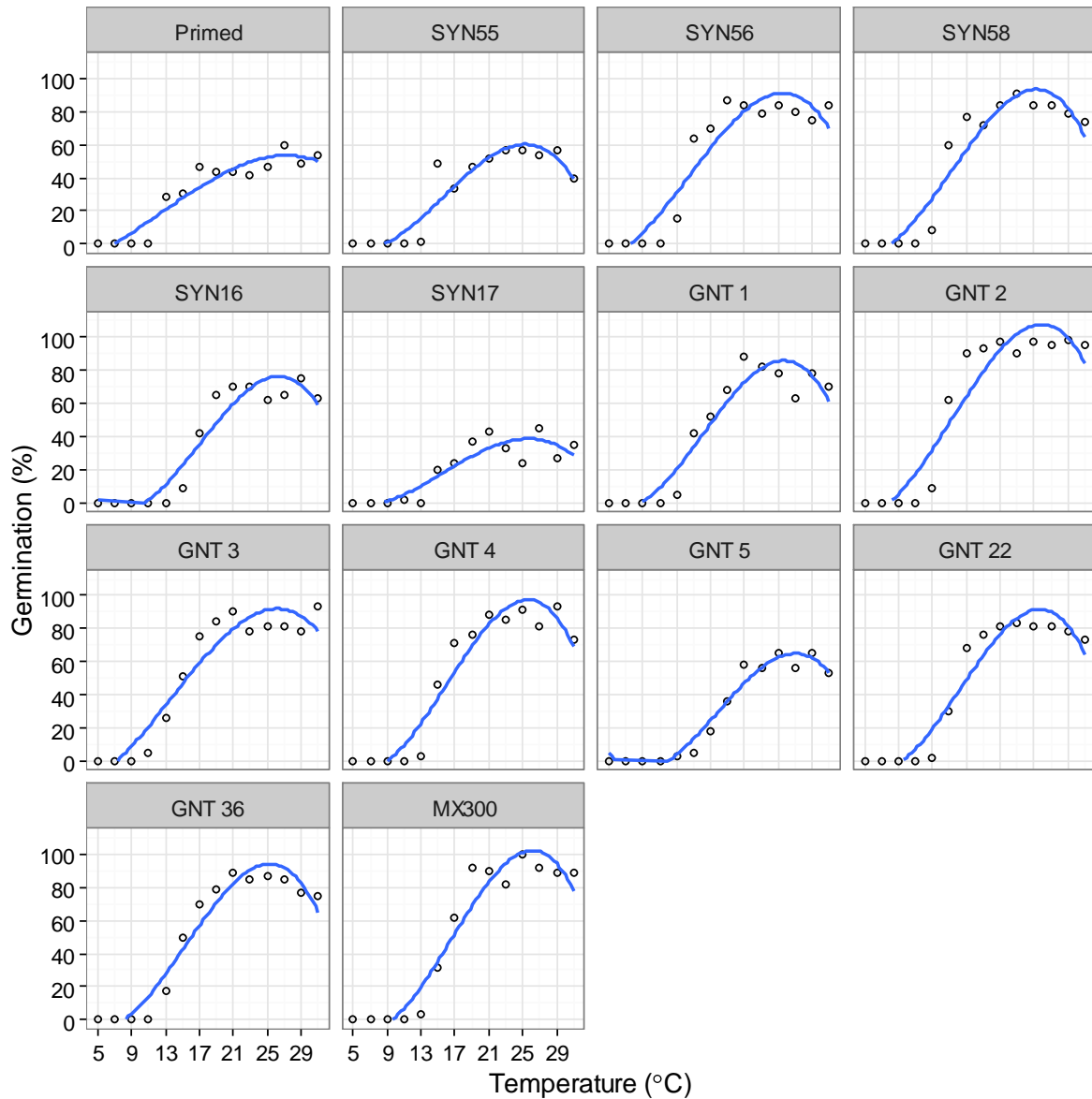


Figure 6-34: The germination of the seed lots at 7 days on the thermal gradient plate. The points have a third order polynomial added.

Figure 6-35 shows there was a lot of variation in total viability of the seed, dependent on genotype; mostly as total viability increases so does germination. The ratio between total viable seeds and germinated seeds indicates the temperature range for germination in that genotype.

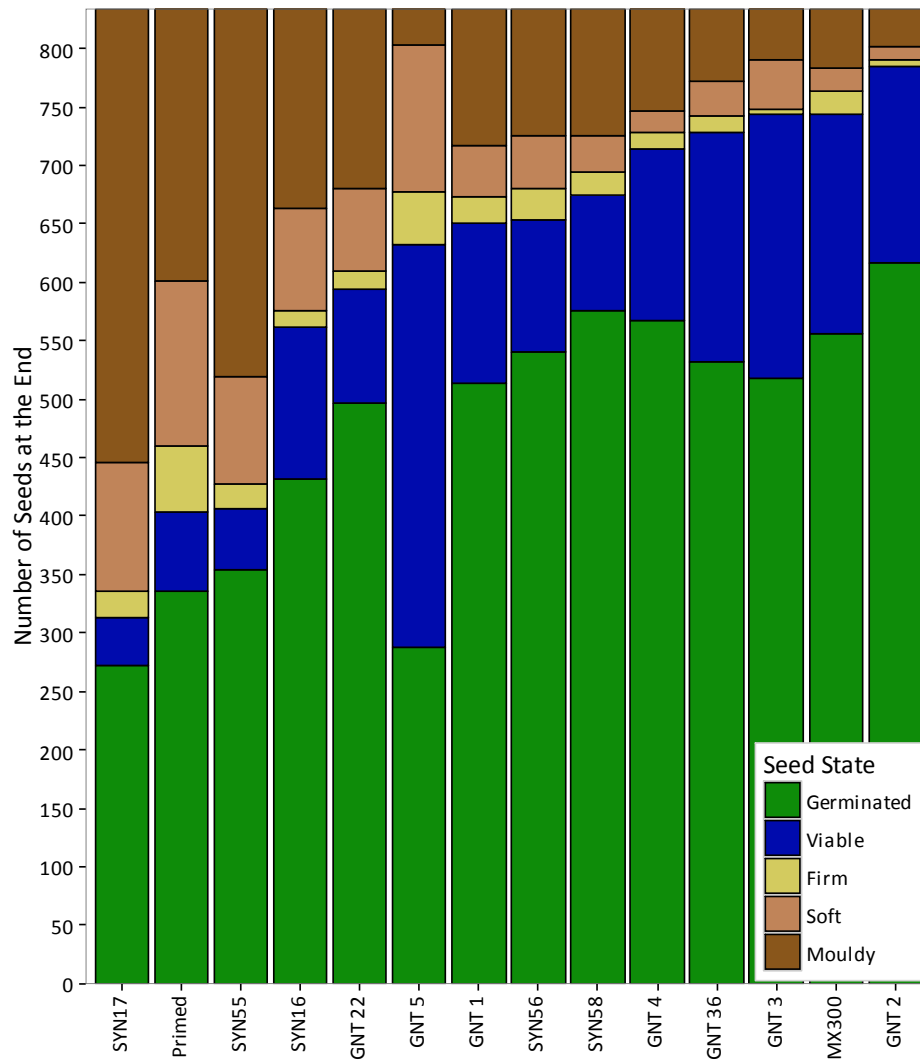


Figure 6-35: The status of each seed that had been on the thermal gradient plate at the end of the test, ordered by total germinated plus viable. Viable seed was seed that germinated when the temperature was increased, while firm seed was un-germinated at the end of the test.

GNT5 germinated better when kept cold and wet on the thermal gradient plate before having the temperature increased, than it did at a constant 25°C (Figure 6-35). The primed seed has less total germinated seeds than unprimed SYN55, and more seeds in the firm (unchanged) category by the end of the thermal gradient plate experiment (Figure 6-35).

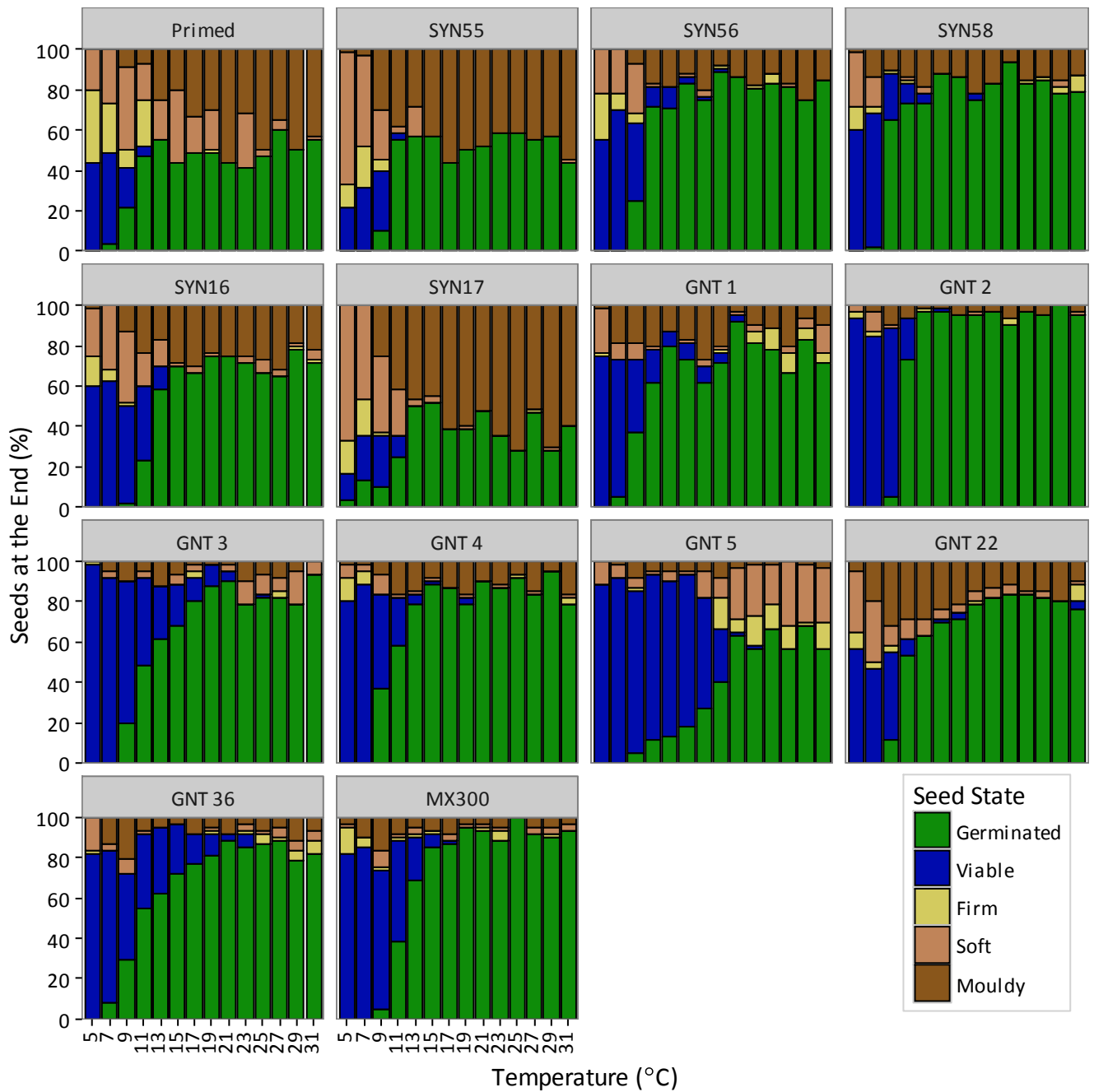


Figure 6-36: The end state for each seed lot at each temperature.

In Figure 6-36, primed seed also has the most firm seed at the conclusion of the experiment. There appears to be little negative effect from priming seed on germination in Figure 6-36; however, primed has a small uptick at 7°C that un-primed SYN55 does not. When only primed and un-primed SYN55 were tested against each other using a generalized linear model with a negative binomial

distribution across all times and temperatures, priming did significantly affect the model ($P < 0.0001$). However, the benefit was to the unprimed seed as seen in Figure 6-36.

Figure 6-36 clearly shows where genotypes thrive and what the rates of mould are; notable is that some seed lots experience the higher rates of mould at hotter temperatures, such as GNT5 and to a lesser extent GNT1, while most seed lots have more mould at the colder temperatures.

Several GNT's such as 3, 5, 2 and 36, show excellent recovery and germination after being kept cold and wet for 40 days while older seed lots, such as SYN55 and SYN17 particularly, go mouldy at these cold temperatures (Figure 6-36).

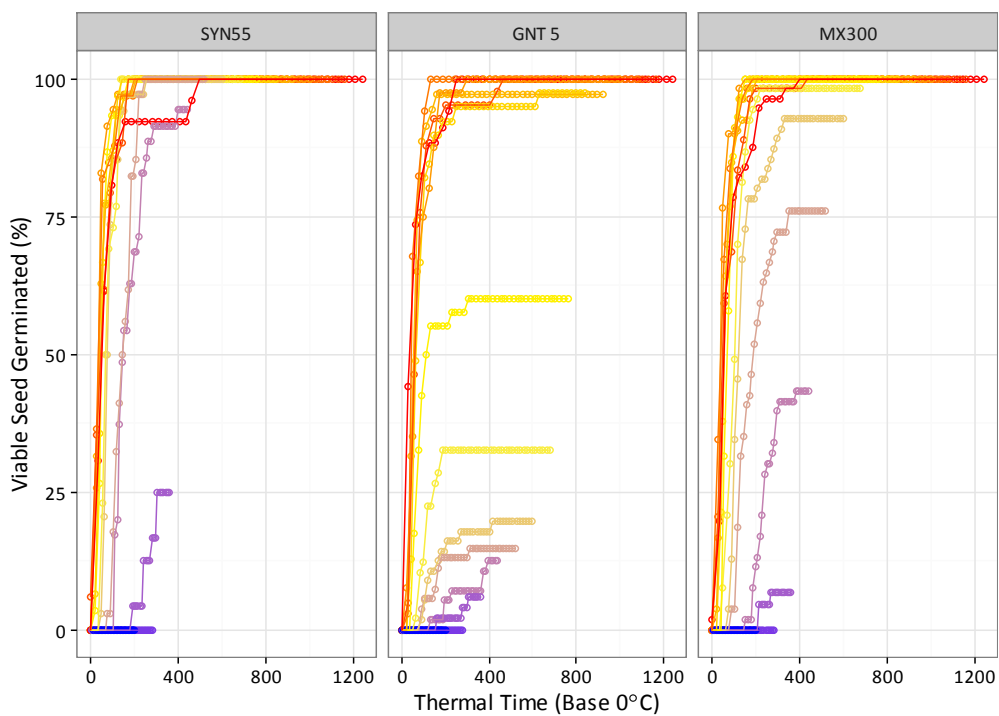


Figure 6-37: Three exemplar seed lots show the calculation of germination of viable seed by thermal time using a default base of 0°C [for all plots see Appendix Q]. Lines have been coloured to represent the thermal gradient plate temperatures.

Figure 6-37 shows the thermal time (base 0°C) for viable seed germination rates for three very different seed lots; the logistic model was fitted to these curves. The logistic models' starting parameters were found by fitting a self-starting model then taking the values and refining them through iteration.

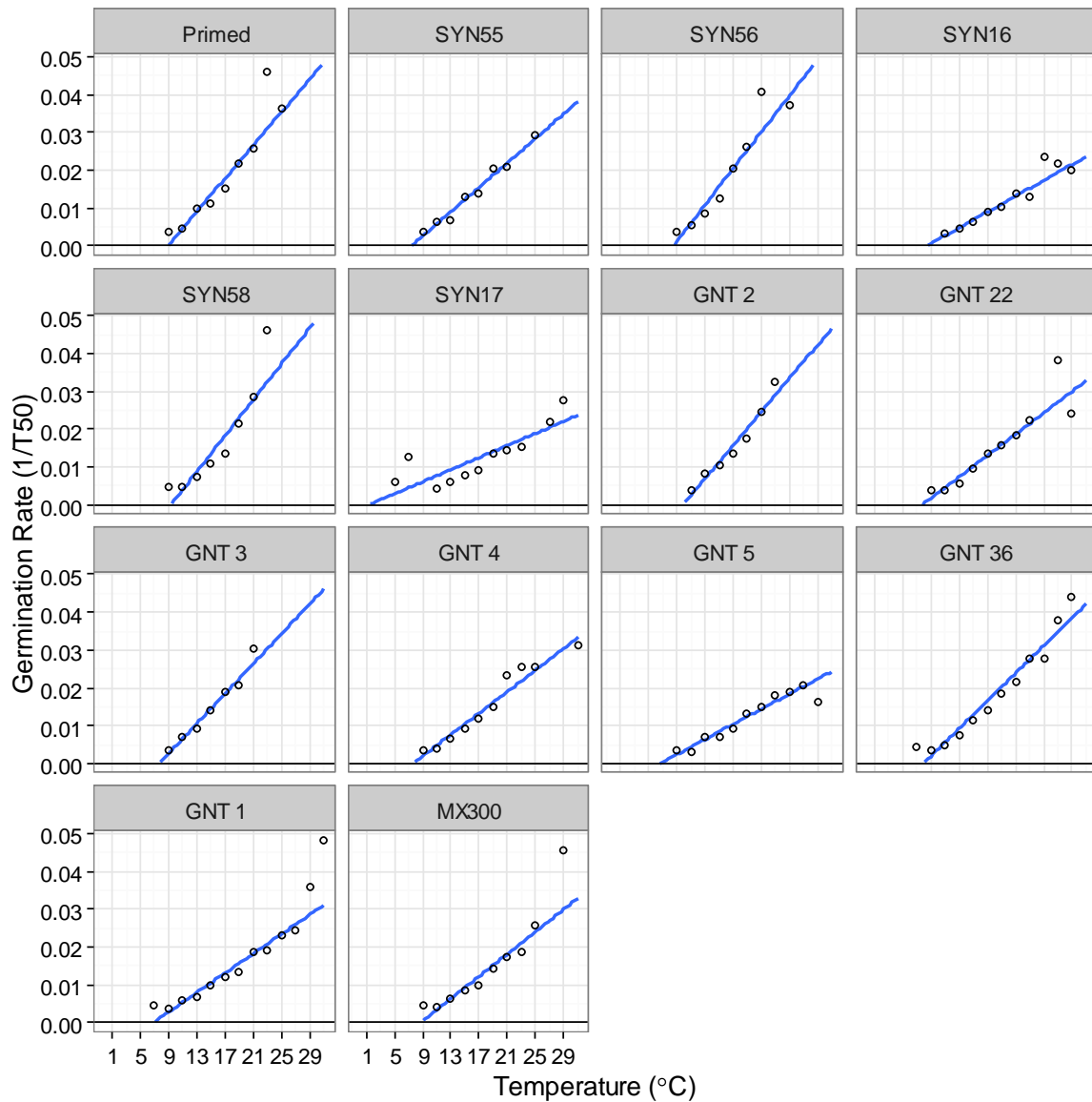


Figure 6-38: The robust linear models plotted on the germination rates of each seed lot over the thermal gradient plate. The x-intercept represents the estimation of base temperature.

The lowest calculated base temperature was SYN17, which was 1.39°C (Table 6–4). SYN17's estimate in Figure 6-38 was disrupted by higher than expected germination rates at 5 and 7°C; however, because germination was observed at

these temperatures, and the rates were not unrealistically high, the points were left in.

Table 6–4: Base temperatures calculated for each seed lot on the thermal gradient plate, with confidence intervals.

Seed Lots	Estimated Base Temperature (°C)	Bootstrapped 95% Confidence interval	
		Lower	Higher
Primed	8.88	6.61	10.22
SYN55	7.39	5.52	8.6
SYN56	8.51	6.09	9.84
SYN58	9.33	6.25	9.68
SYN16	8.50	6.19	12.5
SYN17	1.39	-6.13	24.27
GNT1	6.86	7.62	11.65
GNT2	9.92	4.73	10.74
GNT3	7.66	5.61	8.51
GNT4	7.59	5.48	9.03
GNT5	6.57	4.72	11.34
GNT22	7.85	5.91	10.41
GNT36	7.97	3.0	9.32
MX300	8.67	4.78	11.62

The estimates of base temperature for seed germination averaged at 7.65°C across all the seed lots (Table 6–4).

6.3f THERMAL GRADIENT FOR SEEDLING STEM ELONGATION

The thermal gradient plate was again utilised, this time to parameterise stem elongation in two genotypes. A stable temperature was again maintained (for graph see Appendix S). The elongation of the seeds increased with time and temperature (Figure 6-39), the fastest elongating temperature over all times was

25°C, where seedlings grew at 0.095 mm per hour. 25°C also had the peak elongation change at 11 days (Figure 6-40); at the end of the experiment this peak was 29°C with 25°C second. Peak growing time over all temperatures was from 24 to 48 hours, elongating at 0.052 mm per hour. Overall elongation peaks at 25°C and between 24 to 48 hours with seedlings elongating an average of 0.127 mm per hour.

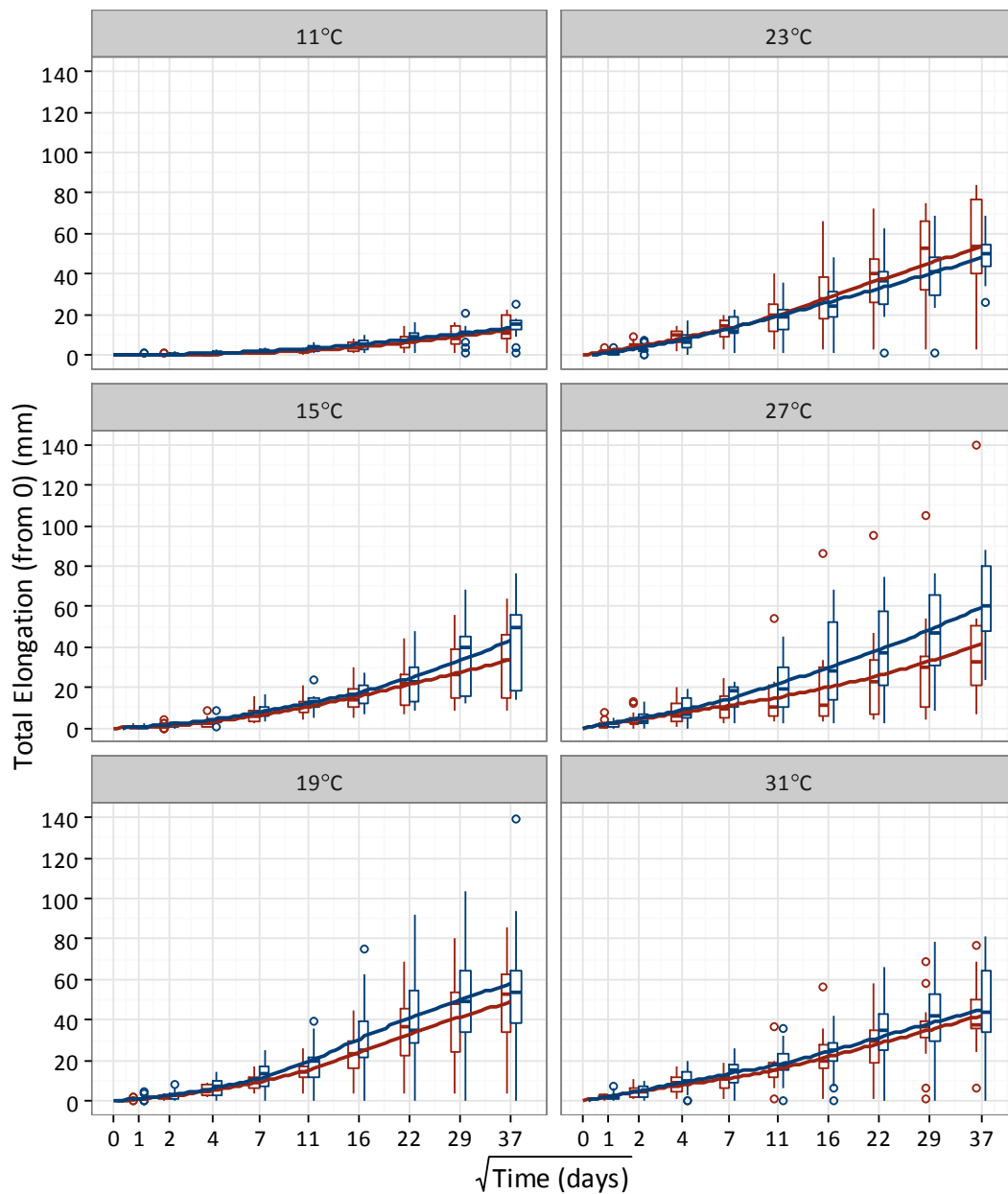


Figure 6-39: Seedling elongation over time between the two seed lots, at six temperatures chosen to show the differences between the seed lots over a range of temperatures. The boxes represent the difference in the fourteen seed of each genotype at each temperature. SYN55 (blue) and MX300 (red) with time plotted on a square rooted scale, with fitted loess curves.

SYN55 seed elongated more than MX300 at most temperatures (Figure 6-39). However, the difference between the seed lots was not significant when this range of six temperatures was tested at the final measurement time with a t-test (except 15°C which was tested with a Kruskal-Wallis rank sum) ($P = 0.67, 0.23,$

0.19, 0.65, 0.21, 0.86 for 11, 15, 19, 23, 27, & 31°C). When a generalized linear model with a negative binomial distribution tested all time points, there was not a significance in the difference between the likelihood functions of the model ($P = 0.087$).

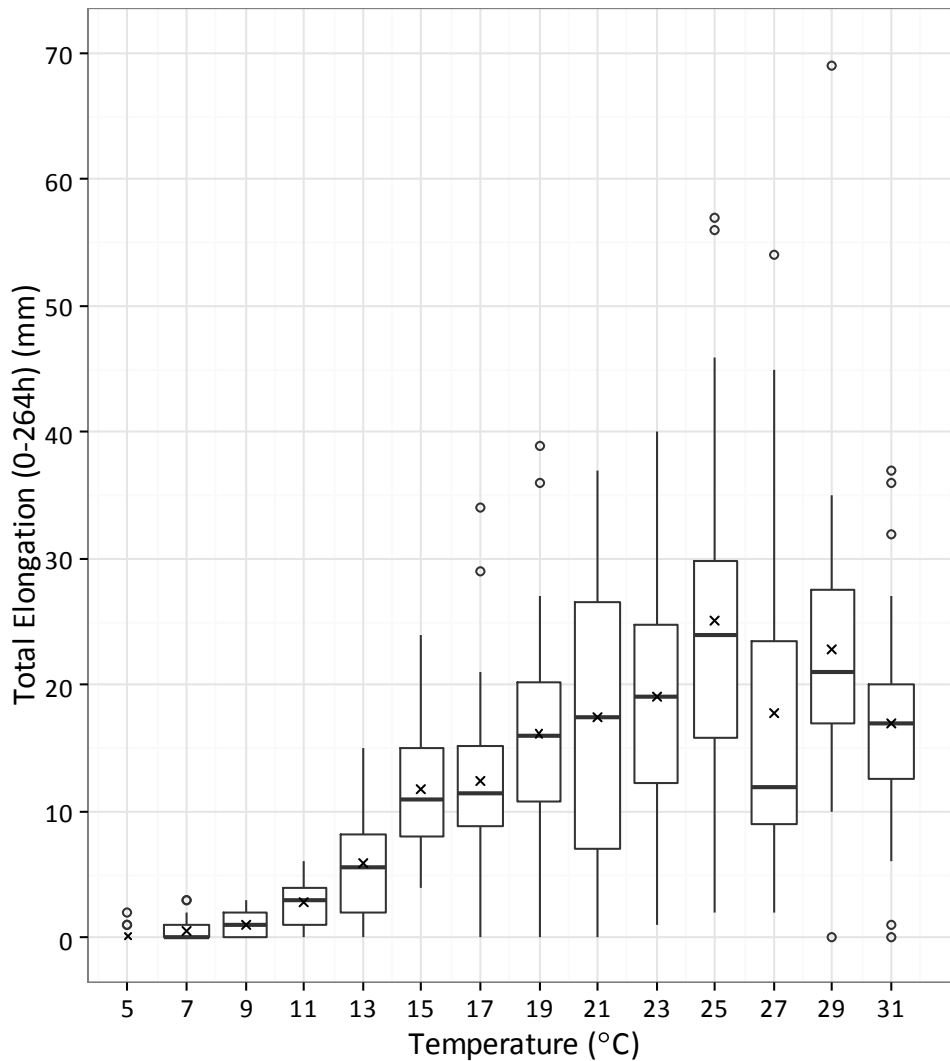


Figure 6-40: Total change in seedling elongation on the thermal gradient plate by the eleventh day (264 hours). MX300 and SYN55 elongations have been combined into twenty-eight seeds per temperature.

Germination rate as shown in Figure 6-41 shows no visually apparent trend, with MX300 or SYN55 growing faster overall. The elongation rate for all seeds has twin peaks at both 25 and 29°C, with similar low points at 23, 27, and

31°C. The curve shown in Figure 6-41 could be smoothed to level out after 21°C and before 9°C, with the major influence of temperature on elongation rate between these points.

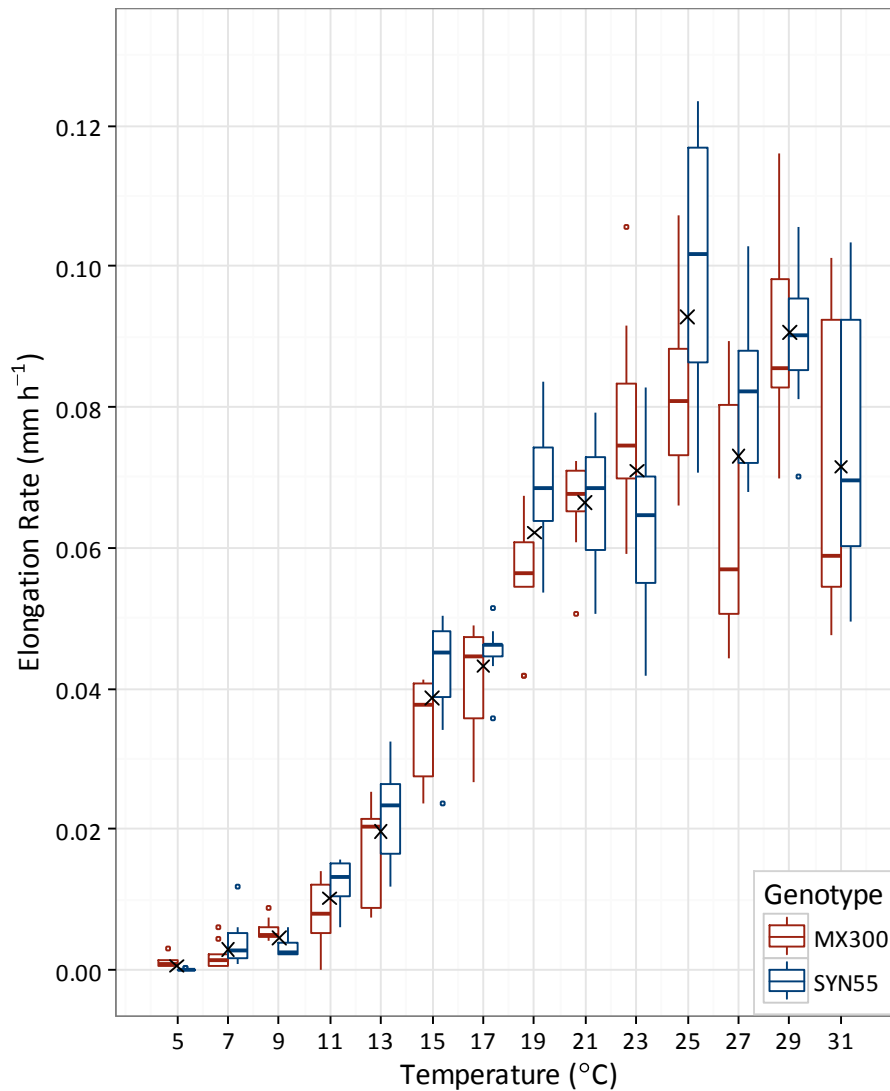


Figure 6-41: A boxplot of the mean hourly elongation rate for seedlings by seed type and temperature. The replicates were averaged; errors represent the elongation rate variation over the nine time points in the experiment. Black x's show the average elongation rate of each temperature.

When elongation rate (see Figure 6-41) was compared with a three-way ANOVA for factors of time, temperature, and seed lot (all logged for normality) there was a significant effect of seed lot ($P < 0.05$), with the SYN55 elongating $2.5 \mu\text{m h}^{-1}$

faster than MX300. Genotype did not produce any significant interactions with time ($P = 0.65$) or temperature ($P = 0.45$). Temperature had a highly significant effect on elongation rate ($P < 0.0001$); this effect can be expressed as a difference of $4.6 \mu\text{m h}^{-1} \text{ } ^\circ\text{C}^{-1}$. Time also had a significant effect on the rate of germination ($P < 0.05$). However, time was more significant as an interaction with temperature ($P < 0.0001$).

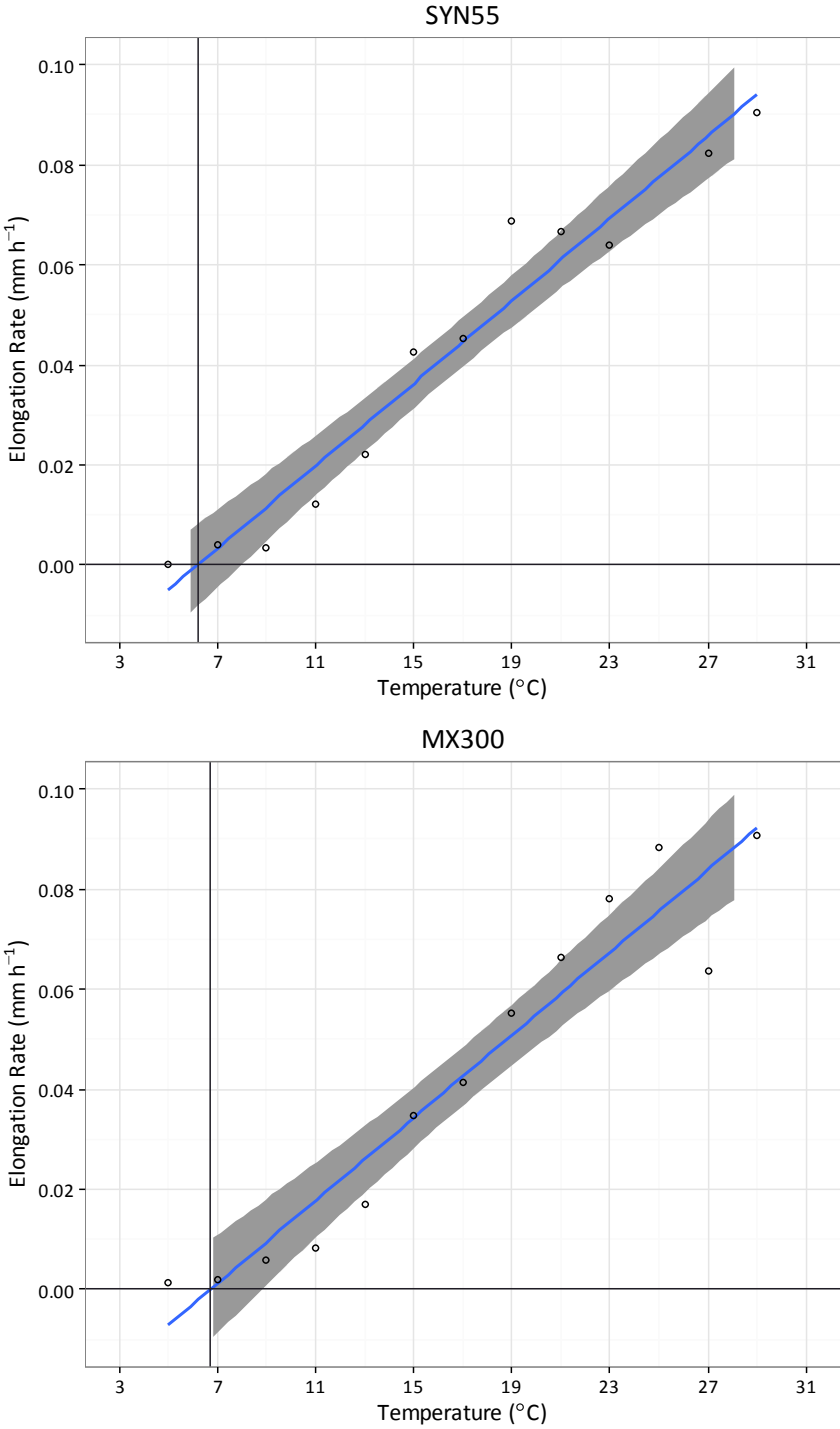


Figure 6-42: Models of the thermal gradient plate elongation per hour for SYN55 and MX300, averaged over the number of hours between readings, then averaged over all.

The mean rates of germination (seen in Figure 6-41) were averaged, and then the result was modelled using a linear model (Figure 6-42) to calculate a minimum temperature for elongation. The minimum temperature for elongation was

5.62°C for SYN55 and 6.73°C for MX300 using this calculation. Removing outliers using Cooks distance only affected the base estimate for MX300 (from 5.96°C).

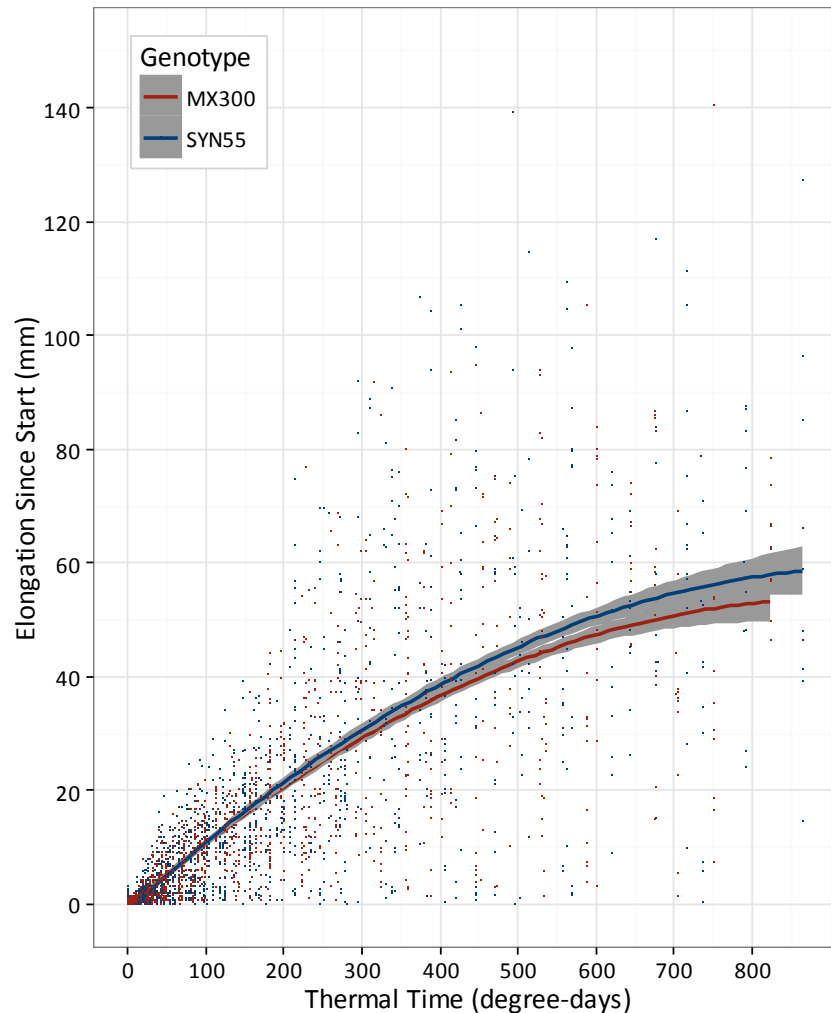


Figure 6-43: The total elongation plotted against thermal time measured from the base temperature for each genotype, plotted with a second order polynomial curve with a confidence interval in grey.

When the base temperature was used to calculate thermal time in degree-days (Figure 6-43), elongation was linear with thermal time levelling out after 500°Days. SYN55 still showed higher elongation but the slopes were the same during the linear part of the curve.

It was observed at the end of the experiment that when the heat was turned up to 25°C across the thermal gradient bar most of the seeds in the low (5 and 7°C) temperatures, that had not become mouldy, revived and started growing.

6.3g COMPUTATIONAL MODELLING OF GERMINATION

A computational model was produced based on SimPIE and parameterised for *Miscanthus* seed using the experiments in this study. The model was tested at three scales by graphing the result and comparing with Wilcoxon tests between the real germination and emergence, and the model results; the comparisons are always done with data from experiments that were not used to parameterise the model. The three scales of testing were the laboratory level (Figure 6-44), controlled environment (Figure 6-45), and field (Figure 6-48 & Figure 6-47).

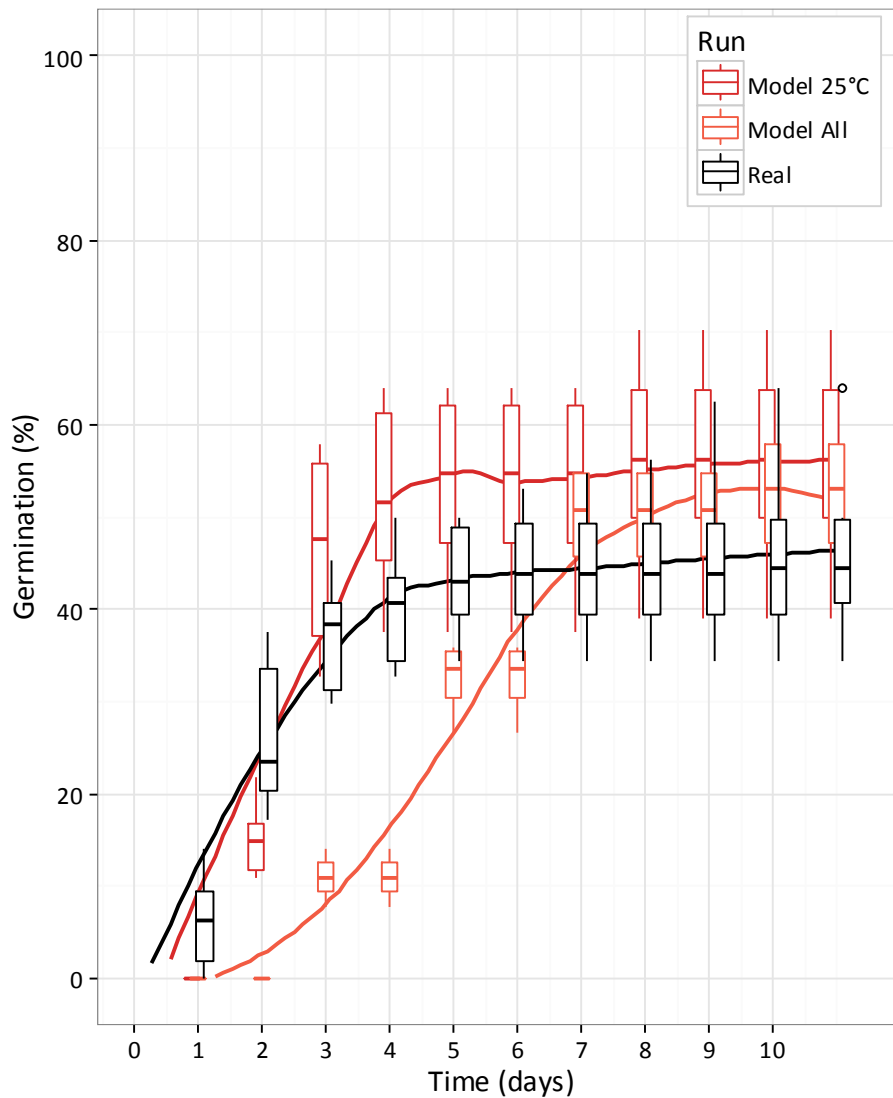


Figure 6-44: Testing the model against the mean value of laboratory germination testing (from the controls in six of the Physical & Chemical Germination Factors results 4.4) (black). The model was run with 64 seed on a none water limited surface at 25°C twelve times, six at using the ‘Thermal Gradient for Seed Germination’ 25°C data (red) and six times using all the input thermal data (as would be used in the field) (orange). A 95% loess line has been added to highlight the trends while boxes characterise the variability between six replicates.

Two models were used to simulate the laboratory germination results in chapter 4, these were compared to the real laboratory germination results at the last time point (the eleventh day). The first model was a restricted temperature model, only parameterised with the 25°C data from the ‘Thermal Gradient for Seed Germination’ experiment; the second model used all the thermal gradient

data (Figure 6-44). A t-test was used for this comparison, because the data for the last day was centred on a mean. Both for the main model and the 25°C model there was no significant difference to the real laboratory germination ($P = 0.23$ & $P = 0.16$). In order to compare across all the times a Friedman test was used for both models, this showed no significant difference for the all temperature model ($P = 0.76$), but there was significance for the 25°C configured model ($P < 0.05$).

Overall, as apparent in Figure 6-44, the 25°C model better matches the germination rate over the first four days, but both models capture the seed germination percentage by day eleven. The elongation at day eleven when it was measured in the real test was 10.95 ± 1.6 mm (SE); this was lower than, but close to, the day eleven elongation for all temperature modelling (14.27 ± 0.3 mm SE). The model using 25°C data produced taller day eleven seedlings (20.09 ± 1.6 mm SE); however, the real seed was growing in the confines of a Petri dish. When tested with two Wilcoxon tests the overall model was not significantly different from the real elongation ($P = 0.065$), while the 25°C model was ($P < 0.01$).

The next scale tested was germinating in soil in a controlled environment. This was modelled and tested against the results of the 'Sowing Methods Experiment'. Two models were run using the soil water and temperature data from the 'Sowing Methods Experiment', one for seed under a soil covering and one for uncovered seed.

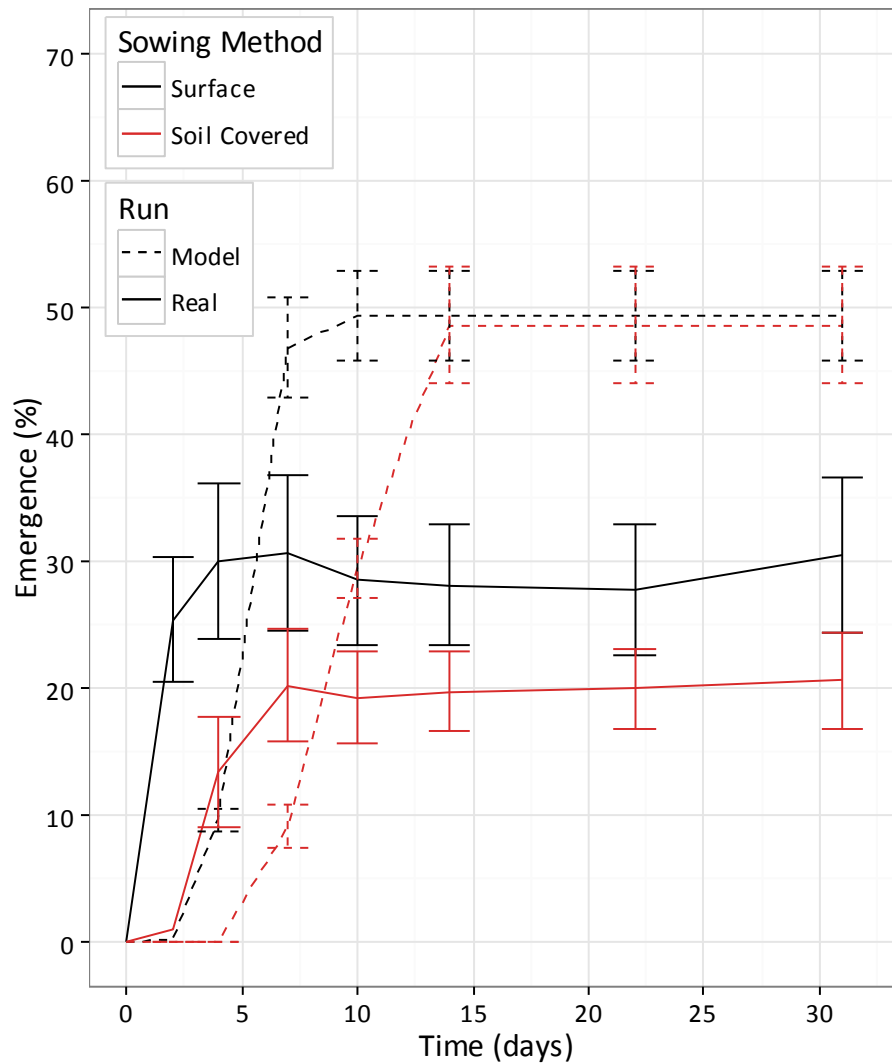


Figure 6-45: Emergence of seed sown in the control environment (from section 6.3c above), compared against the models. Modelling was done with 200 seed and was repeated four times (the number of replicates in the 6.3c experiment), at both surface sown and 5 mm covered with soil. The groove effect was not modelled, because it was not significant. Standard Deviation bars have been added.

As is clear from Figure 6-45 there was a significant difference between the modelled results and the real emergence by the final time point for both soil covered and surface sown seed ($P < 0.01$). The model overestimates germination in the soil by ~20%; this is with the added model factor of a 1% chance of mortality per day. When emergence was tested with the generalized linear model, the effect of whether the data was real or from the model produced a

significant difference ($P < 0.0001$). The model emergence lagged ~ 4 days behind the real data (Figure 6-45). The rate of emergence was about the same between the model and the real data.

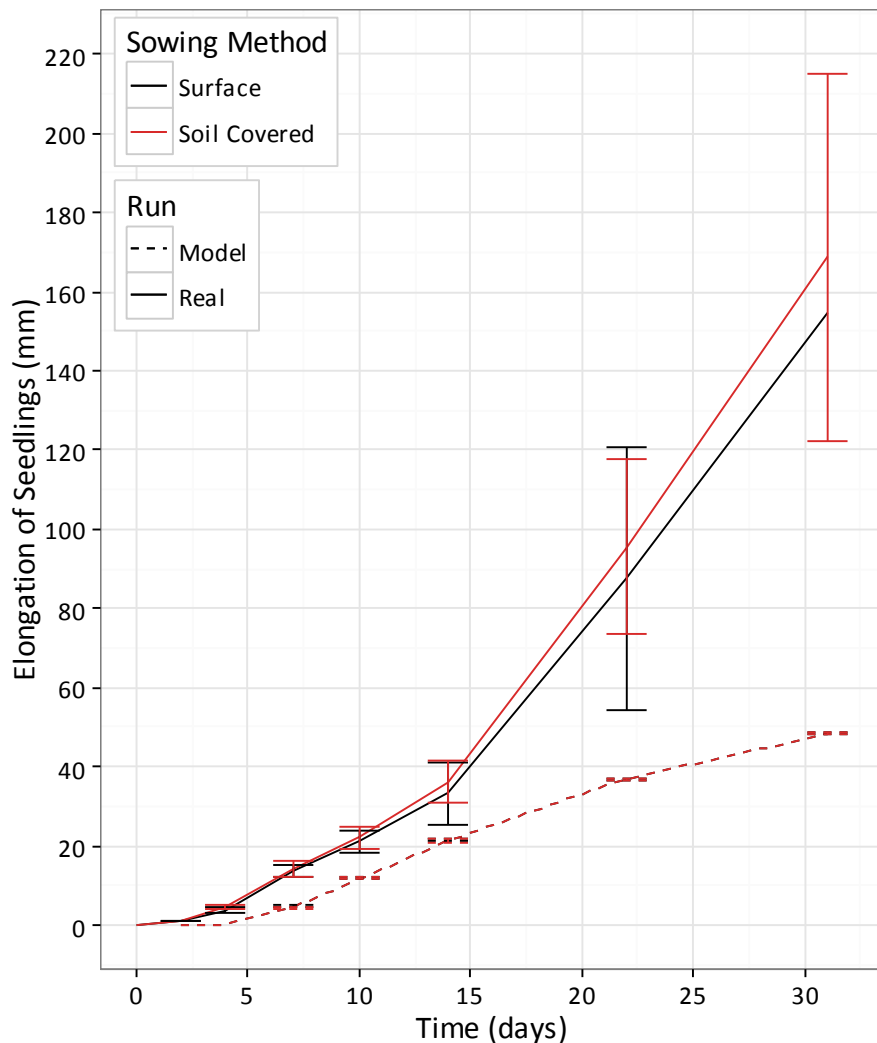


Figure 6-46: Model elongation of seedlings compared to the seedlings in the 6.3c experiment, with standard error bars demonstrating the variability of the four replicates. Two distinct sowings, soil covered (orange) and surface (black), were used.

Emergence produced similar results, with the model lagging behind reality but producing a similar slope (Figure 6-46); however, the slope diverged around 5 cm elongation where the real sowings started a second growth phase and the model did not keep up. The elongation in the real test compared to the model seedling height was significantly different at the end ($P < 0.01$) and overall ($P <$

0.0001). It can also be seen in Figure 6-46 that the model has no difference in the heights of the under soil seed compared to the surface sown seed, because this is not currently a parameter in the model.

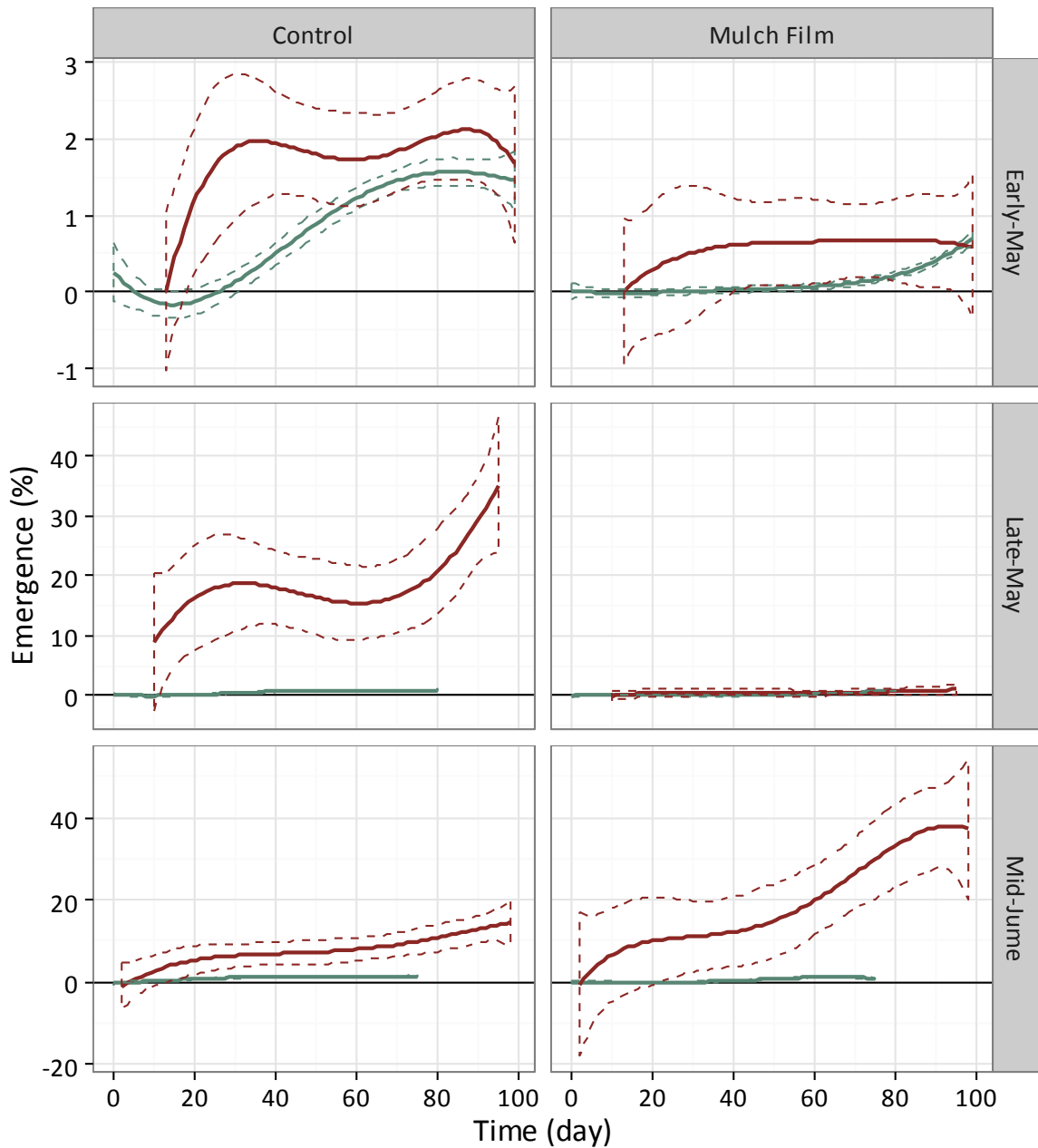


Figure 6-47: Modelling (green) compared by climate to the Direct Sowing Agronomy Trial experiment (section 6.3a) (red). Fourth order polynomial lines with added confidence intervals (dotted) over the six plots. Mean temperature between all film and no film sensors was used. The real data (green) was combined between priming treatments.

The third scale tested was field sowings. The model was run on the 'Direct Sowing Agronomy Trial' environmental data and modelled emergence using the same numbers of seed and replicates. This model predicted the germination with an excessive lag in early-May but was within the confidence interval at 100 days for both film and control (Figure 6-47). In the late-May sowing the model did predict the under film failure but did not predict the success in the control. Lastly, when modelling the mid-June sowing the model predictions were too low for both film and control but were worse under film (Figure 6-47).

When tested with a generalized linear model there was a strong significance to the result for if the data was modelled or not ($P < 0.0001$). However, testing the 45-day counts (the closest field measurement to 45 days was chosen) for each sowing using Kruskal-Wallis rank sum tests gave a better picture; here only the test for the mid-June sowing under film was significantly different between the model and reality ($P < 0.05$). This may be due to the high field variability and low germination (Figure 6-47).

The comparison to the 'Multi Genotype Direct Sowing Trial', shown in Figure 6-48, also has the model under-predicting germination. However, after 45 days in the field the seedling tillering may have artificially inflated real germination counts. It is also clear the soil moisture may have a large effect on the model, particularly because the film sowings always do worse in the model, but in reality, the film improved all the sowings. This will be affected by the data the model used, because it used the environment data for film and control from the 'Direct Sowing Agronomy Trial' (Section 6.3a), where the film was laid 3 weeks earlier.

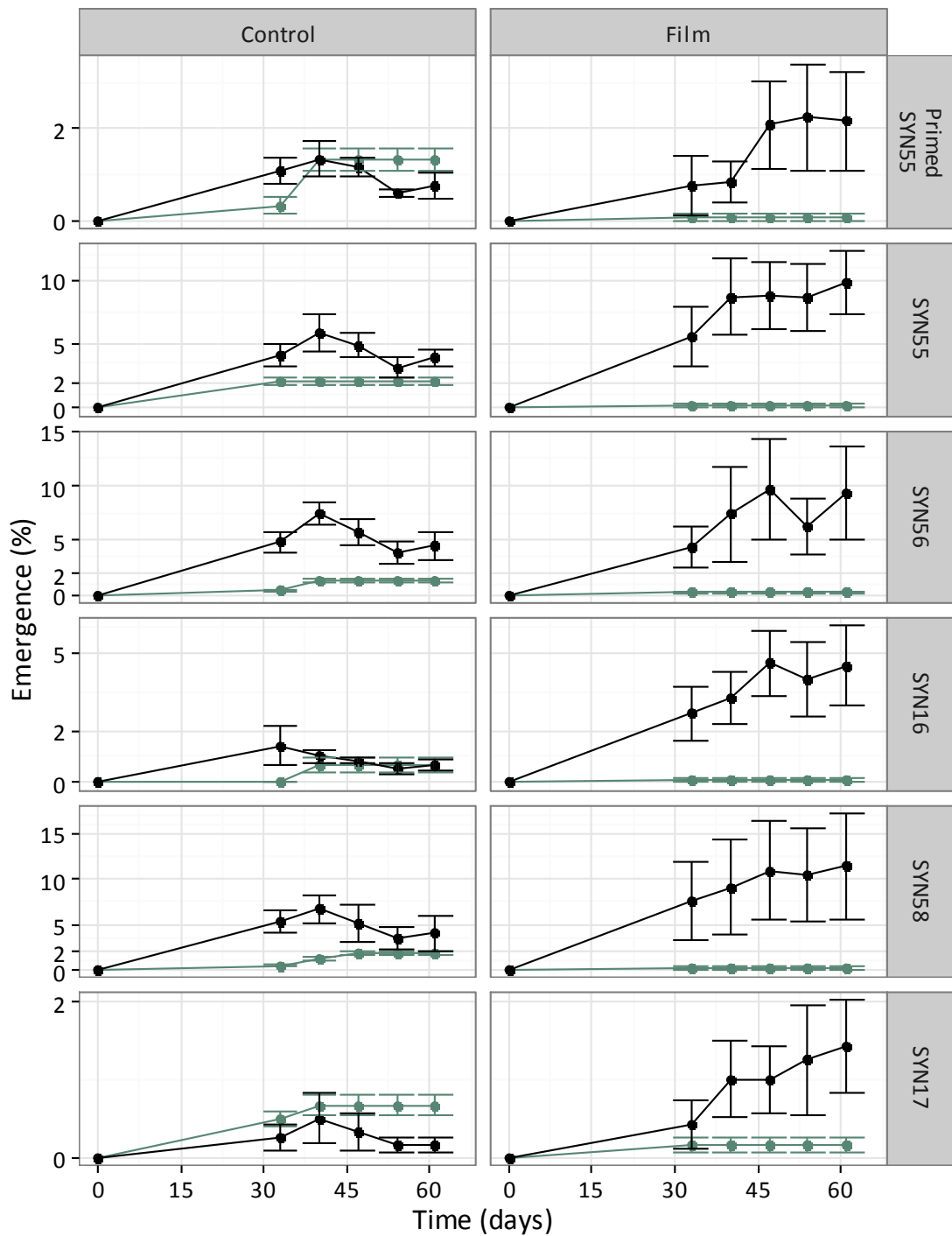


Figure 6-48: Modelling against the results from the 'Multi Genotype Direct Sowing Trial' (section 6.3b) with and without the film treatment, using five genotypes (and primed seed) that were calibrated for the model in the 'Thermal Gradient for Seed Germination' (section 6.3e above). The model (green) was run four times for each film seed combination (the same as the number of field replicates); standard error bars have been added.

The model does predict the relative success of the different seed lots, with SYN55, SYN56, and SYN58 performing best, while primed seed, SYN16 and SYN17 performed worse (Figure 6-48). When tested, the generalized linear model showed a significant difference the model and the real data ($P < 0.0001$). At 40-days, there were only significant differences in the model compared to reality for film sowings of SYN55 and SYN16, and control sowings of SYN56 and SYN58.

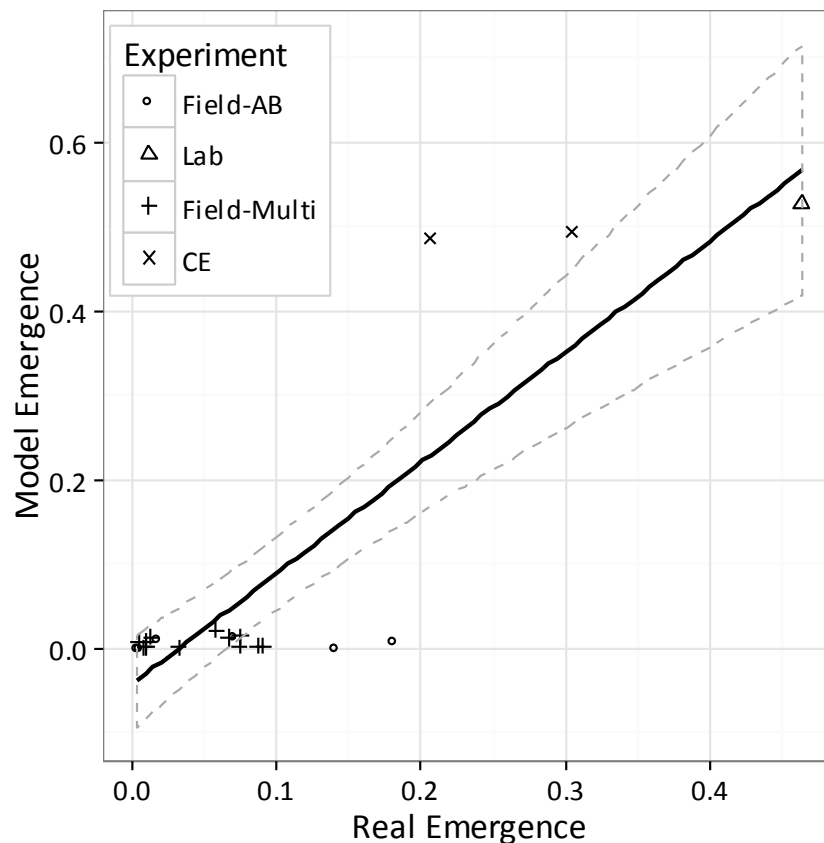


Figure 6-49: A comparison between the final measurements of the model and all of the real experiments, which have been labelled.

Across all the results of each experiment for the single time point used in the analysis, the R^2 between the model and the real tests was 0.73. This was heavily influenced by if the experiment was from the lab, controlled environment, or field (Figure 6-49).

6.4 DISCUSSION

The model and the experiments informing the parametrisation of the model are discussed below. This has been separated into the influences on germination and emergence as tested for in this chapter; therefore, the results of relevant experiments are addressed together. After this, the modelling of germination and emergence is discussed, which was done using the results from the other experiments.

6.4a PRIMING

The first indication of the effect priming had on *Miscanthus* seed was in the lab tests in Chapter 4, which showed only negative effects from priming (Section 4.5b). There was not a significant effect on seed germination, but there were negative effects on stem elongation and Fv/Fm florescence. In this chapter, priming had overwhelmingly negative effects on seed germination and performance.

In the 'Direct Sowing Agronomy Trial', priming had a significant negative effect on emergence overall in both Aberystwyth and Blankney; in Aberystwyth priming resulted in lower emergence in all sowings and treatments. First year stem counts were lower in Aberystwyth for primed seed and second year tiller counts increased significantly less in Aberystwyth. In Blankney, priming had a significant negative effect on stem thickness and second year stem counts, but did not affect first year stem number. There was also a small interaction with first year senescence in Blankney between sowing time and priming; this did not appear to endorse any biological hypothesis.

The priming did significantly interact with sowing in the second year number of leaves (Aberystwyth), this interaction showed earlier sowings producing more

leaves from primed seed and later sowings producing more leaves from un-primed seed. This is probably not an important result by itself but shows as discussed in Section 4.5b that priming can have an effect on later growth.

The main effects of priming at the end of this trial were that priming significantly reduced the chance a plot had sufficiently established to be harvestable in Aberystwyth and when it was harvested final dry weight was significantly lower for primed seed.

The increased aging of primed seed (Hacisalihoglu et al., 1999) could be partially responsible for this result because the sowings happened throughout the year. However, primed seed performed very similarly to unprimed seed on the thermal gradient plate experiment done at the beginning of the next year. In addition, the 'Multi Genotype Direct Sowing Trial' was conducted with freshly primed seed and found only negative effects from the priming of SYN55. In this trial primed seed emerged at approximately 40 days, which was significantly later than un-primed SYN55, and whilst not significantly different (due to the variability in the harvest weight of SYN55) primed seed produced only 12.4% of the mean harvest weight for un-primed seed.

Priming on the thermal gradient plate produced a statistically significant negative effect across most temperatures and times. Despite the positive effect of priming in other crops (Ellis & Butcher, 1988; Elsoms Seeds, 2013), all but the 9°C thermal gradient plate data showed priming to have a negative or neutral effect on germination in a *Miscanthus sinensis* hybrid. This may have resulted from the exact method of priming i.e. over priming which can easily reduce germination (Arif et al., 2008). Even correct priming was expected to kill some of the weaker seeds (Sathish et al., 2011). The positive affect at around 9°C was after 800 hours; this may indicate primed seed can accumulate thermal time

better at lower temperatures, possibly due to some residual metabolic activity. However, this did not make the primed seed any faster to germinate at higher temperatures on the thermal gradient. Further investigation of the settings for priming *Miscanthus* seed would be required at a lab scale before further field experimentation with priming would be advisable.

6.4b FILM

The first sowing in the 'Direct Sowing Agronomy Trial' (Section 6.3a), showed that film degrades over 2-3 months, this would be faster later in the year when there is a higher irradiance of UV. The effect the film had on soil moisture as measured by soil water reflectometers was negative in Aberystwyth and positive in Blankney, possibly indicating that the film protected dry soil from rewetting by rain. Film was expected to raise the soil temperature and increase the yield particularly in a colder climate (Farrell & Gilliland, 2011). There was 2.7°C and 4.2°C increase in mean daily temperature in Aberystwyth and Blankney respectively. This is consistent with a small experiment in a controlled environment (Appendix N), which found film to raise temperature by around 4.5°C. On average between both field trial locations, the change in temperature under film was around 3.46°C (Section 6.3a) during the emergence phase (2 months).

Film did not significantly affect emergence at the Aberystwyth site but did in Blankney; this was probably due to the extra 1.5°C mean daily temperature Blankney seed received under film. Film took Blankney seed from 15 to 19°C while in Aberystwyth the change was from 10 to 13°C. Comparing field temperatures with the 'Thermal Gradient for Seed Germination' experiment showed that the effect of film at Blankney was to increase the temperature along a steep part of the germination response curve almost to the point where germination approaches an asymptote. If film does not rapidly stimulate germination of

Miscanthus then weed seeds may be stimulated to germinate and have a negative impact on *Miscanthus* growth.

The effect on film-covered seed in Blankney resulted in significantly more stems at the end of the first year, which was a direct result of significantly more emergence, but the film did not produce any other benefits. This was possibly due to the good environmental conditions. In Aberystwyth film treatment resulted in significantly taller plants after the first year, possibly showing that the thermal boost was of benefit to the seeds that did emerge resulting in faster metabolism and growth. The interaction with first year senescence in Aberystwyth and mulch film increased the senescence where the film was on longer (at both ends of the year). This may have been due to weaker plants that were stuck in a warm environment to long.

By the second year in Aberystwyth, tiller number increased significantly in plots treated with mulch film, showing a delayed improvement to the establishment. The increased height in the first year may have improved competition or resulted in more nutrient capture for rhizome production and overwintering, leading to more tillering of surviving plants the next year. However, the significant difference in plant height seen in the first year did not carry over to the second year. The final dry weight per plot harvested at Aberystwyth was not significantly different with or without film nor was the rate of plot establishment significantly affected. Mulch film had some small statistically significant effects on plant morphology at Aberystwyth but these were not sufficient to have a measurable impact on yield. A longer study would be needed to test if there is a longer-term cumulative effect due to the early growth.

At Blankney, the mulch film treated plots had significantly more tillers in the second year but this was not due to a significant increase in the rate of tillering,

only an effect carried over from the previous year. At Blankney, the effect of mulch film on final biomass index was not significant per plot but there was a significant film effect on plot survivability giving film plots a 33% higher chance of survival. This again originates from improved emergence in the first year.

The 'Multi Genotype Direct Sowing Trial' again found that film had no significant effect on emergence, but film plots had more variable emergence counts for all genotypes. However, the film treated plots in this Aberystwyth trial did produce significantly more harvested biomass, and more mean harvest mass across all seed lots. For example, germinating SYN16 under mulch film produced more than a 1000% increase in harvested biomass, and across the five seed the average increase was 547% (this took account of failed plots as zero).

The benefit of mulch film for soil water on dry soils (Zhou et al., 2009), was not seen in either field trial. However, this was primarily because the trials in this study were not semi-arid and film was laid when the soil was dry for a short time and subsequently sheltered the seeds from the precipitation that fell on the field in the weeks following the sowing.

In both experiments there were also observed issues with weeds (e.g. black grass) taking advantage of the climate under the film to out-compete the *Miscanthus* seedlings.

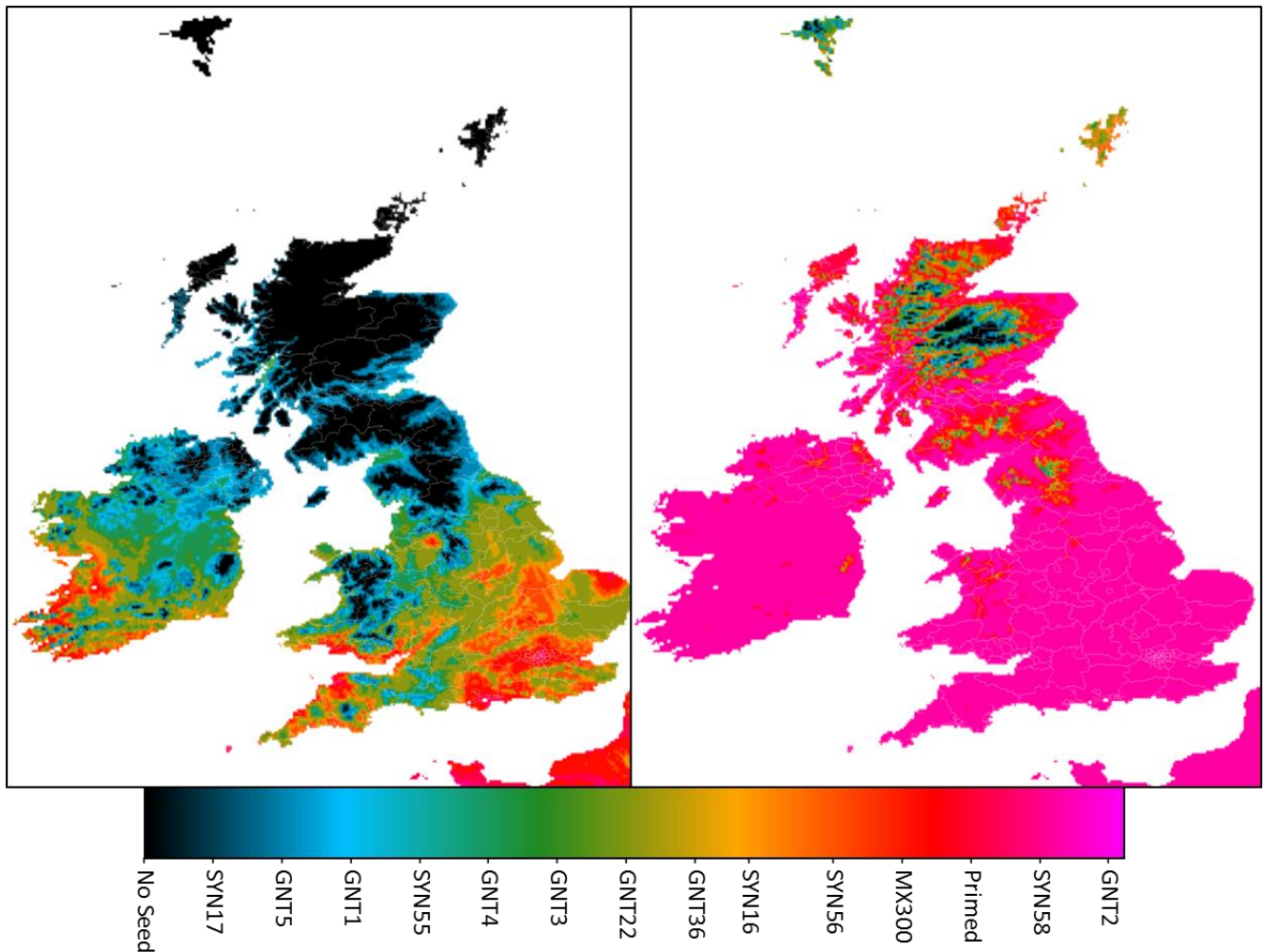


Figure 6-50: Base temperatures of different *Miscanthus* genotypes applied to an April UK thermal map (Klein Tank et al., 2002). The map on the left represents base germination temperatures and the map on the right adding the mean 3.46°C resulting from sowing under mulch film. Each increment away from the no seed [black] includes all previous genotypes.

Applying the 3.46°C thermal boost to a climate map (Figure 6-50), shows there is an impressive potential expansion of the UK area covered by the improved base germination temperatures provided by mulch film treatment. However, the thermal boost that the film produces was only measured on the first sowing making it difficult to generalise the effect for all times of year. The average 3.46°C was used to approximate the benefit of film, this does not account for the smoothing of temperature fluctuations that film conveys to the plot. In addition, because the film was only monitored in the first sowing the tempera-

tures under the film for the sowings at the height of summer were not recorded. The June and July sowings under film may have received a huge thermal boost; however, film degraded the quickest after summer sowings, which may lessen the total the mean thermal impact but it is possible that high and even inhibitory short-term temperatures were generated under film in summer.

It was previously not known if the film could survive the winter from an October planting, this experiment showed the last film from October survived through to spring. In Blankney during the 'Direct Sowing Agronomy Trial', there was a small chance that some plots sown in September and October that did not have any recorded seedlings in the first year, did have seedlings in the second year. Rather than result from overwintered seeds it may be that small seedlings were missed in the first year, survived due to Blankney's milder climate, and were then counted in the second year. The success of later sowings is an indication that film could accelerate late sown seed during the first year; this would extend the sowing season.

6.4c SOWING TIME

The time of year for sowing was tested in the 'Direct Sowing Agronomy Trial'. For the westerly (Aberystwyth) site the optimal sowing time for dry weight was sowing 2 (late-May), but the best chance of a plot surviving was sowing 3 (mid-June). This could have been due to drier soils in the first two sowings or just temperature dips that did not give the *Miscanthus* the best chance against competition from weeds. In the easterly (Blankney) site, the optimal final biomass index was at sowing 1 (early-May), because the better climate afforded the seeds a longer growing season. However, the best chance of survival was again later at sowing 2 (late-May). This shows that despite the better early season conditions in Blankney losses were seen; but overall the optimal sowing time

was advanced by three weeks. This indicates that thermal time is most important for germination and establishment; however, the specific circumstances of this trial and the one year it was done in, may limit how much can be said about this effect; particularly because soil moisture, on the days before and after sowing may have a large effect.

In Aberystwyth, emergence peaked at sowing 5 (late-July); this coincided with high temperatures and rainfall. Despite these ideal conditions mean emergence was only around 8% and because the seeds had less time to establish, their final growth was reduced. Late sowings, as late as sowing 8 (late-September), did emerge in the first year; however, they mostly did not survive into the late autumn for phenotyping. Sowing 6 (Mid-August) was the last sowing to have plants survive into the second year at Aberystwyth.

The idea of late sowing to allow the seed to overwinter (especially with the protection of film) and germinate when ready the next year did not find any support in Aberystwyth. In Blankney, only two late sown plots with no seedlings in the first year had seedlings present in the second year and these plots were not large. Sowing 5 (late-July) was the last sowing to have an increase in tiller number from the first to second year. Therefore, there is no reason to conclude that an autumn sowing of *Miscanthus* may lead to a crop that establishes in the latter half of the year before overwintering, or germinating and growing strongly in the second year.

The *Miscanthus* crop loses biomass (mostly leaf) in the last part of the year (Caslin et al., 2011; Lewandowski & Heinz, 2003) and translocates nutrients to the rhizome. This could account for up to 42% under the right conditions (Lewandowski et al., 2000). Therefore, whilst some plants may have grown to a

reasonable size mid-season this top growth may not have allowed them to produce sufficient rhizome for the next year.

Most of the differences in the first year phenotyping between sowings derive from differences in the maturity of the emerged plants in the first year, but by the end of the second year, there were still significant differences in stem height, stem thickness, and number of leaves. At both locations sowings 1-3 normally produced the highest values for these traits, the exception was stem thickness in sowing 2 (late-May), which in both locations stems were thinner than in the other early sowings.

6.4d SOWING METHODS

It was first observed that *Miscanthus* seeds germinated more readily in tractor tracks than in well-tilled plots, this was some combination of the seed being washed down by precipitation in the plot and the availability of water in the groove. This is difficult to replicate on fields with a variety of soils and soil moisture, but film may solve part one while a light covering of soil solves part two.

The sowing methods tested in the controlled environment (Section 6.3c) were only a short 30-day study so the significant improvement in non-groove sown biomass may not have led to a long-term improvement. Both field trials in this chapter clearly show early success as reasonable indicator of success in the first and second years; this is mostly because early growth allows the plant to utilise more light energy. However, the experiment suggests that groove surface sowing of seeds may be better than using a groove, which could benefit low-till sowing techniques.

The germination/emergence was not significantly affected by the groove (at the comparison of 96 h); it was faster on the surface. Root and stem growth was

better on the surface but this was not a significant improvement. Germination overall in the soil was lower than in laboratory on paper germination experiments, yet was still ~40%, rather than ~8% detected in field experiments. This shows that either due to hydraulic contact or potential of mould the seed is naturally less able to germinate in soil conditions.

Whilst the seeds emerged significantly better when not covered by soil, the difference was not large and the benefits to seed/seedling water availability in the variable environment of the field by sowing beneath a layer of soil may be more significant if tested across different years. Christian et al. (2005) found drilling seed into soil to be more successful than direct sowing in *Miscanthus* and speculated this was due to the direct seed soil contact. Therefore, seedling power, the force with which a seedling can move stones or aggregates out of its way, would be necessary to understand; because the shoot will not grow downwards to reach the soil surface once it has grown into a cavity (Dürr & Aubertot, 2000).

A thorough investigation with different substrates and different depths may be required; this could also be extended to include the moisture content of aggregates. Further investigation of seedling emergence force could easily be re-integrated with the model (Brunel-Muguet et al., 2011; Dürr & Aubertot, 2000), and used to update our sowing techniques in light of the results of this experiment for under soil sowings. A field trial would be the only way to fully test if the negative effects of a soil covering demonstrated in Section 6.3c would be outweighed by better hydraulic contact and availability of water.

6.4e GENOTYPE

The 'Thermal Gradient for Seed Germination' experiment showed a range of responses to temperature, these were a combination of genotypic and seed lot effects; as only one year's seed was available the primary affect is presumed to be genotypic. GNT3 and GNT5 responded better to being kept cold before being germinated, as opposed to SYN16, SYN17, SYN55, and GNT22 that had a higher proportion of mould in the colder sowings. GNT2 had a near binary germination response to temperature, while GNT3 and GNT36 which both had similar germination at high temperatures, had shallow response curves at low temperatures. The total proportion of seeds germinated and total proportion of mould-infected seed did not seem to follow a trend based on type of cross or age of seed.

The 'Multi Genotype Direct Sowing Trial' showed a significant effect of genotype on field emergence, emergence was highest for SYN55, then SYN56 and SYN58 and lowest for SYN17 then SYN16. While SYN17 performed worst. Interestingly this would not be the order predicted by overall performance on the thermal gradient; which would be SYN58, SYN56, SYN16, SYN55, and then SYN17, i.e. SYN55 does much better than would be expected in the field. If calculated base temperature were used to predict order, it would be SYN17, SYN16, SYN55, SYN56, and then SYN58. This correctly places SYN55 compared to SYN56 and SYN58; SYN17 has the lowest base temperature but cannot overcome its low germination percentage. The field demonstrates that SYN55 uses its modest germination and lower base temperature to out germinate SYN56 and SYN58 with higher germination potential.

In the 'Thermal Gradient for Seedling Stem Elongation' experiment, the final stem elongations from the two seed lots (SYN55 & MX300) were not significantly

different but the growth rate was (SYN55 grew faster). The base temperature for elongation was lower for SYN55 than MX300 seed as was seen for the base temperature for germination on the thermal gradient plate. This possibly shows that the base temperature for germination and elongation are physiologically linked, and involve the same tolerance for cold temperatures, so seed that germinate in colder conditions do also grow in colder conditions.

In the 'Multi Genotype Direct Sowing Trial', SYN55 seed had the highest harvest weight, closely followed by SYN56, and SYN58 (the best performing single plot was a SYN56 plot); these are all synthetic crosses from 2011-2012. This is the same order as field emergence; sowings with higher levels of emergence had more biomass at the end of the second year. SYN17 was still the lowest performing genotype despite the lower temperature germination found on the thermal gradient; SYN17 low germination curve may show that while it can germinate under cold stress the advantage this gives is only applicable to a narrow range of field temperatures.

This work can be greatly expanded upon with the use of other varieties of *Miscanthus* and other climatic locations, particularly parents of seed based hybrids. To clarify why certain genotypes perform better in the field than expected, and why all perform worse than on, paper or soil, in a controlled environment; there are clearly factors that need to be identified and incorporated in to our understanding of what is required for germination and seedling establishment that explain the difference between controlled and field experiments. These experiments indicate that the primary factor influencing this drop is the soil water or the seeds inability to access the soil water.

6.4f SOIL WATER AND TEMPERATURE

6.4f-i WATER CONTENT OF SOIL

The lab experiment showed that water content of the soil was crucial in germination and few seedlings emerged at Aberystwyth when water levels in the field were low. The base value of 6.2% W/V water required in the soil for germination is specific to the soil type, because water availability to the seed (Ψ) will vary depending on soil composition, and would require better characterisation of the substrate such as a soil release curve to calculate (Forcella et al., 2000). In the sandy loam from Aberystwyth this would approximate to -1 MPa (Brady, 1990, p. 133). However, imperfect seed soil contact would reduce water availability. This base value of 6.2% implies seed would likely not germinate in a timely fashion in field conditions due to water stress. Blankney often dropped below ~10% water with peaks at ~20%, while Aberystwyth is normally around 10% with peaks at ~15%.

All of the experiments with substrate of less than 24% water had a linear not sigmoid germination curve suggesting water stress is responsible for the difference between linear and log-linear germination rates. The biggest difference in germination was between 20 and 24% soil water. As field soil water was usually between 8-12%, germination should be between 4 and 12% given ideal temperature and good seed. This is close to the poor emergence seen in the 'Direct Sowing Agronomy Trial' and 'Multi Genotype Direct Sowing Trial'; showing emergence was less due to temperature than soil water, possibly supporting Anderson et al. (2015) in the need for direct sown *Miscanthus* to be irrigated. Future work could compare different soils effect on *Miscanthus* seeds germination, and seed soil hydraulic contact. This would also show if the model's

representation of soil waster needed to record soil type and calculate water potential.

6.4f-ii GERMINATION TEMPERATURE

The base temperature for germination on the thermal gradient plate was around 7.7°C, while Clifton-Brown et al., 2011 recorded a range from 9.6 to 11.6°C; however, that experiment had diurnal temperature cycles and a different *Miscanthus* genotype. The genotypic variation in the thermal gradient experiment ranged from a base of 9.9°C for GNT2, down to SYN17 and GNT5 at 1.4°C and 6.6°C respectively. The genotypic variation in the new hybrid GNT seed lots implies that breeding could greatly improve establishment in a UK climate even though temperature *per se* was not a target trait for the production of these hybrids. On top of this film adds temperature to the growing conditions, particularly reducing cold snaps; adding a blanket 3.46°C change due to mulch film use when modelled across the UK illustrates the exciting potential for *Miscanthus* seed propagation (Figure 6-51). However, the actual impact of mulch film is more complicated than this due to the effects of film on moisture, subsequent exclusion of precipitation, over temperature effects on germination and the impact on weed seed germination and subsequent competition. When the film was used in the field on the multi-location trial (Section 6.3b) the optimum genotype changed from SYN55 to SYN56 between control and film covered sowings, in both emergence and final harvest. This change was because SYN56 had a higher base temperature, and was allowed to germinate and grow due to the film providing a temperature boost.

Germination peaked at an average optimal temperature of 25°C as was recommended by Aso (1976) for *Miscanthus* germination. Only a few genotypes underwent a notable decline in germination above 25°C, which suggests that

peak temperatures under film will not have a significant impact due to high temperature inhibition of germination in *Miscanthus* seeds in the UK. The optimal temperature also suggests that in the UK climate optimal germination would not be possible, even with film. So genotypes with more than 90% germination at an optimal temperature are often germinating at just half that, at 10°C (as well as the lower optimal temperature germination in soil). However, the optimal curve is flattened for some of the varieties providing a wide range of 'optimal' conditions, particularly GNT2 where germination was at 73% at 11°C, 97% at 13°C, 95% at 25°C, and 100% at 29°C. It is also important that seed sets have an ability to remain in the cold wet conditions without a high prevalence of spoilage from overgrowth by mould. Field success based on temperature may have been better in genotypes that stopped germinating sharply; it was observed that a slower slope up to peak germination tended to lead to more mouldy and less viable seeds. The mould could be an effect of threshing damage and not necessary genotypically dependent.

6.4f-iii ELONGATION TEMPERATURE

Base elongation temperature was slightly lower at 5.62°C in SYN55 than the 6.73°C of MX300; this is similar to the base germination temperature for the seed types, which were 7.39°C and 8.67°C respectively. This may indicate that the seed activity requires a temperature above its germination base to grow. However, it does not indicate the seed dies when exposed to temperatures lower than this, and in both the germination and elongation thermal gradient plate experiments (Sections 6.3e & 6.3f), when the temperature was turned up at the end, the not growing or not germinated seeds that had not obviously died usually started to grow/germinate. In addition, this does not indicate whether seed normally need more temperature to germinate or grow. From this limited study, it would appear increase needed from base germination temperature may

be genotype dependent, or because the germination and elongation studies were done a year apart, changes in the seed during cold storage may have affected its base temperatures.

The elongation rate peaked at 25°C, but mostly levelled out after 19°C showing the seedlings do not need unrealistically high field temperatures to achieve a high elongation rate. The increase in total elongation followed an exponential curve over time for most temperatures, showing the fastest elongation happened in the first three days. There appears to be some instances where the elongation noticeably changes rate, this may correspond with leaf extension stages because this was what elongation primarily measured (Clifton-Brown & Jones, 1997). It would be logical if elongation rate slowed during new leaf production. Leaf production was not recorded for seedlings in this experiment, because the time the seedling first leaf was fully extended was difficult to judge. Therefore, this cannot be confirmed but there were two times (~200 and 500 h) where seeds appeared to move their growth into a slower stage. This would be consistent with stages of photosynthetic production and seed storage. The approximate slowing times were similar to observed times of slowing that occurred in the time-lapses of seed growth (data not shown). This may indicate that the slowing was more to do with seed energy storage necessitating a slower form of growth, as the seed switched to a photosynthetic energy source.

There was a small but significant difference in elongation rate between the genotypes. It is not known if this was dependent on base temperature; however, elongation as measured by a change in thermal time did not account for all of the difference in elongation (as an offset). Both seeds did have the same elongation rate during the linear phase (first 500 degree-days). This implies that seed with a similar base temperature, which most seed lots had, would elongate at the same rate until 5 cm. This should be useful in the field because seeds of

varying genotypes should be able to establish to a point where the seed is growing and photosynthesising in the same thermal time.

6.4g LOCATION

The environmental differences between the locations studied in the 'Direct Sowing Agronomy Trial' were that the easterly site (Blankney) was warmer than the westerly site (Aberystwyth) during the sowings in 2013. Plant size, particularly height, was larger in the warmer location. However, there was a higher incidence of failed sowings in a warmer location. This could have been due to the soil structure allowing less hydraulic contact; the Aberystwyth soil was stonier so hydraulic contact should have been worse. However, the fine soil in Blankney allowed better drainage than in the stony Aberystwyth site. The Blankney site often had a drier soil surface; it also had higher spikes in the surface soil moisture from precipitation than the Aberystwyth site. This may have been important to certain sowings succeeding in Blankney and performing better, while more often than in Aberystwyth the sowings failed. Sowings that did not coincide with precipitation were poor at both sites but in Aberystwyth, the soil measure was ~3% higher in-between precipitation.

Stem heights at the end of the first year were similar in both locations while stem counts were higher at the Aberystwyth site; however, by the end of the second year the Blankney site was higher in all measurements. Overall, by the final assessments of success, the Aberystwyth site was best sown in late-May while the Blankney was best-sown one sowing date earlier in early-May, giving a 3-week difference in optimal sowing time. The better overwintering and some late sowings emerging in the second year in the easterly site, is consistent with the milder winter on the easterly side of the UK.

6.4h COMPUTATIONAL MODELLING

6.4h-i GERMINATION PERFORMANCE

The model is required to predict germination over a wide variety of sowing conditions. Early iterations of the model predicted lab germination very accurately, particularly when running the model using only the 25°C thermal gradient data; this was due to the similarities between the thermal gradient plate data from which was used to train the model, and the lab conditions. However, the success of the lab prediction did not account for the field sowing results, where emergence was over estimated by more than 10x. After introducing parameters for the probability of seed loss in the soil and the probability of seedling death, as well as a properly parameterised soil water model, the results presented were more accurate. This model is still inaccurate, particularly in predicting the controlled environment experiments, which sits between the lab and the field (where the germination was over-estimated by ~20%). However, while the slope of germination lagged behind reality, it was still a similar overall germination rate. With further parameterisation of why the seed does not germinate in soil conditions, it may be possible to lower total germination in the controlled environment soil while reducing the lag. The field is now somewhat under-represented for emergence, but the model predicted germination best in the first sowing, which had the most accurate data. Again, further parameterisation could be of benefit in the field, but would need to account for the controlled environment as well.

The model will likely remain unsatisfactory until the causes of the lower field germination are better understood. The model also remains inaccurate in non-soil conditions. A lag to germination for the lab-based experiments was noticeable; if the field death factor were not switched off the lab germination, would

have also been too low, because the seed loss factor proves too extreme for a lab environment. This imprecise way of controlling the model's behaviour based on conditions seems non-optimal but is practical and identifies a need to identify the factor or factors that are currently not accounted for in field experiments. This model should be seen as an experimental method for modelling establishment that will lead to better models, as has been the case in other *Miscanthus* models that have gone through several iterations (Hastings et al., 2009a).

This seed death/loss effect in the field is probably due to at least two factors: The hydraulic contact between the surface and the seed is not as good with soil as it is with paper in the lab, and there is a much lower average water potential (Section 6.3d above) in the field from the lab or controlled environment. The lower hydraulic contact between seed and soil than with wet paper is also in effect for the controlled environment soil experiment. This possibly explains the result, because seeds with good water contact would germinate very quickly due to the optimal temperature, while seeds without it would quickly dry out due to the temperature.

The lag in the rate of germination in both the controlled environment soil and the lab may be caused by the humidity at the 25°C temperature. When the lab was compared to a 25°C based model, the lag disappeared. This may be because lower temperatures on the thermal gradient had slower germination not just due to thermal time but also the effect of the humidity surrounding the seed.

6.4h-ii ELONGATION PERFORMANCE

The elongation of the seedlings, not the height, is what the model predicts, this was because the early growth model is always calculating elongation of small seedlings (less than 5 cm), and may be dealing with pre-emergent seedlings. The secondary growth model is currently quite crude and predicts elongation up to

~20 cm; this was not designed for predicting a whole year's growth. For these reasons, the model was not compared to the field's end of year height measurements.

In the lab testing, the main model non-significantly over estimated the elongation of the seedlings (3.32 mm); this could have been an effect of the seedlings being in a contained Petri dish. With the controlled environment, the model closely mirrored elongation of seedlings under ~2 cm. However, longer seedlings, rapidly diverged from the model ending up at 31 days only ~30% the size of the real seedlings, this shows a failing in the secondary growth model. Although, the elongation reached by the modelled seeds of ~5 cm when real seeds had elongated to ~16 cm, may show a better estimation of height to the last ligule, because a height of 5 cm would correlate to an elongation of ~20 cm in the 'Seed Competition Soil Experiment within a Controlled Environment' (Section 5.3b above).

Overall, the model well represents early growth, but is less accurate in later growth. However, the early model has not been fully tested with *Miscanthus* seedlings growing up through soil. While it may be difficult, on a thermal gradient, a time lapse of seedlings growing up through soil could be used to better calculate pre-emergence seedling growth. This may not be necessary, unless it becomes routine agronomic practice to sow *Miscanthus* under soil.

6.4h-iii FUTURE IMPROVEMENTS

Further investigation of field sowing methods as used in Section 6.2c & 5 above could provide data that could be integrated as a germination curve with the existing model or as a modifier to the germination process if the model was updated.

The model original model by Dürr et al. (2001) used water potential to calculate whether the seed had a chance of germinating (only as a minimum that a seed with adequate thermal time to germinate must be above). This is a more accurate measure of water useable by the seed than percentage soil water; however, this is not directly measurable in the field without knowing the composition of the soil. The model for *Miscanthus* uses a soil water percentage (W/V) calculated from the 'Soil Water Content' (Section 6.2d), as minimum water for germination. The model may be improved by utilising a more complicated equation, because the soil water experiment, conducted at 25°C (Section 6.2d), did not have a sudden point at which no germination took place but a decrease in germination with soil water percentage. However, this effect may be due to the decreased chance of good soil to seed hydraulic contact and not a factor of the amount of water in the soil. This would require further investigation. The model could also take account of water potential and chance of good hydraulic contact where more detailed soil data was available.

Future improvements to the model should include a better growth model, which accounts for *Miscanthus* height in its secondary phase. A parametrised seed loss and seedling death model would be useful, because the current estimates are only estimations used to limit growth to more realistic field conditions. The model testing seems to suggest the seed loss/death estimation in the soil should be increased to account for the over estimation of germination in the controlled environment, while the seedling death chance should be decreased to account for the under estimation of surviving seeds in the field. The growth of a seedling around a clod should be better defined if below soil sowings are used in the future, as should the chance of a seedling making it through a crusty soil both of which are part of the original model. Eventually information from the time of

year could be used for day length when the effect of diurnal cycles on *Miscanthus* germination and growth is better understood.

Factors that are using estimates currently are coded into the Python part of the model; these should be added to the user interface as data becomes available for them.

6.4h-iv MAPPING

Data from both trials was used in the 'SimPIE' Model (Dürr et al., 2001) to produce results that could be used to better predict the effect of climate variation on *Miscanthus*. By using the model, the data collected can be expanded to tell a broader story about the use of film and priming in different climates within Britain. This would provide a much better idea of when to plant, and of what seed and sowing treatments may be necessary depending on other climates.

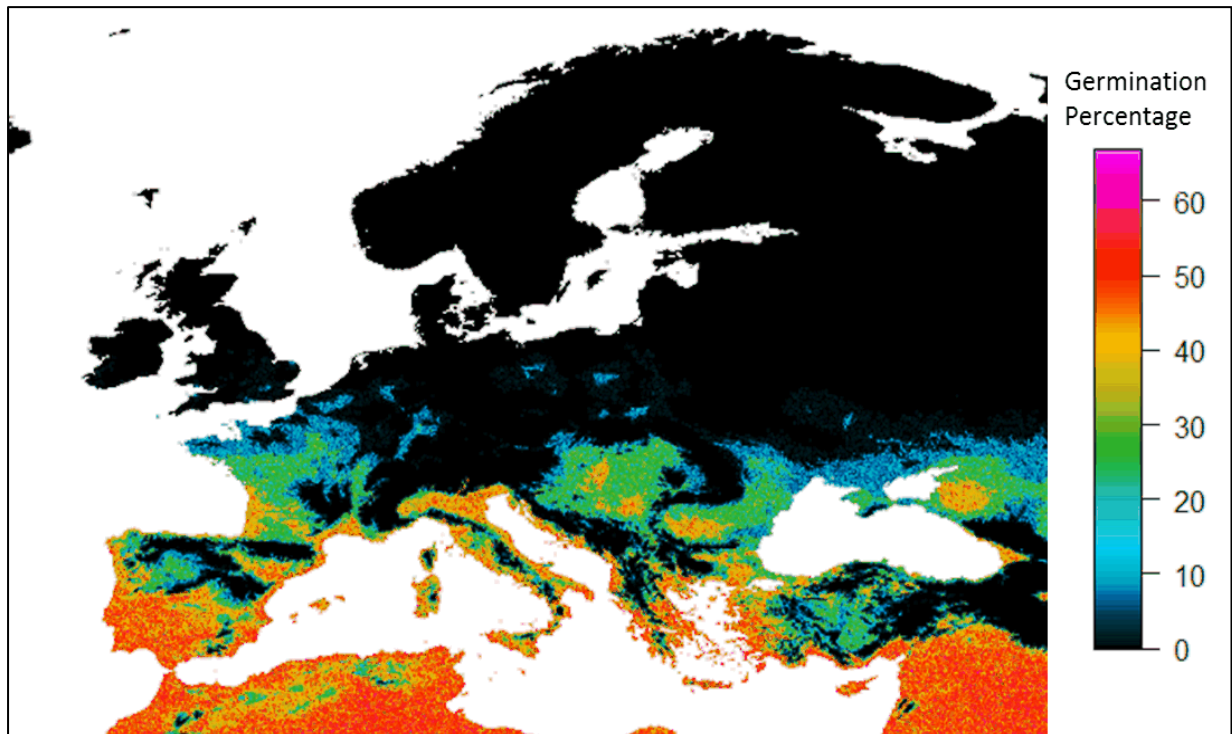


Figure 6-52: Modelled map using April EU temperatures (Klein Tank et al., 2002). The percentage of seeds germinated in the month was calculated, by sowing 100 SYN55 seeds at each pixel and running the SimPIE model. Soil water data was not known so this was calculated as germination on wet soil.

In the future, the model could (with R package ‘raster’ (Hijmans, 2016)) be run over maps (e.g. Figure 6-52), though this would require making assumptions about the soil temperature and water. This could be achieved by adding a simple soil water balance equation to the model so the rainfall can be modelled for soil water or with soil data water potential that the seed experiences.

7 CONCLUSIONS

Decarbonising the economy is an important global target and dedicated biomass crops can contribute to this goal as discussed in Chapter 1. Such crops are new and require improved understanding of traits that are important for successful growth and yield of the crop. Seed biology is a critical area of research for improving the agronomy and economics of the *Miscanthus* crop. The main methods and seed used to address the challenge of improving seed agronomy were outlined in Chapter 2. After demonstrating the problems with human imaging of seed, Chapter 3 explored the application of imaging in improving the scoring of germination. A number of factors may contribute to germination and many of these have been studied in other systems. In Chapter 4 *Miscanthus* seed germination was scored by a combination of images and human assessment across a variety of potential factors including light, stratification, and water stress as well as important plant hormones to determine the extent that these factors are also important in germination of *Miscanthus* seed. In the final part of Chapter 4, these factors were used in a Taguchi experiment to gain greater insight into the impact and interaction of factors contributing to *Miscanthus* germination. Laboratory experiments may not be easily transferable to field conditions and chapter 5 explored cluster sowing over many levels and approaches from laboratory to field studies to determine the potential for direct sowing and refinements to sowing for improved establishment rates. Germination in *Miscanthus* may be impacted by agronomic treatments such as mulch film, priming, sowing time, and by variation in meteorological conditions plus the impact of genotypic variation. In chapter 6, experiments to gather funda-

mental seed parameters in response to a number of different treatments and factors were carried out. These were then utilised to parameterise a model of *Miscanthus* germination and emergence.

This investigation into *Miscanthus* seed focused on the optimal conditions for germination and establishment, with a view to direct sowing of *Miscanthus* seed. Establishment by rhizome is intrinsically expensive (£1500+ per hectare) (Christian et al., 2005; Clifton-Brown et al., 2016), and difficult to upscale commercially due to the large nurseries required to grow rhizomes (Road & Malling, 2009). Seed, with an upscaling multiplication of 1500 times (Clifton-Brown et al., 2016), compared to 50-100 times for rhizomes (Huisman, Kasper, & Venturi, 1996; Pari, 1996) cited (Scurlock, 1999), can produce plants at a much higher ratio of parent plant to propagule than other methods (Xue et al., 2015) and improve the rapid deployment and upscaling of new varieties. Therefore, improved understanding of the seed biology and agronomy associated with implementing seed-based *Miscanthus* crops is vital for successful deployment of *Miscanthus* as a crop. Sowing seed directly in the field is commercially cheaper than sowing in a nursery and then planting plugs of the crop (Anderson et al., 2015), as is used with some fruit and vegetable crops (Marr & Jirak, 1990; Poling, 1993). Plug planting is the current focus in *Miscanthus* as the cost is close to that of rhizome planting, and plug planting allows up-scaling of new seed based varieties (Clifton-Brown et al., 2016). By understanding, testing, and modelling the germination and early establishment of *Miscanthus* from seed, this study aimed to expand and parameterise the existing knowledge in this area. This research provides improved understanding of *Miscanthus* seed establishment potentially reducing the costs associated with crop establishment, and improving commercial opportunities in *Miscanthus*.

PHYSICAL EFFECTS

Water and temperature are major physical factors affecting the germination of seed for many species. In order to understand the germination of *Miscanthus*, experiments explored the impact of temperature and water on germination. It has been suggested that *Miscanthus* requires a high optimal temperature of 25°C for germination (Aso, 1976) and high base temperatures for growth (Clifton-Brown et al., 2011). It has also been suggested that *Miscanthus* seed would require irrigation to grow down to soil moisture when germinated in dry environments (Anderson et al., 2015). This study demonstrates *Miscanthus* can establish in the UK climate from seed but confirms soil moisture is a major factor affecting germination success. Endorsing existing findings from Aso (1976) the optimal temperature for germination in most genotypes tested was 25°C. However, base temperatures for many seed based hybrids were lower than those previously reported in *M. sinensis* by Clifton-Brown et al. (2011). Water severely limited germination in the field (Section 6.3a), both in Aberystwyth (West UK), where early sowings were onto dry soil, and in Blankney (East UK), where the climate was drier resulting in fewer surviving plots. When examined in a small-scale experiment (Section 6.3d) water dramatically reduced germination at around the 20% soil water level or lower. Base soil water was calculated to be 6.2%, and therefore if direct sowing in the field is to be successful the crop may require added hydration to successfully germinate confirming observations of Anderson et al. (2015).

The water available for the seed to germinate is also determined by the water potential of the soil; this is an issue particularly if *Miscanthus* is intended to be sown on marginal land, as 3.9% of European soils are affected by salt which represents a large potential area for cultivation of biomass crops (FAO, 2015). Saline environments may have a greater effect on seed germination than on the

growth of mature plants. It was found that seed could germinate in saline environments similar to those that affected growth of mature plants for example Stavridou et al. (2016) found an adult plant can grow at -1 MPa in NaCl; therefore, direct sowing may be extended to such stressed environments. Without the ion toxicity effect of NaCl, it was expected that polyethylene glycol (PEG) water stress would only affect the seeds by limiting water availability. While this was the case for PEG 8000, PEG 4000 had some effects in common with NaCl implying that the PEG 4000 was able to penetrate the seed and affect more than water potential alone. There was some expectation that smaller molecular mass PEGs could leach into the sample (Lagerwerff et al., 1961; Lawlor, 1970).

Light is another important physical factor for germination and breaking dormancy in many species. It was expected that light would not be required for breaking dormancy in *M. sinensis* (Christian et al., 2014); it was discovered that although as expected *M. sinensis* genotypes did not require light for germination, light did play a significant part in germination of *M. sacchariflorus* seed. This effect was less in a *M. sacchariflorus* × *M. sinensis* hybrid but not totally absent, showing a distinct species effect of light as was suggested by Ellis et al. (1989) in *Lactuca sativa*.

In addition to any part played in germination, light is clearly important for plant growth, and higher light intensity early after germination was expected improve seedling growth. This may also improve root growth, because the plant would acquire adequate light from fewer leaves allowing it to allocate more resources for root development. The Taguchi experiment (Section 4.4d) unexpectedly showed reduced light improved all the metrics used to determine germination and seedling success. While this result merits further investigation because it could have been due to lower temperature under lower light, or insulation from

the neutral density filter, it is a notable finding and could indicate inhibition of seedling germination and growth from strong light.

CHEMICAL EFFECTS

To go beyond the main physical effects and understand the germination of *Miscanthus* in relation to hormones, the broad effects on seed germination and early growth of widely used hormones were assessed. The hormones chosen to test were abscisic acid (ABA), gibberellic acid (GA), auxin, and brassinosteroid (BR). There was a general hypothesis that *Miscanthus* seed would react to hormone treatment in a similar way to other seed tested in the literature.

ABA is well known to maintain seed dormancy in many species (Baskin & Baskin, 2004; Finch-Savage & Leubner-Metzger, 2006; Grappin et al., 2000; Shu et al., 2016). When observed in an experiment to determine the active range of ABA over which germination was affected (Chapter 4), it was concluded that germination did decrease with ABA concentration and time to germination increased, and there was a small and unpredicted benefit to stem elongation of small quantities of ABA of less than 5 mg L⁻¹. However, although the trend was consistent the impact of ABA was not statistically significant.

ABA and GA are often perceived as working in opposition to control dormancy (Baskin & Baskin, 2004; Cadman et al., 2006; Shu et al., 2016; Steber & McCourt, 2001), in this role GA should down-regulate seed dormancy, and promote stem growth (Aso, 1976; Yaldagard et al., 2008). There was no detectable positive effect on germination from GA treatment across a range of concentrations (chapter 4). This may indicate a lack of dormancy, which was unexpected as previous studies on *Miscanthus* with GA had shown an improvement in germination in GA treated seed, indicating the presence of dormancy in *M. sinensis* (Christian et al., 2014). There was a large increase to stem lengths

for even small concentrations (1 mg L^{-1}). The long stemmed seedlings affected by GA were weak as had been reported by Aso (1976).

Auxin was hypothesised to positively affect root growth (Müssig et al., 2003). While there was a small positive effect on root growth from auxin (Chapter 4), there was a greater positive effect on stem elongation, and while both effects were lost at higher concentrations of auxin, the root effect disappeared at a 1000x lower concentration. Auxin may have increased root hair production allowing shorter roots to be more effective allowing stem elongation.

It was predicted that BR would inhibit root elongation (Steber & McCourt, 2001). BR can also promote germination because BR can either stimulate or enhance the signalling of GA (Kucera et al., 2005; Shu et al., 2016). After treatment with a range of different concentrations of BR, root growth in *Miscanthus* was inhibited by BR and germination was slowed at higher concentrations (Chapter 4), possibly because GA did not have the expected increase on germination.

OPTIMISATION OF GERMINATION AND EARLY GROWTH

The next step was to determine the optimal conditions for in-laboratory germination, this was done using the Taguchi method (Taguchi, 1986); this tested combinations of physical factors and hormone effects on germination using a Taguchi orthogonal array. The Taguchi analysis was done in conjunction with Dr Sreenivas Rao Ravella. The activities of hormones often balance each other to provide tipping points (Karssen & Lacka, 1986), such as is often seen with activities of ABA and GA in germination (Cadman et al., 2006; Shu et al., 2016; Steber & McCourt, 2001), therefore it is useful to test them in combinations. The experiment in Chapter 4 tested the four hormones each at four levels (from very low to very high concentrations) and three physical factors (water stress

[low / moderate], priming [primed / control], light [high / low]) at two levels. This determined the optimal levels for eight metrics of seed success and one combination of all metrics. The unexpected outcomes of this were the low effect that GA had on influencing germination, indicating a lack of dormancy, and that there was a greater effect from BR than expected, supporting a regulatory effect for BR (Kucera et al., 2005; Shu et al., 2016). GA hardly affected stem elongation at all, with ABA dominating this effect.

The next step was to optimise the seed before germination, by stratifying the seed, storing the seed cold, and priming the seed.

Priming was included in the Taguchi experiment because this treatment should have positive effects on germination and performance of the seed (Ellis & Butcher, 1988; Elsoms Seeds, 2013), while increasing seed ageing (Hacisalihoglu et al., 1999), and possibly killing weaker seeds (Sathish et al., 2011). In the Taguchi experiment, it had a notable negative effect on germination percentage, median Fv/Fm chlorophyll fluorescence level and root elongation. The primed *Miscanthus* seed has been used elsewhere in this study, and while it is a popular treatment for commercial seed (Sathish et al., 2011) it was not proved useful in this study. In the other Chapter 4 priming experiments, priming showed no positive effects with some significant negative effects in fluorescence and root elongation. In Chapter 6, primed seed were tested in both field trials and on a thermal gradient plate. The thermal gradient experiment showed a mostly negative effect of priming seed on germination with some small increases in germination over unprimed seed at lower temperatures after 30 days. In the 'Multi Genotype Direct Sowing Trial', primed seed had significantly worse emergence than unprimed seed, while in the 'Direct Sowing Agronomy Trial', the primed seed had lower emergence at all sowing times and in film and no film plots. The plants from the primed seed in the trial had

significantly thinner stems and fewer tillers resulting in significantly lower harvest mass, as well as a higher plot failure rate.

It was expected that stratification would improve seed germination in a similar way to priming. Stratification is used to stop the seeds being dormant and is used in some crops such as Switchgrass (Walker, 2009). Seeds were stratified in Chapter 4 by keeping the seeds wet at a low sub-lethal temperature before either germinating them or drying the seed then germinating (Shen et al., 2001). Stratification had previously been shown to increase germination in *Miscanthus* seed (Christian et al., 2014). The stratification treatment was not entirely successful; there was not the expected increase in germination, but the time taken for the seed to germinate decreased in both the wet and dried seed stratification methods. The proportion of seedlings observed to be 'Thriving' by the end of the test were highest in 7 day stratified then dried seed.

The temperature the seed is kept at prior to germinating affects the viability of the seed (Ellis & Butcher, 1988). Seed was stored cold before germination or threshing, then germinated without priming or stratification (Chapter 4). It was hypothesised that storing the seed in the cold would improve germination. While this was not proven, there was a large increase in the variability of germination in stored seed and an increase in the mean percentage of germination.

Before germination seed could be sorted into sub sets by size, larger seeds would be expected to perform better, both producing healthier seedlings and having more resources to survive longer in periods of dark. This was not the case with seedlings assessed in the 'Dark Burnout Seed Testing' where larger seeds did not produce larger seedlings. When tested, seed status was affected by inactive seeds being significantly larger than seeds that were dead or that produced a seedling.

AGRONOMIC IMPROVEMENTS

This study tested important agronomic methods for improved seed establishment. By optimising the conditions in which the seed are sown, it was hoped that germination and establishment of *Miscanthus* could be brought to a commercially viable level. In-field temperature and water improvements were tested. Mulch film should increase water availability to the seed on even semi-arid soil (Zhou et al., 2009). It was also expected that the thermal boost mulch film would provide would help establishment in a cooler temperate climate such as in the UK (Farrell & Gilliland, 2011).

Film application was tested in two field trials on direct sown seed (Chapter 6). It was found that by applying mulch film field temperatures increased but the impact that mulch film had on soil water depended on whether the film was laid before or after wet periods. It was also expected that mulch film would affect the earliest sowing time by providing frost protection (Easson & Fearnough, 2000). Film did allow successful sowings later into the year but did not have the same effect on early sowings due to low starting soil moisture.

In the 'Direct Sowing Agronomy Trial' the largest plants were produced from seed sown in late-May for the colder site and early-May for the warmer site; in both locations the best survival of plots was in the sowing three weeks later than the sowing dates that produced the largest plants. In warmer locations this supports Clifton-Brown et al. (2011), who suggested a mid-April sowing to give seed adequate time to establish; however, sowing was not tested quite this early and the survival peak three weeks later suggests extensive oversowing may be needed to de-risk earlier sowings for farmers. The 'Direct Sowing Agronomy Trial' revealed that even in the warmer location the establishment was poor, as by the second year plants were less than 1 meter tall in the most successful sowing, and height was half that in the colder location. While this shows UK

direct sowing establishment is possible, the slow establishment also makes direct sowing *Miscanthus* seed in the UK an economic problem for farmers because economically harvestable yield will be delayed.

Hydraulic contact is important for seed germination, therefore sowing the seed into the soil should improve germination (Christian et al., 2005). However, by sowing the seed into the soil, there are more obstacles for the seedling to overcome before it can reach the surface to begin photosynthesis. *Miscanthus* seed was germinated in soil using a controlled environment to test the sowing methods in an ideal environment. To replicate observations in the field that seed had germinated well in tractor indents seed was sown within a groove in the field trials (Sections 6.2a & 6.2b), when this method was tested in a controlled environment seeds produced the same emergence as surface sowing but a smaller plant after 30 days (Section 6.3c). A light covering of soil lowered emergence, but resulted in no other significant effect. This suggested that while observations of germination pointed to a firm base being needed to stop the seed being washed into the soil, it is better if that base is on the surface rather than in a groove. In a water-limited environment, a light covering of soil may improve hydraulic contact.

Direct sowing trials of *Miscanthus* seed (Chapter 6) proved somewhat successful but lines of seed were patchy. Cluster-sowing seed as a means of oversowing was hypothesised to be a possible solution in field sowings. Depending on cluster size, this would improve the probability of at least one plant from each cluster surviving, and the plants produced would be evenly distributed based on a spaced sowing. However, it was also expected that the germinating seed could produce some allelopathic effects on nearby seed, and the plants may be weakened by germinating and growing in close proximity. This was investigated in Chapter 5; which concluded there was evidence for inhibitory effects of seed

within sown clusters from the in-water germination and lower germination percentages when seed was in clusters above 20, and evidence of root competition in soil in a controlled environment. Due to the current variability in the tested *Miscanthus* seed-based hybrid, sowing multiple seed did provide a better chance of a large plant. However, until other agronomic techniques or combinations are tried the numbers of seed required to ensure an acceptable probability of a plant in the field may be economically impractical.

MODELLING

Computational modelling could be used in *Miscanthus* germination, as in *Miscanthus* yield predictions (Clifton-Brown et al., 2000; Hastings et al., 2009a), to predict suitable sowing times, treatments, and locations. A version of the SimPIE model (SIMulation of PLant Emergence) (Dürr et al., 2001, 2003) was replicated and modified for *Miscanthus* and parametrised. The parametrisation used data from several experiments that identified the impacts of parameters affecting germination, primarily those conducted in Chapter 6. The purpose of this model was to estimate in particular the germination and emergence of seed but also the subsequent impact on early growth of *Miscanthus*.

The thermal requirements for germination of thirteen *Miscanthus* genotypes were parametrised from a thermal gradient plate experiment. A laboratory test provided the base soil-water content required to germinate *Miscanthus*. The thermal gradient elongation experiment on two genotypes parameterised early growth. The controlled environment cluster sowing into soil experiment provided later stem elongations. The basic functions of clods and soil surface crusting were added to the model but were not parameterised for *Miscanthus* as none of the sowings were under the soil surface. Further parametrisation can be added

to account for more effects of field sowing on the seed or the effect of sowing the seed below the soil surface.

The model was compared to germination on paper in controlled environment soil testing and the main field trials. Its performance at the three levels was mixed, it predicted germination percentage well on germination paper as this was similar to the parametrisation; however, the model overestimated emergence in the controlled environment soil after showing the correct rate of emergence early on, and the field models were low for many sowings. Real soil germination rates in soil, even in the wet controlled environment conditions, were lower than expected by the model, probably because of unaccounted variation in seed soil hydraulic contact. This was despite soil water parameterisation and a random death chance having been added into the model to limit emergence. More parameterisation may be needed to resolve these differences of scale fully. The field modelling also struggled with an environment with so many variables. Across all environments tested, the model was good at predicting germination/emergence; elongation could only be compared properly at one level for which it was accurate at the lower scale of less than 25 mm.

IMAGE ANALYSIS

The increasing use of image analysis for high throughput assessment of biological samples will become more widely used in germination testing. It was found in Chapter 3 and Section 4.2 that unassisted human counting lacked repeatability. This was particularly noticeable when monitoring the same seed over time, where the seed's status as germinated or not could fluctuate for slow growing seeds. Therefore, a technique to reproducibly score the germination of individual seeds over time was developed using an image-assisted method. Algorithmic methods for testing the point of germination for individual seeds

were developed in Chapter 3, these achieved ~70% trueness on an individual seed basis, the trueness was higher (~90%) with selected seed images (when compared to a human scorer). While this was not high enough for use in this study due to the low throughput of seeds, the methods would prove useful for evaluating the time individual seeds germinate in future research.

As well as trialling the methods in Chapter 3, it may also be useful to attempt to observe pre-germination signs in *Miscanthus* using a hyper-spectral camera (Eliceiri et al., 2012) or fluorescence, by detecting chemical changes in the seed prior to radicle emergence (Wagner et al., 2011). This could provide useful information concerning the seed's speed of reaction to different conditions, for example dormancy breaking treatments. It could also be useful for following up on observations made during the study, such as that inactive seeds were sometimes observed to fluoresce weakly (Chapter 4), which could indicate under ripe seed which would have higher levels of chlorophyll in the seed coat (Jalink et al., 1998).

Image analysis at a larger scale was tested in Chapter 3, as it was hypothesised it would be possible to take quick photographs of first year plots, which were accurate enough to predict second year dry weight as well as existing first year measurements. Field photographs and image analysis was found to be at least as accurate as the much more time-consuming stem and height counts.

7.2 RECOMMENDATIONS FOR FURTHER STUDIES

In order to best utilise and expand on this research in the future, there are some suggested experimental directions.

The image analyses experiments could be expanded upon. The seed image dataset provides a basis for testing more complex machine learning methods. The germination scoring methods used in this study could also be tested against empirical ground truths such as seedling establishment for the same seed set. The field pixel scoring on *Miscanthus* in this study was tested on plots, testing this method on individual first year plants may demonstrate its efficacy for future automation of biomass scoring.

The ideal levels for hormone effects on germination within a laboratory environment were optimised in this study. Investigation into hormones and their interactions in more complex soil conditions may help define methods for optimal sowing of *Miscanthus*, as the effects of hormones in soil conditions were not examined in this study. In addition to this, a deeper study is needed, either in the laboratory or in the field, in order to understand the interactions between GA and BR, and GA and auxin, as seen in the Taguchi experiment.

Screening a wide selection of *Miscanthus* seed for light dependency during germination should help to refine further the optimal germination conditions for differing *Miscanthus* genotypes. Genotypic analysis could aid in determining the extent of this dependency. An investigation into light level variations would also assist in explaining the effect seen in the Taguchi experiment, where lower light was optimal for germination and early growth. This could utilise chlorophyll fluorescence imaging to identify seedling response to light intensity. Additional experiments into the relationship between seed size and growth, and the longer-

term effect of this relationship on the plant produced, could aid in determining whether there is a practical advantage to not sowing larger seed, which were found in this study to have a larger likelihood of being inactive.

One element that has not been investigated in this study is the use of diurnal cycles to improve germination. This is a complex topic involving the interaction of many variables including temperature and light, and merits extensive exploration in itself. A large range of experiments would need to be carried out in order to determine fully the potential benefits of diurnal cycles in *Miscanthus*. A possible alternative method would be to design a Taguchi experiment similar to that used in this study. This would reduce the experimental overheads involved with investigating such a complex area.

Wider ranging field direct sowing methods could be investigated to address the issue of soil-seed contact and water availability. For example, hydroseeding (mixing seed with water and fibre than sowing as a slurry) which has showed promising results in *Miscanthus* (Anderson et al., 2015), or other methods of adding water to the seed when sown, have not been explored in this study. By testing pelleting of the seed using a water absorbing aggregate, it may be possible to solve the problem of hydraulic contact when *Miscanthus* is sown in the soil.

In addition, a longer-term study into cluster sowing seed could determine the number of seed required to ensure a successful plot, and if first year competition would affect harvest yield. Further experiments into direct sowing in the field could also lead to refinement of the model by supplementing the parameterisation, as more is understood about field germination. The model itself could be improved through the addition of parameters. For example, if drilling seed into the soil was considered, a measure of seedling emergence force could be

added to better estimate emergence. This could be done by expanding on the techniques of Tamet et al. (1996) and Dürr & Aubertot (2000). In the model, germination and establishment is largely effected by water; determining the germination of *Miscanthus* in more than the one soil type used in this study would improve the model and define the effect of changing the hydraulic contact.

DIRECT SOWING RECOMMENDATIONS

Plug planted *Miscanthus* is currently the most favoured technology for seed based hybrids (Clifton-Brown et al., 2016). Many of the findings in this study can inform the sowing of plug plants, but direct sowing remains a possible cheaper establishment method. In order to attempt direct sowing of *Miscanthus* seed in the UK it is recommended to facilitate the seeds' hydraulic contact with the soil, which is vital for effective germination particularly in field conditions. Therefore, seed should be sown on compacted ground to prevent loss of the seed and drainage of water. Current methods do this by sowing in grooves; this could be improved by adding a light top layer of soil and sowing on a compacted soil surface. This was not tested in water-limited soil, but both should increase the water availability in the field. Care should be taken when adding a layer of soil with *M. sacchariflorus* based seed hybrids as they may have some light requirement for optimal germination.

Sowings should also use film for improved establishment for the thermal increase, but care should be taken in not to sowing under film in dry conditions as this prevents the seed from accessing water from precipitation to replenish dry soil. Optimal time for seed sowing is in mid to late spring dependent on climate, in order to establish enough growth in the first year to allow successful overwintering.

Based on the findings of this study, seed should not be primed but storing the seed cold, possibly with a short period of stratification, could benefit the establishment from seed. The addition of hormones is difficult in the field, but with technology such as seed pelleting, a hormone beneficial to germination and growth such as auxin could be added to help stem elongation and possibly improve root hair growth.

FUTURE

Miscanthus uptake by farmers is low due to the financial uncertainty of the market (McCalmont et al., 2015) because there is a large degree of variation in yields and prices (Witzel & Finger, 2016). The harvest price for *Miscanthus* increased to £70 per tonne in 2013 and rises with demand and support for renewable energy (Farmers Guardian, 2013); this had risen to £74 per tonne by 2015 (Terravesta, 2017). Modelling of *M. x giganteus* has suggested that dry-matter yields over most of Europe would be over 15 Mg ha⁻¹ Y⁻¹ (Clifton-Brown et al., 2000). Due to the perennial nature of the crop, markets need to be stable long term for farmers to choose *Miscanthus* (Lewandowski et al., 2016). Terravesta (Lincoln, UK) is stabilising price fluctuations for farmers over the commercial life of the crop (Clifton-Brown et al., 2016). As prices rise, and more is known about the establishment and potential yield of *Miscanthus* as a bioenergy crop, the uncertainty should reduce; with associated costs such as transport falling due to technological improvements (Clifton-Brown et al., 2016). As usage of the crop increases more customised or custom made machinery will be economic to produce (Lewandowski et al., 2016). These factors should reduce the risks associated with *Miscanthus* and allow farmers to invest in the crop, which should in turn help create infrastructure to drive down the costs of growing what is currently a novel crop. Having more *Miscanthus* biomass supply

should also lead to more demand (McCalmont et al., 2015). This study forms a basis for further investigations into seed based *Miscanthus*.

KEY FINDINGS

This provides a brief summary of the key findings and impactful results found during this study, and outputs that could be used elsewhere.

IMAGE ANALYSIS OF GERMINATION

- Two automated methods of germination detection were developed, using FIJI alone and using FIJI with a k -Nearest Neighbour machine-learning algorithm added in R.
- High throughput image-based automated germination counting was successful at a level of around 70 to 90% trueness dependent of the level of mould.
- An image-assisted germination counting method was developed to improve repeatability if scoring individual seed.
- Seed size found by image area can be used as a proxy for seed mass. The combination of size and mass appears to be genotype dependent.
- Fluorescence imaging was very effective when using the blue germination paper – this can be used to count germination automatically.
- An image and data set has been produced in this study of 6,000 human scored seed germination time-sequences that can be used for future refinements to potential methodologies.

LIGHT DEPENDENCY ON GERMINATION

- There appears to be a species effect of light on germination with *M. sinensis* not responding to light while *M. sacchariflorus* requires light for germination.

- Seedlings survived for 10 plus days after being deprived of light from germination (growth stopped at 6~8 days).
- There appears to be a small boost to seedling health after being germinated in the dark for ~48 hours.
- Larger seeds did not grow into bigger seedlings without light, compared with smaller seed.
- Bigger *Miscanthus* seeds were on average more likely to be inactive.

WATER STRESS AND COLD STORAGE

- A water potential of -1 MPa limits germination and below -2.2 MPa stops germination.
- The concentration of NaCl required to stop germination was similar to the concentration required to stop adult *Miscanthus* from growing.
- Cold storage increased the variability in germination percentage.
- Stratification for 7 days with drying before germination decreased the mean time required for seeds to germinate.

HORMONE EFFECTS ON GERMINATION

- Abscisic acid reduced germination and increased stem length.
- Gibberellic acid alone did not significantly affect germination in *Miscanthus*, but did disproportionately increase stem length.
- Auxin treated seedlings did not produce longer roots.
- Small quantities of auxin increased stem length.
- Brassinosteroid negatively affected root growth.
- The optimal conditions for germination and early growth were produced using an efficient Taguchi design, for hormones, water stress, light, and priming.

DIRECT SOWING OF *MISCANTHUS* SEED

- *Miscanthus* appears to compete for resources when sown in clusters, and may inhibit the germination of seeds sharing the same medium.
- Sowing 40 *M. sinensis* seeds in a cluster was not enough to ensure a plant.
- Mid-August was found to be the latest date that seed could be sown in an Aberystwyth climate that would allow plants to overwinter.
- Water appears to limit germination more than temperature when sown in the UK in spring/summer.

SEED PRIMING

- Priming was found to have little to no positive effect.

SOWING METHODS

- Film improves germination/tillering in sowings in wet conditions.
- The film does overwinter, because solar radiation is lower.
- Film can extend the sowing times later into the year, but not over winter with a spring emergence.
- Film may not extend the growing season forwards for direct sown *Miscanthus* despite frost protection.
- Film can result in fewer plants if sowing is done under dry conditions.
- Surface-sown seed produced larger plants than groove-sown seed in a controlled environment.

Soil covering did significantly reduce germination/emergence percentage but did not affect dry weight.

MINIMUM REQUIREMENTS FOR GERMINATION

- A base water requirement of 6.2% W/V was found for *Miscanthus* seed to germinate in soil.
- Base temperature for germination was found to be 7.7°C on average over 13 *Miscanthus* genotypes.
- 25°C was confirmed to be on average the optimum temperature for *Miscanthus* seed germination.

MODELLING

- A version of the SimPIE model was produced and parameterised for a range of *Miscanthus* genotypes.
- The model with UI will be available to use and improve with more data sets, this collaborative process should help *Miscanthus* agronomic modelling more broadly.
- The model predicted field germination, controlled environment germination, and laboratory germination with an R^2 of 0.73.

REFERENCES

- Abadikhahdeh Ali, D., & Gholam, R. Z. (2011). Effect of cyanide potassium on sorghum and wheat germination. In *IPCBE International conference on environment and industrial innovation* (Vol. 12). Singapore: IACSIT Press.
- Abràmoff, M. D., Magalhães, P. J., & Ram, S. J. (2004). Image Processing with ImageJ. *Biophotonics International*, 11(7), 36–41.
- Achigan-Dako, E. G., Avohou, E. S., Linsoussi, C., Ahanchede, A., Vodouhe, R. S., & Blattner, F. R. (2015). Phenetic characterization of *Citrullus* spp. (Cucurbitaceae) and differentiation of egusi-type (*C. mucosospermus*). *Genetic Resources and Crop Evolution*, 62(8), 1159–1179.
- Achigan-Dako, E. G., Fuchs, J., Ahanchede, A., & Blattner, F. R. (2008). Flow cytometric analysis in *Lagenaria siceraria* (Cucurbitaceae) indicates correlation of genome size with usage types and growing elevation. *Plant Systematics and Evolution*, 276(1–2), 9–19.
- Acosta, J. M., Bentivegna, D. J., Panigo, E. S., Dellaferrera, I., & Perreta, M. G. (2013). Factors affecting seed germination and emergence of *Gomphrena perennis*. *Weed Research*, 53(1), 69–75.
- 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(5), 1075–1083.
- Anderson, E. K., Voigt, T. B., Bollero, G. A., & Hager, A. G. (2010). *Miscanthus × giganteus* Response to Preemergence and Postemergence Herbicides. *Weed Technology*, 24(4), 453–460.
- Andrews, T. S., Jones, C. E., & Whalley, R. D. B. (1997). Factors affecting the germination of Giant Parramatta grass. *Australian Journal of Experimental Agriculture*, 37, 439–446.
- Angus, A., Burgess, P. J., Morris, J., & Lingard, J. (2009). Agriculture and land use: Demand for and supply of agricultural commodities, characteristics of the farming and food industries, and implications for land use in the UK. *Land Use Policy*, 26, S230–S242.
- Arif, M., Jan, M., Marwat, K. B., & Khan, M. A. (2008). Seed Priming Improves Emergence and Yield of Soybean. *Pakistan Journal of Botany*, 40(3), 1169–1177.
- Arnoult, S., & Brancourt-Hulmel, M. (2015). A Review on *Miscanthus* Biomass Production and Composition for Bioenergy Use: Genotypic and Environmental Variability and Implications for Breeding. *BioEnergy Research*, 8(2), 502–526.
- Arundale, R. A., Dohleman, F. G., Heaton, E. A., Mcgrath, J. M., Voigt, T. B., & Long, S. P. (2014). Yields of *Miscanthus × giganteus* and *Panicum virgatum* decline with stand age in the Midwestern USA. *GCB Bioenergy*, 6(1), 1–13.
- Aso, T. (1976). Studies on the germination of seeds of *Miscanthus sinensis* Anderss. *Science Reports of the Yokohama National University. Section II, Biological and Geological Sciences*, 23, 27–37.
- Ayott, M., & McDermott, F. (2012). *Domestic Energy Crops; Potential and Constraints Review*. (L. Hopwood, Ed.) NNFFCC. York.

- Bais, H. P., Vepachedu, R., Gilroy, S., Callaway, R. M., & Vivanco, J. M. (2003). Allelopathy and exotic plant invasion: from molecules and genes to species interactions. *Science*, *301*(5638), 1377–80.
- Bajji, M., Kinet, J.-M., & Lutts, S. (2002). Osmotic and ionic effects of NaCl on germination, early seedling growth, and ion content of *Atriplex halimus* (Chenopodiaceae). *Canadian Journal of Botany*, *80*(3), 297–304.
- Bao, F., Shen, J., Brady, S. R., Muday, G. K., Asami, T., & Yang, Z. (2004). Brassinosteroids interact with auxin to promote lateral root development in *Arabidopsis*. *Plant Physiology*, *134*(4), 1624–1631.
- Barney, J. N., & DiTomaso, J. M. (2008). Nonnative Species and Bioenergy: Are We Cultivating the Next Invader? *BioScience*, *58*(1), 64–70.
- Barney, J. N., Mann, J. J., Kyser, G. B., & DiTomaso, J. M. (2012). Assessing habitat susceptibility and resistance to invasion by the bioenergy crops switchgrass and *Miscanthus × giganteus* in California. *Biomass and Bioenergy*, *40*, 143–154.
- Barua, D., Butler, C., Tisdale, T. E., & Donohue, K. (2012). Natural variation in germination responses of *Arabidopsis* to seasonal cues and their associated physiological mechanisms. *Annals of Botany*, *109*(1), 209–226.
- Baskin, J. M., & Baskin, C. C. (2004). A classification system for seed dormancy. *Seed Science Research*, *14*(1).
- Bates, D. M., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using {lme4}. *Journal of Statistical Software*, *67*(1), 1–48.
- Belin, C., Megies, C., Hauserová, E., & Lopez-Molina, L. (2009). Abscisic acid represses growth of the *Arabidopsis* embryonic axis after germination by enhancing auxin signaling. *The Plant Cell*, *21*(8), 2253–68.
- Benor, S., Fuchs, J., & Blattner, F. R. (2011). Genome size variation in *Corchorus olitorius* (Malvaceae s.l.) and its correlation with elevation and phenotypic traits. *Genome*, *54*(7), 575–585.
- Bewley, J. D. (1997a). Breaking down the walls - A role for endo- β -mannanase in release from seed dormancy? *Trends in Plant Science*, *2*(12), 464–469.
- Bewley, J. D. (1997b). Seed germination and dormancy. *The Plant Cell*, *9*(July), 1055–1066.
- Bilder, C. R., & Loughin, T. M. (2014). *Analysis of categorical data with R*. CRC Press.
- Bishop, C. M. (2006). *Pattern Recognition and Machine Learning*. (M. Jordan, J. Kleinberg, & B. Scholkopf, Eds.). New York: Springer Science.
- Boakye-boaten, N. A., Kurkalova, L., & Xiu, S. (2017). Biomass and Bioenergy Techno-economic analysis for the biochemical conversion of *Miscanthus x giganteus* into bioethanol. *Biomass and Bioenergy*, *98*, 85–94.
- Bollen, K. A., & Jackman, R. W. (1985). Regression Diagnostics: An Expository Treatment of Outliers and Influential Cases. *Sociological Methods & Research*, *13*(4), 257–291.
- Borza, J. K., Westerman, P. R., & Liebman, M. (2007). Comparing Estimates of Seed Viability in Three Foxtail (*Setaria*) Species Using the Imbibed Seed Crush Test with and Without Additional Tetrazolium Testing. *Weed Technology*, *2*(21), 518–522.
- Bradford, K. J. (1990). A water relations analysis of seed germination rates. *Plant Physiology*, *94*(2), 840–849.

References

- Brady, N. C. (1990). *The nature and properties of soils*. (10th ed.). New York: Macmillan publishing company.
- Brown, R. F., & Mayert, D. G. (1988). Representing Cumulative Germination. 1. A Critical Analysis of Single-value Germination Indices. *Annals of Botany*, *61*, 117–125.
- Brunel-Muguet, S., Aubertot, J.-N., & Dürr, C. (2011). Simulating the impact of genetic diversity of *Medicago truncatula* on germination and emergence using a crop emergence model for ideotype breeding. *Annals of Botany*, *107*(8), 1367–76.
- Brunel, S., Teulat-Merah, B., Wagner, M.-H. M.-H., Huguet, T., Prosperi, J. M., & Dürr, C. (2009). Using a model-based framework for analysing genetic diversity during germination and heterotrophic growth of *Medicago truncatula*. *Annals of Botany*, *103*(7), 1103–17.
- Burgass, R. W., & Powell, A. A. (1984). Evidence for Repair Processes in the Invigoration of Seeds by Hydration. *Annals of Botany*, *53*, 753–757.
- Cadman, C. S. C., Toorop, P. E., Hilhorst, H. W. M., & Finch-Savage, W. E. (2006). Gene expression profiles of *Arabidopsis Cvi* seeds during dormancy cycling indicate a common underlying dormancy control mechanism. *The Plant Journal*, *46*(5), 805–822.
- Caffarra, A., Donnelly, A., Chuine, I., & Jones, M. B. (2011). Modelling the timing of *Betula pubescens* budburst. I. Temperature and photoperiod: A conceptual model. *Climate Research*, *46*(2), 147–157.
- Cai, Q., Aitken, K. S., Fan, Y. H. H., Piperidis, G., Jackson, P., & McIntyre, C. L. L. (2005). A preliminary assessment of the genetic relationship between *Erianthus rockii* and the “*Saccharum complex*” using microsatellite (SSR) and AFLP markers. *Plant Science*, *169*(5), 976–984.
- Caslin, B., Finnan, J., & Eason, L. (2011). *Miscanthus Best Practice Guidelines*. Agriculture and Food Development Authority: Prepared by Teagasc and the Agri-Food and Bioscience Institute.
- Chang-Hung, C., & Yi-Feng, L. (1991). Allelopathic Dominance of *Miscanthus Transmorrisonensis* in An Alpine Grassland Community In Taiwan. *Journal of Chemical Ecology*, *17*(11), 2267–2281.
- Chou, C.-H., & Chung, Y.-T. (1974). The Allelopathetic Potential of *Miscanthus Floridulus*. *Botanical Bulletin of Academia Sinica*, *15*, 14–27.
- Christian, D. G., Yates, N. E., & Riche, A. B. (2005). Establishing *Miscanthus sinensis* from seed using conventional sowing methods. *Industrial Crops and Products*, *21*(1), 109–111.
- Christian, E. J., Goggi, A. S., & Moore, K. J. (2014). Temperature and Light Requirements for *Miscanthus sinensis* Laboratory Germination Test. *Crop Science*, *54*, 1–5.
- Clark, R. T., Famoso, A. N., Zhao, K., Shaff, J. E., Craft, E. J., Bustamante, C. D., ... Kochian, L. V. (2013). High-throughput two-dimensional root system phenotyping platform facilitates genetic analysis of root growth and development. *Plant, Cell and Environment*, *36*(2), 454–466.
- Clifton-Brown, J. C., Breuer, J., & Jones, M. B. (2007). Carbon mitigation by the energy crop, *Miscanthus*. *Global Change Biology*, *13*(11), 2296–2307.
- Clifton-Brown, J. C., Hastings, A. F., Mos, M., McCalmont, J. P., Ashman, C., Awty-Carroll, D., ... Flavell, R. (2016). Progress in upscaling *Miscanthus* biomass production for the European bio-economy with seed based hybrids. *GCB Bioenergy*, 1–12.
- Clifton-Brown, J. C., & Jones, M. B. (1997). The thermal response of leaf extension rate in genotypes of the C 4 –grass *Miscanthus* : an important factor in determining the potential productivity of different genotypes. *Journal of Experimental Botany*, *48*(8), 1573–1581.

- Clifton-Brown, J. C., & Lewandowski, I. (2000). Overwintering problems of newly established *Miscanthus* plantations can be overcome by identifying genotypes with improved rhizome cold tolerance. *New Phytologist*, *148*(2), 287–294.
- 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*(2), 97–110.
- Clifton-Brown, J. C., Lewandowski, I., Andersson, B., Basch, G., Christian, D. G., Kjeldsen, J. B., ... Teixeira, F. (2001). Performance of 15 *Miscanthus* Genotypes at Five Sites in Europe. *Agronomy Journal*, *93*, 1013–1019.
- Clifton-Brown, J. C., Lewandowski, I., & Jones, M. B. (2000). *MiscanMod: a model for estimating biomass production from Miscanthus throughout Europe*.
- Clifton-Brown, J. C., Robson, P. R. H., Sanderson, R., Hastings, A. F., Valentine, J., & Donnison, I. S. (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*(5), 375–386.
- Clouse, S. D., Sasse, J. M., Carolina, N., & Sasse, J. M. (1998). BRASSINOSTEROIDS: Essential Regulators of Plant Growth and Development. *Annual Review of Plant Physiology and Plant Molecular Biology*, *49*(1), 427–451.
- Constantin, J., Dürr, C., Tribouillois, H., & Justes, E. (2015). Catch crop emergence success depends on weather and soil seedbed conditions in interaction with sowing date: a simulation study using the SIMPLE emergence model. *Field Crops Research, Submitted*, 22–33.
- Covell, S., Ellis, R. H., Roberts, E. H., & Summerfield, R. J. (1986). The Influence of Temperature on Seed Germination Rate in Grain Legumes. *Journal of Experimental Botany*, *37*(178), 705–715.
- Cover, T. M., & Hart, P. E. (1967). Nearest Neighbor Pattern Classification. *IEEE*, *13*(1), 21–27.
- Davison, A. C., & Hinkley, D. V. (1997). *Bootstrap methods and their application* (Vol. 1). Cambridge university press.
- De Jong, S. M., Pebesma, E. J., & Lacaze, B. (2003). Above-ground biomass assessment of Mediterranean forests using airborne imaging spectrometry: the DAIS Payne experiment. *International Journal of Remote Sensing*, *24*(7), 1505–1520.
- de Kroon, H., Mommer, L., & Nishiwaki, A. (2003). Root Competition: Towards a Mechanistic Understanding. In H. de Kroon & E. J. W. Visser (Eds.), *Root Ecology* (Vol. 168, pp. 215–234). Berlin, Heidelberg: Springer Berlin Heidelberg.
- de Vries, S. C., van de Ven, G. W. J., & van Ittersum, M. K. (2014). First or second generation biofuel crops in Brandenburg, Germany? A model-based comparison of their production-ecological sustainability. *European Journal of Agronomy*, *52*, 166–179.
- DEFRA. (2007). *Planting and Growing Miscanthus. Policy for Energy Crops Scheme*. London.
- DEFRA. (2013). *Area of Crops Grown For Bioenergy in England and the UK: 2008-2011. Government Statistical Service*.
- Dell' Aquila, A. (2004). Application of a computer-aided image analysis system to evaluate seed germination under different environmental conditions. *Italian Journal of Agronomy*, *5583400*, 51–62.
- Dell' Aquila, A. (2005). The use of image analysis to monitor the germination of seeds of broccoli (*Brassica oleracea*) and radish (*Raphanus sativus*). *Genetics*, 1–6.

References

- Dell' Aquila, A. (2009). Digital imaging information technology applied to seed germination testing. A review. *Agronomy for Sustainable Development*, 29(1), 213–221.
- Department of Trade and Industry. (2007). Economic analysis of biomass energy. *UK Biomass Strategy 2007*, 1–82.
- Deuter, M. (2000). Breeding approaches to improvement of yield and quality in *Miscanthus* grown in Europe. In I. Lewandowski & J. Clifton-Brown (Eds.), *European Miscanthus Improvement* (pp. 28–52). University of Hohenheim, Germany.
- Dodd, G. L., & Donovan, L. A. (1999). Water Potential and Ionic Effects on Germination and Seedling Growth of Two Cold Desert Shrubs. *American Journal of Botany*, 86(8), 1146–1153.
- Dong, Y., Kamiuten, H., Yang, Z., Lin, D., Ogawa, T., Luo, L., & Matsuo, H. (2006). Mapping of quantitative trait loci for gibberellic acid response at rice (*Oryza sativa* L.) seedling stage. *Plant Science*, 170(1), 12–17.
- Dorsainvil, F., Dürr, C., Justes, E., & Carrera, A. (2005). Characterisation and modelling of white mustard (*Sinapis alba* L.) emergence under several sowing conditions. *European Journal of Agronomy*, 23(2), 146–158.
- Ducournau, S., Feutry, A., Plainchault, P., Revollon, P., Vigouroux, B., & Wagner, M.-H. (2005). Using computer vision to monitor germination time course of sunflower (*Helianthus annuus* L.) seeds. *Seed Science and Technology*, 33(2), 329–340.
- Ducournau, S., Feutry, A., Plainchault, P., Revollon, P., Vigouroux, B., & Wagner, M.-H. M.-H. (2004). An image acquisition system for automated monitoring of the germination rate of sunflower seeds. *Computers and Electronics in Agriculture*, 44(3), 189–202.
- Dürr, C., & Aubertot, J.-N. (2000). Emergence of seedlings of sugar beet (*Beta vulgaris* L.) as affected by the size, roughness and position of aggregates in the seedbed. *Plant and Soil*, 219, 211–220.
- Dürr, C., Aubertot, J.-N., Dubrulle, P., Richard, G., Dorsainvil, F., Justes, E., ... Boiffin, J. (2003). SIMPLE (SIMulation of PLant Emergence): a model for predicting crop emergence. In G. Nicolas (Ed.), *Biology of seeds: Recent Research Advances. 7th International Workshop on Seed Biology* (pp. 225–231). Salamanca, Spain: CABI.
- Dürr, C., Aubertot, J.-N., Richard, G., Dubrulle, P., Duval, Y., & Boiffin, J. (2001). Division S-6-Soil & Water Management & Conservation - SIMPLE : A Model for SIMulation of PLant Emergence Predicting the Effects of Soil Tillage and Sowing Operations. *Soil Science Society of America Journal*, 65, 414–423.
- Dürr, C., & Boiffin, J. (1995). Sugarbeet seedling growth from germination to first leaf stage. *Journal of Agricultural Science*, 124, 427–435.
- Easson, D. L., & Fearnough, W. (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(3), 221–231.
- Eliceiri, K. W., Berthold, M. R., Goldberg, I. G., Ibáñez, L., Manjunath, B. S., Martone, M. E., ... Carpenter, A. E. (2012). Biological imaging software tools. *Nature Methods*, 9(7), 697–711.
- Ellis, R. H., & Butcher, P. D. (1988). The Effects of Priming and “Natural” Differences in quality amongst Onion seed lots on the response of the rate of germination to temperature and the Identification of the characteristics under genotypic control. *Journal of Experimental Botany*, 39(204), 935–950.

- Ellis, R. H., Hong, T. D., & Roberts, E. H. (1985a). *Handbook of seed technology for genebanks. Volume I. Principles and methodology. Handbooks for Genebanks No. 2, International Board for Plant Genetic Resources.*
- Ellis, R. H., Hong, T. D., & Roberts, E. H. (1985b). Handbook of seed technology for genebanks. Volume II. Compendium of specific germination information and test recommendations. In *Handbook of seed technology for genebanks. Volume II. Compendium of specific germination information and test recommendations.* (pp. 210–667).
- Ellis, R. H., Hong, T. D., & Roberts, E. H. (1989). Response of Seed Germination in Three Genera of Compositae to White Light of Varying Photon Flux Density and Photoperiod. *Journal of Experimental Botany*, 40(210), 13–22.
- Ellis, R. H., Simon, G., & Covell, S. (1987). The Influence of Temperature on Seed Germination Rate in Grain Legumes. *Journal of Experimental Botany*, 38(191), 1033–1043.
- Elsoms Seeds. (2013). Seed Treatments and Services Guide. *Seed Treatments and Services Guide.* Retrieved July 16, 2013, from <http://www.elsoms.com>
- Ervin, G. N., & Wetzel, R. G. (2000). Allelochemical autotoxicity in the emergent wetland macrophyte *Juncus effusus* (Juncaceae). *American Journal of Botany*, 87(6), 853–860.
- Evans, M. L., & Ray, P. M. (1969). Timing of the Auxin Response in Coleoptiles and Its Implications Regarding Auxin Action. *The Journal of General Physiology*, 53, 1–20.
- Everman, W. J., Lindsey, A. J., Henry, G. M., Glaspie, C. F., Phillips, K., Mckenney, C., & Phillips, K. (2011). Response of *Miscanthus × giganteus* and *Miscanthus sinensis* to Postemergence Herbicides. *Weed Technology*, 25(3), 398–403.
- FAO. (2015). Food and Agriculture Organization of the United Nations. *FAO SOILS PORTAL.* Retrieved December 1, 2016, from www.fao.org/soils-portal/soil-management/management-of-some-problem-soils/salt-affected-soils/en/
- Farmers Guardian. (2008a, March 21). Finding the best maize type. *Farmers Guardian*, p. 79.
- Farmers Guardian. (2008b, March 21). New varieties and plastic help marginal maize production plus an earlier harvest. *Farmers Guardian*, p. 79.
- Farmers Guardian. (2013, February 21). Price for energy crop miscanthus at all-time high. *Farmers Guardian*.
- Farrell, A. D., & Gilliland, T. J. (2011). Yield and quality of forage maize grown under marginal climatic conditions in Northern Ireland. *Grass and Forage Science*, 66(2), 214–223.
- Fawcett, T. (2006). An introduction to ROC analysis. *Pattern Recognition Letters*, 27(8), 861–874.
- Finch-Savage, W. E., & Leubner-Metzger, G. (2006). Seed dormancy and the control of germination. *New Phytologist*, 171(3), 501–23.
- Fonteyne, S., Muylle, H., De Swaef, T., Reheul, D., Roldán-Ruiz, I., & Lootens, P. (2016). How low can you go?—Rhizome and shoot frost tolerance in miscanthus germplasm. *Industrial Crops and Products*, 89, 323–331.
- Forcella, F., Benech Arnold, R. L., Sanchez, R., & Ghera, C. M. (2000). Modeling seedling emergence. *Field Crops Research*, 67(2), 123–139.
- French, A. N., Ubeda-Tomás, S., Holman, T. J., Bennett, M. J., & Pridmore, T. (2009). High-Throughput Quantification of Root Growth Using a Novel Image-Analysis Tool. *Plant Physiology*, 150(August), 1784–1795.

References

- Garcia-Huidobro, J., Monteith, J. L., & Squire, G. R. (1982). Time, Temperature and Germination of Pearl Millet (*Pennisetum typhoides* S. & H.). *Journal of Experimental Botany*, *33*(133), 288–296.
- Gardarin, A., Guillemin, J.-P., Munier-Jolain, N. M., & Colbach, N. (2010). Estimation of key parameters for weed population dynamics models: Base temperature and base water potential for germination. *European Journal of Agronomy*, *32*(2), 162–168.
- Gegas, V. C., Nazari, A., Griffiths, S., Simmonds, J., Fish, L., Orford, S., ... Snape, J. W. (2010). A genetic framework for grain size and shape variation in wheat. *The Plant Cell*, *22*(4), 1046–1056.
- Geneve, R. L., & Kester, S. T. (2001). Evaluation of Seedling Size Following Germination Using Computer-aided Analysis of Digital Images from a Flat-bed Scanner. *HortScience*, *36*(6), 1117–1120.
- Glasbey, C. A., & Horgan, G. W. (1995). *Image analysis for the biological sciences*. Wiley Chichester (1st ed., Vol. 1). New York: John Wiley & Sons, Inc.
- Godfray, H. C. J., Crute, I. R., Haddad, L., Lawrence, D., Muir, J. F., Nisbett, N., ... Whiteley, R. (2010). The future of the global food system. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *365*(1554), 2769–77.
- gov.uk. (2015). Power stations in the United Kingdom, May 2015. gov.uk. Retrieved from www.energy-uk.org.uk/energy-industry/coal-generation.html
- Grappin, P., Bouinot, D., Sotta, B., Miginiac, E., & Jullien, M. (2000). Control of seed dormancy in *Nicotiana glauca*: post-imbibition abscisic acid synthesis imposes dormancy maintenance. *Planta*, *210*, 279–285.
- Greco, M., Chiappetta, A., Bruno, L., & Bitonti, M. B. (2012). In *Posidonia oceanica* cadmium induces changes in DNA methylation and chromatin patterning. *Journal of Experimental Botany*, *63*(2), 695–709.
- Greef, J. M., & Deuter, M. (1993). Syntaxonomy of *Miscanthus × giganteus* Greef et Deu. *Angewandte Botanik*, *67*, 87–90.
- GTA Sensorik GmbH. (n.d.). MARVIN – New Analyser/Counter for Vegetable and Flower Seeds.
- Gummerson, R. J. (1986). The Effect of Constant Temperatures and Osmotic Potentials on the Germination of Sugar Beet. *Journal of Experimental Botany*, *37*(179), 729–741.
- Hacisalihoglu, G., Taylor, A. G., Paine, D. H., Hilderbrand, M. B., & Khan, A. A. (1999). Embryo elongation and germination rates as sensitive indicators of lettuce seed quality: Priming and aging studies. *American Society for Horticultural Science*, *34*(7), 1240–1243.
- Hastings, A. F., Clifton-Brown, J. C., Wattenbach, M., Mitchell, C. P., & Smith, P. (2009a). The development of MISCANFOR, a new *Miscanthus* crop growth model: towards more robust yield predictions under different climatic and soil conditions. *GCB Bioenergy*, *1*(2), 154–170.
- Hastings, A. F., Clifton-Brown, J. C., Wattenbach, M., Mitchell, C. P., Stampfl, P., & Smith, P. (2009b). Future energy potential of *Miscanthus* in Europe. *GCB Bioenergy*, *1*(2), 180–196.
- Heaton, E. A., Boersma, N., Caveny, J. D., Voigt, T. B., & Dohleman, F. G. (2012). *Miscanthus* (*Miscanthus × giganteus*) for Biofuel Production. *Iowa State University: University Extension*. Retrieved September 13, 2013, from <http://www.extension.org/pages/26625/miscanthus-miscanthus-x-giganteus-for-biofuel-production>
- Heaton, E. A., Dohleman, F. G., & Long, S. P. (2008). Meeting US biofuel goals with less land: the potential of *Miscanthus*. *Global Change Biology*, *14*(9), 2000–2014.

- Heaton, E. A., Voigt, T. B., & Long, S. P. (2004). A quantitative review comparing the yields of two candidate C4 perennial biomass crops in relation to nitrogen, temperature and water. *Biomass and Bioenergy*, 27(1), 21–30.
- Hijmans, R. J. (2016). raster: Geographic Data Analysis and Modeling.
- Hodgson, E. M., Lister, S. J., Bridgwater, A. V., Clifton-Brown, J. C., & Donnison, I. S. (2010). Genotypic and environmentally derived variation in the cell wall composition of *Miscanthus* in relation to its use as a biomass feedstock. *Biomass and Bioenergy*, 34(5), 652–660.
- Hodkinson, T. R., & Renvoize, S. A. (2001). Nomenclature of *Miscanthus x giganteus* (Poaceae). *Kew Bulletin*, 56(3), 759–760.
- Hodkinson, T. R., Renvoize, S. A., & Chase, M. W. (1997). Systematics of *Miscanthus*. *Aspects of Applied Biology*, (49), 189–198.
- Hsiao, A. I., & Vidaver, W. (1989). Effects of Repetitive Acid Immersion , Red Light , and Gibberellin A 3 Treatments on Phytochrome- mediated Germination Control in Skotodormant Lettuce Seeds. *Journal of Experimental Botany*, 40(210), 23–31.
- Hsu, F. H., Nelson, C. J., & Chow, W. S. (1984). A Mathematical Model to Utilize the Logistic Function in Germination and Seedling Growth. *Journal of Experimental Botany*, 35(160), 1629–1640.
- Huisman, W., Kasper, G. J., & Venturi, P. (1996). Technical and economical feasibility of the complete production-transport chain of *Miscanthus x giganteus* as an energy crop.
- Hunt Jr, E. R., Cavigelli, M., Daughtry, C. S. T., McMurtrey III, J. E., & Walthall, C. L. (2005). Evaluation of digital photography from model aircraft for remote sensing of crop biomass and nitrogen status. *Precision Agriculture*, 6(4), 359–378.
- Hussain, S., Siddiqui, S. U., Khalid, S., Jamal, A., Qayyum, A., & Ahmad, Z. (2007). Allelopathic potential of senna (*Cassia angustifolia* Vahl.) on germination and seedling characters of some major cereal crops and their associated grassy weeds. *Pakistan Journal of Botany*, 39(4), 1145–1153.
- Hussian, I., Ahmad, R., Farooq, M., Rehman, A., Amin, M., & Bakar, M. A. (2014). Seed priming: a tool to invigorate the seeds. *Scientia Agriculturae*, 3(3), 122–128.
- Iqbal, Y., Gauder, M., Claupein, W., Graeff-Hönninger, S., Lewandowski, I., & S., G.-H. (2015). Yield and quality development comparison between miscanthus and switchgrass over a period of 10 years. *Energy*, 89, 268–276.
- Jalink, H., van der Schoor, R., Frandas, A., van Pijlen, J. G., & Bino, R. J. (1998). Chlorophyll fluorescence of *Brassica oleracea* seeds as a non-destructive marker for seed maturity and seed performance. *Seed Science Research*, 8(4), 437–443.
- Jensen, E. F., Farrar, K., Thomas-Jones, S., Hastings, A. F., Donnison, I. S., & Clifton-Brown, J. C. (2011). Characterization of flowering time diversity in *Miscanthus* species. *GCB Bioenergy*, 3(5), 387–400.
- Jensen, E. F., Robson, P. R. H., Norris, J., Cookson, A., Farrar, K., Donnison, I. S., & Clifton-Brown, J. C. (2013). Flowering induction in the bioenergy grass *Miscanthus sacchariflorus* is a quantitative short-day response, whilst delayed flowering under long days increases biomass accumulation. *Journal of Experimental Botany*, 63(2), 541–552.
- Jeong, C. S., Murthy, H. N., Hahn, E. J., Lee, H. L., & Paek, K. Y. (2009). Inoculum size and auxin concentration influence the growth of adventitious roots and accumulation of ginsenosides in suspension cultures of ginseng (*Panax ginseng* C.A. Meyer). *Acta Physiologiae Plantarum*, 31(1), 219–222.

References

- Joosen, R. V. L., Kodde, J., Willems, L. A. J., Ligterink, W., van der Plas, L. H. W., & Hilhorst, H. W. M. (2010). GERMINATOR: a software package for high-throughput scoring and curve fitting of Arabidopsis seed germination. *The Plant Journal*, *62*(1), 148–59.
- Jørgensen, U. (2011). Benefits versus risks of growing biofuel crops: the case of Miscanthus. *Current Opinion in Environmental Sustainability*, *3*(1–2), 24–30.
- Jørgensen, U., & Schwarz, K. (2000). Why do basic research? A lesson from commercial exploitation of Miscanthus. *New Phytologist*, *148*(2), 190–193.
- Kaehler, A., & Bradski, G. (2014). Learning OpenCV.
- Karssen, C. M., & Lacka, E. (1986). A revision of the hormone balance theory of seed dormancy: studies on gibberellin and/or abscisic acid-deficient mutants of Arabidopsis thaliana. In *Plant Growth Substances 1985* (pp. 315–323). Springer.
- Keeley, J. E., & Fotheringham, C. J. (1998). Mechanism of smoke induced seed germination in a post fire chaparral annual. *Journal of Ecology*, *86*(1), 27–36.
- Kiesel, A., Wagner, M., & Lewandowski, I. (2016). Environmental Performance of Miscanthus, Switchgrass and Maize: Can C4 Perennials Increase the Sustainability of Biogas Production? *Sustainability*, *9*(1), 5.
- Klein Tank, A. M. G., Wijngaard, J. B., Können, G. P., Böhm, R., Demarée, G., Gocheva, A., ... Petrovic, P. (2002). Daily dataset of 20th-century surface air temperature and precipitation series for the European Climate Assessment. *International Journal of Climatology*, *22*(12), 1441–1453.
- Knight, P. R., Coker, C. E., Posadas, B., & Anderson, J. M. (2004). Weed Control and pest Management - poster Session 1. In *101st Annual International Conference of the American Society for Horticultural Science* (Vol. 39, pp. 745–840).
- Koçar, G., & Civaş, N. (2013). An overview of biofuels from energy crops: Current status and future prospects. *Renewable and Sustainable Energy Reviews*, *28*, 900–916.
- Koger, C. H., Reddy, K. N., & Poston, D. H. (2004). Factors affecting seed germination, seedling emergence, and survival of texasweed (*Caperonia palustris*). *Weed Science*, *52*(6), 989–995.
- Koornneef, M., Alonso-Blanco, C., Blankestijn-de Vries, H., Hanhart, C. J., & Peeters, A. J. M. (1998). Genetic interactions among late-flowering mutants of Arabidopsis. *Genetics*, *148*, 885–92.
- Koornneef, M., Bentsink, L., & Hilhorst, H. W. M. (2002). Seed dormancy and germination. *Current Opinion in Plant Biology*, *5*(1), 33–36.
- Kucera, B., Cohn, M. A., & Leubner-Metzger, G. (2005). Plant hormone interactions during seed dormancy release and germination. *Seed Science Research*, *15*(4), 281–307.
- Lagerwerff, J. V., Ogata, G., & Eagle, H. E. (1961). Control of Osmotic Pressure of Culture Solutions with Polyethylene Glycol. *Science*, *133*(3463), 1486–7.
- Lawlor, D. W. (1970). Absorption of Polyethylene Glycols by Plants and their Effects on Plant Growth. *New Phytologist*, *69*(2), 501–513.
- Lecarme, O., & Delvare, K. (2013). Lecarme, O., & Delvare, K. (2013). The Book of GIMP: A Complete Guide to Nearly Everything. In *User Manual, Edge-Detect Filters, Sobel, The GIMP Documentation Team* (First., pp. 547–560). San Francisco: No Starch Press.
- Lesur, C., Bazot, M., Bio-Beri, F., Mary, B., Jeuffroy, M.-H., & Loyce, C. (2013). Assessing nitrate leaching during the three-first years of Miscanthus × giganteus from on-farm measurements and modeling. *GCB Bioenergy*, n/a-n/a.

- Lewandowski, I., Clifton-Brown, J. C., Scurlock, J. M. O., & Huisman, W. (2000). Miscanthus : European experience with a novel energy crop. *Biomass and Bioenergy*, *19*, 209–227.
- Lewandowski, I., Clifton-Brown, J. C., Trindade, L. M., van der Linden, G. C., Schwarz, K.-U., Müller-Sämman, K., ... Kalinina, O. (2016). Progress on Optimizing Miscanthus Biomass Production for the European Bioeconomy: Results of the EU FP7 Project OPTIMISC. *Frontiers in Plant Science*, *7*(November), 1–23.
- 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*(1), 45–63.
- Lewis, G. N. (1908). The Osmotic Pressure of Concentrated Solutions, and the Laws of the Perfect Solution. *Journal of the American Chemical Society*, *34*(5), 668–683.
- Li, C. H., & Lee, C. K. (1993). Minimum cross entropy thresholding. *Pattern Recognition*, *26*(4), 617–625.
- Li, C. H., & Tam, P. K. S. (1998). An iterative algorithm for minimum cross entropy thresholding. *Pattern Recognition Letters*, *19*(8), 771–776.
- Li, W., Khan, M. A., Yamaguchi, S., & Kamiya, Y. (2005). Effects of heavy metals on seed germination and early seedling growth of Arabidopsis thaliana. *Plant Growth Regulation*, *46*(1), 45–50.
- Liu, W., Mi, J., Song, Z., Yan, J., Li, J., & Sang, T. (2014). Long-term water balance and sustainable production of Miscanthus energy crops in the Loess Plateau of China. *Biomass and Bioenergy*, *62*, 47–57.
- Lockhart, J. A. (1956). Reversal of the light inhibition of pea stem growth by the gibberellins. *Biology*, *42*, 841–848.
- Lou, L., Liu, Y., Han, J., & Doonan, J. H. (2014). Accurate Multi-View Stereo 3D Reconstruction for Cost-Effective Plant Phenotyping. In A. Campilho & M. Kamel (Eds.), *Image Analysis and Recognition: 11th International Conference, ICIAR 2014, Vilamoura, Portugal, October 22-24, 2014, Proceedings, Part II* (pp. 349–356). Cham: Springer International Publishing.
- Lovett, A. A., Sünnerberg, G. M., Richter, G. M., Dailey, A. G., Riche, A. B., & Karp, A. (2009). Land use implications of increased biomass production identified by gis-based suitability and yield mapping for miscanthus in england. *Bioenergy Research*, *2*(1–2), 17–28.
- Malinowska, M., Donnison, I. S., & Robson, P. R. H. (2016). Phenomics analysis of drought responses in Miscanthus collected from different geographical locations. *GCB Bioenergy*, *78*–91.
- Marr, C. W., & Jirak, M. (1990). Holding Tomato Transplants in Plug Trays. *HortScience*, *25*(2), 173–176.
- MATLAB. (2010). *version 7.10.0 (R2010a)*. Natick, Massachusetts: The MathWorks Inc.
- McCalmont, J. P., Hastings, A. F., McNamara, N. P., Richter, G. M., Robson, P. R. H., Donnison, I. S., & Clifton-Brown, J. C. (2015). Environmental costs and benefits of growing Miscanthus for bioenergy in the UK. *GCB Bioenergy*, 1–18.
- McCalmont, J. P., McNamara, N. P., Donnison, I. S., Farrar, K., & Clifton-Brown, J. C. (2016). Partitioning of ecosystem respiration of CO₂ released during land-use transition from temperate agricultural grassland to Miscanthus × giganteus. *GCB Bioenergy*, 1–15.
- McClendon, J. H. (1981). The osmotic pressure of concentrated solutions of polyethylene glycol 6000, and its variation with temperature. *Journal of Experimental Botany*, *32*(4), 861–866.

References

- Melville, A. H., Galletta, G. J., Draper, A. D., & Ng, T. J. (1980). Seed germination and early seedling vigor in progenies of inbred strawberry selections. *HortScience*.
- Merriam-Webster. (2006). Cotyledon. *Merriam-Webster Dictionary*. Retrieved September 11, 2013, from <http://www.merriam-webster.com/dictionary/cotyledon>
- Michel, B. E. (1983). Evaluation of the water potentials of solutions of polyethylene glycol 8000 both in the absence and presence of other solutes. *Plant Physiology*, *72*(1), 66–70.
- Midgley, O. (2012). Renewables Choice to grow miscanthus has revolutionised business Features Farmers Guardian. *Farmers Guardian*.
- Money, N. P. (1989). Osmotic Pressure of Aqueous Polyethylene Glycols : Relationship between Molecular Weight and Vapor Pressure Deficit. *Plant Physiology*, *91*(2), 766–769.
- Mościcki, K. J., Niedźwiecki, Ł., Owczarek, P., & Wnukowski, M. (2014). Commoditization of biomass: Dry torrefaction and pelletization-a review. *Journal of Power Technologies*, *94*(4), 233–249.
- Muetzelfeldt, R., & Massheder, J. (2003). The Simile visual modelling environment. *European Journal of Agronomy*, *18*, 345–358.
- Müssig, C., Shin, G.-H., & Altmann, T. (2003). Brassinosteroids promote root growth in Arabidopsis. *Plant Physiology*, *133*(November), 1261–1271.
- Nafziger, E. D., Carter, P. R., & Graham, E. E. (1991). Response of Corn to Uneven Emergence. *Crop Science*, *31*(3), 811.
- Naidu, S. L., Moose, S. P., Al-shoabi, A. K., Raines, C. A., & Long, S. P. (2003). Cold Tolerance of C4 photosynthesis in Miscanthus x giganteus: Adaptation in Amounts and Sequence of C4 Photosynthetic Enzymes. *Plant Physiology*, *132*, 1688–1697.
- Nixon, P., & Bullard, M. (2001). Planting and Growing For Applicants to DEFRA'S Energy Crops Scheme. *DEFRA*.
- Olsen, J. K., & Gounder, R. K. (2001). Alternatives to polyethylene mulch film — a field assessment of transported materials in capsicum (Capsicum annum L.). *Australian Journal of Experimental Agriculture*, *41*, 93–103.
- Oluoch, M. O., & Welbaum, G. E. (1996). Viability and vigor of osmotically primed muskmelon seeds after nine years of storage. *Journal of the American Society for Horticultural Science*, *121*(2), 408–413.
- Pari, L. (1996). First trials on Arundo donax and Miscanthus rhizomes harvesting. In P. Chartier, G. L. Ferrero, U. M. Henius, S. Hultberg, H. Sachau, & M. Wiinblad (Eds.), *Biomass for energy and the environment: Proceedings of the Ninth European Bioenergy Conference* (pp. 889–94). Copenhagen, Denmark: New York: Pergamon.
- Parisi, L., Majica, M. M., & Arturi, M. J. (1991). Variability in the germination of Lotus tenuis (Waldst et Kit).
- Paruelo, J. M., Lauenroth, W. K., & Roset, P. A. (2000). Estimating Aboveground Plant Biomass Using a Photographic Technique. *Journal of Range Management*, *53*(2), 190–193.
- Pérez-Harguindeguy, N., Díaz, S., Lavorel, S., Poorter, H., Jaureguiberry, P., Bret-Harte, M. S., ... Cornelissen, J. H. C. (2013). New Handbook for standardized measurement of plant functional traits worldwide. *Australian Journal of Botany*, *23*(34), 167–234.
- Pinheiro, J., Bates, D. M., DebRoy, S., Sarkar, D., & R Core Team. (2016). {nlme}: Linear and Nonlinear Mixed Effects Models.

- Pinheiro, J. C., & Bates, D. M. (2000). *Mixed-effects models in S and S-PLUS*. (J. Chambers, W. Eddy, W. Hardle, S. Sheather, & L. Tierney, Eds.) *Statistics and computing*. New York: Springer.
- Pitts, R. J., Cernac, A., & Estelle, M. (1998). Auxin and ethylene promote root hair elongation in *Arabidopsis*. *The Plant Journal*, *16*(5), 553–560.
- Pogson, M., Hastings, A. F., & Smith, P. (2013). How does bioenergy compare with other land-based renewable energy sources globally? *GCB Bioenergy*, *5*(5), 513–524.
- Poling, E. B. (1993). Strawberry plasticulture in North Carolina: II. Preplant, planting, and postplant considerations for growing “Chandler” strawberry on black plastic mulch. *HortTechnology*, *3*(4), 383–393.
- Pourjavadi, A., Salimi, H., Amini-Fazl, M. S., Kurdtabar, M., & Amini-Fazl, A. R. (2006). Optimization of synthetic conditions of a novel collagen-based superabsorbent hydrogel by Taguchi method and investigation of its metal ions adsorption. *Journal of Applied Polymer Science*, *102*(5), 4878–4885.
- Purdy, S. J., Maddison, A. L., Jones, L. E., Webster, R. J., Andralojc, J., Donnison, I. S., & Clifton-Brown, J. C. (2013). Characterization of chilling-shock responses in four genotypes of *Miscanthus* reveals the superior tolerance of *M. x giganteus* compared with *M. sinensis* and *M. sacchariflorus*. *Annals of Botany*, *111*(5), 999–1013.
- Pyter, R., Voigt, T. T. B., Heaton, E. A., Dohleman, F. G., & Long, S. P. S. (2007). Giant *Miscanthus* : Biomass Crop for Illinois. *Issues in New Crops and New Uses*, 39–42.
- Python Software Foundation. (2012). *The Python Language Reference Manual*. (G. van Rossum & F. L. Drake Jr, Eds.).
- R Core Team. (2015). *R: A Language and Environment for Statistical Computing*. Vienna, Austria.
- Rahman, A. (2001). Auxin is a Positive Regulator for Ethylene-Mediated Response in the Growth of *Arabidopsis* Roots. *Plant and Cell Physiology*, *42*(3), 301–307.
- Ranal, M. A., & de Santana, D. G. (2006). How and why to measure the germination process? *Brazilian Journal of Botany*, *29*(1), 1–11.
- Rao, N. K., Roberts, E. H., & Ellis, R. H. (1987). The influence of pre and post-storage hydration treatments on chromosomal aberrations, seedling abnormalities, and viability of lettuce seeds. *Annals of Botany*, *60*, 97–108.
- Rao, R. S., Kumar, C. G., Prakasham, R. S., & Hobbs, P. J. (2008). The Taguchi methodology as a statistical tool for biotechnological applications: A critical appraisal. *Biotechnology Journal*, *3*(4), 510–523.
- Ridler, T.W. Calvard, S. (1978). Picture Thresholding Using an Iterative Slection Method. *IEEE Transactions on Systems, Man and Cybernetics*, *8*(8), 630–632.
- Ripley, B. D. (2015). *class: Functions for Classification*. CRAN.
- Road, N., & Malling, E. (2009). *Establishing perennial grass energy crops: a review of current propagation options with a focus on Miscanthus*.
- Robbins, M. P., Evans, G., Valentine, J., Donnison, I. S., & Allison, G. (2012). New opportunities for the exploitation of energy crops by thermochemical conversion in Northern Europe and the UK. *Progress in Energy and Combustion Science*, *38*(2), 138–155.
- Robin, X., Turck, N., Hainard, A., Tiberti, N., Lisacek, F., Sanchez, J.-C., & Müller, M. (2011). pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinformatics*, *12*, 77.

References

- Robson, P. R. H., Farrar, K., Gay, A. P., Jensen, E. F., Clifton-Brown, J. C., & Donnison, I. S. (2013). Variation in canopy duration in the perennial biofuel crop *Miscanthus* reveals complex associations with yield. *Journal of Experimental Botany*, *64*(8), 2373–83.
- Robson, P. R. H., Mos, M., Clifton-Brown, J. C., & Donnison, I. S. (2011). Phenotypic variation in senescence in *Miscanthus*: towards optimising biomass quality and quantity. *BioEnergy Research*, *5*(1), 95–105.
- Rolland, A., Gaudichon, S., Gioia, L. di, Macherel, D., Benamar, A., Pierart, A., ... Macherel, D. (2013). Simple system using natural mineral water for high-throughput phenotyping of *Arabidopsis thaliana* seedlings in liquid culture. *International Journal of High Throughput Screening*, *4*, 1–15.
- Rosquete, M. R., von Wangenheim, D., Marhavý, P., Barbez, E., Stelzer, E. H. K., Benková, E., ... Kleine-Vehn, J. (2013). An auxin transport mechanism restricts positive orthogravitropism in lateral roots. *Current Biology*, *23*(9), 817–822.
- Roy, R. K. (2001). *Design of experiments using the Taguchi approach: 16 steps to product and process improvement*. John Wiley & Sons.
- Roy, R. K. (2010). *A primer on the Taguchi method* (2nd Revise.). Dearborn, United States: Society of Manufacturing Engineers.
- Samco Agricultural Manufacturing Ltd. SAMCO Brochure (2014). Adare, Co Limerick , Ireland: Samco Agricultural Manufacturing Ltd.
- Sami, M., Annamalai, K., & Wooldridge, M. (2001). Co-firing of coal and biomass fuel blends. *Progress in Energy and Combustion Science*, *27*(2), 171–214.
- Sarath, G., Bethke, P. C., Jones, R., Baird, L. M., Hou, G., & Mitchell, R. B. (2006). Nitric oxide accelerates seed germination in warm-season grasses. *Planta*, *223*(6), 1154–1164.
- Sarath, G., Hou, G., Baird, L. M., & Mitchell, R. B. (2007). Reactive oxygen species, ABA and nitric oxide interactions on the germination of warm-season C4-grasses. *Planta*, *226*(3), 697–708.
- Sarath, G., & Mitchell, R. B. (2008). Aged switchgrass seed lot's response to dormancy-breaking chemicals. *USDA Agricultural Research Service*. University of Nebraska.
- Sartorius Mechatronics. (2010). Sartorius M-power Operating Instructions. Sartorius Mechatronics.
- Sathish, S., Sundareswaran, S., & Ganesan, N. (2011). Influence of Seed Priming on Physiological Performance of Fresh and Aged Seeds of Maize Hybrid [COH (M) 5] and it's Parental Lines. *ARPJ Journal of Agricultural and Biological Science*, *6*(3), 12–17.
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., ... Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. *Nature Methods*, *9*(7), 676–682.
- Scott, D. (1975). Effects of seed coating on establishment. *New Zealand Journal of Agricultural Research*, *18*(1), 59–67.
- Scott, J. M. (1989). Seed Coatings and Treatments and Their Effects on Plant Establishment. In N. C. Brady (Ed.), *Advances in Agronomy* (pp. 44–83). London: Academic Press.
- Scurlock, J. M. O. (1999). *Miscanthus: A review of European experience with a novel energy crop*. Oak Ridge, TN: U.S. DEPARTMENT OF ENERGY.
- Sester, M., Dürr, C., Darmency, H., & Colbach, N. (2007). Modelling the effects of cropping systems on the seed bank dynamics and the emergence of weed beet. *Ecological Modelling*, *204*(1–2), 47–58.

- Shalev-Shwartz, S., & Ben-David, S. (2014). *Understanding Machine Learning. Understanding Machine Learning: From Theory to Algorithms*. Cambridge: Cambridge University Press. doi:10.1017/CBO9781107298019
- Sharma, A. D., Rathore, S. V. S., Srinivasan, K., & Tyagi, R. K. (2014). Comparison of various seed priming methods for seed germination, seedling vigour and fruit yield in okra (*Abelmoschus esculentus* L. Moench). *Scientia Horticulturae*, *165*, 75–81.
- Shen, Z.-X., Parrish, D. J., Wolf, D. D., & Welbaum, G. E. (2001). Stratification in Switchgrass Seeds Is Reversed and Hastened by Drying. *Crop Science*, *41*(5), 1546.
- Shrestha, A., Roman, E. S., Thomas, A. G., & Swanton, C. J. (1999). Modeling germination and shoot-radicle elongation of *Ambrosia artemisiifolia*. *Weed Science*, *47*(5), 557–562.
- Shu, K., Liu, X. D., Xie, Q., & He, Z. H. (2016). Two Faces of One Seed: Hormonal Regulation of Dormancy and Germination. *Molecular Plant*, *9*(1), 34–45.
- Siddiqui, S., Bhardwaj, S., Khan, S. S., & Meghvanshi, M. K. (2009). Allelopathic Effect of Different Concentration of Water Extract of *Prosopis Juliflora* Leaf on Seed Germination and Radicle Length of Wheat (*Triticum aestivum* Var-Lok-1). *American-Eurasian Journal of Scientific Research*, *4*(2), 81–84.
- Singh, B. P. (Ed.). (2013). *Biofuel crops: production, physiology and genetics*. Wallingford: CABI. doi:10.1079/9781845938857.0000
- Slavov, G., Robson, P. R. H., Jensen, E. F., Hodgson, E. M., Farrar, K., Allison, G., ... Donnison, I. S. (2013). Contrasting geographic patterns of genetic variation for molecular markers vs. phenotypic traits in the energy grass *Miscanthus sinensis*. *GCB Bioenergy*, *5*(5), 562–571.
- Soltani, E., Ghaderi-Far, F., Baskin, C. C., & Baskin, J. M. (2015). Problems with using mean germination time to calculate rate of seed germination. *Australian Journal of Botany*, *63*(8), 631–635.
- Songstad, D., Lakshmanan, P., Chen, J., Gibbons, W., Hughes, S., Nelson, R., ... Moser, B. R. (2010). *Biofuels: global impact on renewable energy, production agriculture, and technological advancements*. (D. Tomes, P. Lakshmanan, & D. Songstad, Eds.). New York, NY: Springer. doi:10.1007/978-1-4419-7145-6
- Sparks, D. L. (Ed.). (2011). Presowing seed treatment with organic osmolytes. In *Advances in Agronomy* (1st ed., pp. 257–258). London: Elsevier.
- Stavridou, E., Hastings, A. F., Webster, R. J., & Robson, P. R. H. (2016). The impact of soil salinity on the yield, composition and physiology of the bioenergy grass *Miscanthus x giganteus*. *GCB Bioenergy*, 1–13.
- Steber, C. M., & McCourt, P. (2001). A role for brassinosteroids in germination in *Arabidopsis*. *Plant Physiology*, *125*(February), 763–769.
- Steuter, A. A., Mozafar, A., & Goodin, J. O. E. R. (1981). Water potential of aqueous polyethylene glycol. *Plant Physiology*, *67*(1), 64–67.
- Stewart, J. R., Toma, Y., Fernández, F. G., Nishiwaki, A., Yamada, T., & Bollero, G. A. (2009). The ecology and agronomy of *Miscanthus sinensis*, a species important to bioenergy crop development, in its native range in Japan: a review. *GCB Bioenergy*, *1*(2), 126–153.
- Stričević, R., Dželetović, Z., Djurović, N., & Cosić, M. (2015). Application of the AquaCrop model to simulate the biomass of *Miscanthus x giganteus* under different nutrient supply conditions. *GCB Bioenergy*, *7*(6), 1203–1210.

References

- Subba Rao, C., Madhavendra, S. S., Sreenivas Rao, R., Hobbs, P. J., & Prakasham, R. S. (2008). Studies on improving the immobilized bead reusability and alkaline protease production by isolated immobilized bacillus circulans (MTCC 6811) using overall evaluation criteria. *Applied Biochemistry and Biotechnology*, 150(1), 65–83.
- Taguchi, G. (1986). *Introduction to quality engineering: Designing quality into products and processes*. Asian productivity organization. New York: UNIPUB.
- Tamet, V., Boiffin, J., Dürr, C., Souty, N., & Diirr, C. (1996). Emergence and early growth of an epigeal seedling (*Daucus carota* L.): Influence of soil temperature, sowing depth, soil crusting and seed weight. *Soil and Tillage Research*, 40(1–2), 25–38.
- TCI America. (2005). 1-Naphthaleneacetic Acid. Tokyo Chemical Industry Co. Ltd.
- Terravesta. (2017). terravesta.com. *Growing*. Retrieved January 16, 2017, from <http://www.terravesta.com/>
- The DataCamp Team. (2015). Machine Learning in R for beginners. *DataCamp*. Retrieved June 10, 2015, from <http://blog.datacamp.com/machine-learning-in-r/>
- Tomasel, F. G., Paruelo, J. M., Abras, G., Ballarín, V., & Moler, E. (2009). A chromaticity-based technique for estimation of above-ground plant biomass. *Applied Vegetation Science*, 4(2), 207–212.
- Tong, H., Xiao, Y., Liu, D., Gao, S., Liu, L., Yin, Y., ... Chu, C. (2014). Brassinosteroid Regulates Cell Elongation by Modulating Gibberellin Metabolism in Rice. *The Plant Cell*, 26(November), 1–19.
- Tong, L.-I., Su, C.-T., & Wang, C.-H. (1997). The optimization of multi-response problems in the Taguchi method. *International Journal of Quality & Reliability Management*, 14(4), 367–380.
- Trudgill, D. L., Squire, G. R., & Tompson, K. (2000). A thermal time basis for comparing the germination requirements of some British herbaceous plants. *New Phytologist*, 145(1), 107–114.
- Tseng, M.-H., Kuo, Y.-H., Chen, Y.-M., & Chang-Hung, C. (2003). Allelopathic potential of *Macaranga tanarius* (L.) muell.-arg. *Journal of Chemical Ecology*, 29(5), 1269–86.
- Turley, D., Taylor, M., Laybourn, R., Hughes, J., Kilpatrick, J., Procter, C., ... Edgington, P. (2010). *Assessment of the availability of marginal or idle land for bioenergy crop production in England and Wales (Report to DEFRA)* (Vol. NF0444). London.
- Tzilivakis, J., Warner, D. J., May, M., Lewis, K. A., & Jaggard, K. (2005). An assessment of the energy inputs and greenhouse gas emissions in sugar beet (*Beta vulgaris*) production in the UK. *Agricultural Systems*, 85(2), 101–119.
- Unterholzner, S. J., Rozhon, W., Papacek, M., Ciomas, J., Lange, T., Kugler, K. G., ... Poppenberger, B. (2015). Brassinosteroids Are Master Regulators of Gibberellin Biosynthesis in Arabidopsis. *The Plant Cell*, 27, 1–13.
- Valentine, J., Clifton-Brown, J. C., Hastings, A. F., Robson, P. R. H., 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), 1–19.
- Venables, W. N., & Ripley, B. D. (2002). *Modern Applied Statistics with S-plus* (Fourth.). New York: Springer.
- Vogel, K. P. (1987). Seeding Rates for Establishing Big Bluestem and Switchgrass with Preemergence Atrazine Applications. *Agronomy Journal*, 79(3), 509.

- Wagner, M.-H., Demilly, D., Ducournau, S., Dürr, C., & Lechappe, J. (2011). Computer vision for monitoring seed germination from dry state to young seedlings. *Seed Testing International*, (142), 49–51.
- Walker-Simmons, M. (1987). ABA Levels and Sensitivity in Developing Wheat Embryos of Sprouting Resistant and Susceptible Cultivars. *Plant Physiology*, 84, 61–66.
- Walker, J. M. (2009). *Biofuels*. (J. R. Mielenz, Ed.) (Vol. 581). Totowa, NJ: Humana Press. doi:10.1007/978-1-60761-214-8
- Wang, B., Zhang, J., Xia, X., & Zhang, W.-H. (2011a). Ameliorative effect of brassinosteroid and ethylene on germination of cucumber seeds in the presence of sodium chloride. *Plant Growth Regulation*, 65(2), 407–413.
- Wang, L., Hua, D., He, J., Duan, Y., Chen, Z., Hong, X., & Gong, Z. (2011b). Auxin response Factor2 (ARF2) and its regulated homeodomain gene HB33 mediate abscisic acid response in Arabidopsis. *PLoS Genetics*, 7(7), e1002172.
- Wang, L., Uilecan, I. V., Assadi, A. H., Kozmik, C. A., & Spalding, E. P. (2009). HYPOTrace: image analysis software for measuring hypocotyl growth and shape demonstrated on Arabidopsis seedlings undergoing photomorphogenesis. *Plant Physiology*, 149(4), 1632–1637.
- WheatGenetics. (2016). 1KK (Version 1.0.3) [Mobile software application]. google play. Retrieved March 1, 2016, from play.google.com/store/apps/details?id=org.wheatgenetics.onekk
- White, J. W., Andrade-Sanchez, P., Gore, M. A., Bronson, K. F., Coffelt, T. A., Conley, M. M., ... Wang, G. (2012). Field-based phenomics for plant genetics research. *Field Crops Research*, 133, 101–112.
- Wickham, H. (2007). Reshaping Data with the {reshape} Package. *Journal of Statistical Software*, 21(12), 1–20.
- Wickham, H. (2009). *ggplot2: elegant graphics for data analysis*. Springer New York.
- Wickham, H., & Francois, R. (2016). dplyr: A Grammar of Data Manipulation.
- Witzel, C.-P., & Finger, R. (2016). Economic evaluation of Miscanthus production – A review. *Renewable and Sustainable Energy Reviews*, 53, 681–696.
- Xue, S., Kalinina, O., & Lewandowski, I. (2015). Present and future options for Miscanthus propagation and establishment. *Renewable and Sustainable Energy Reviews*, 49, 1233–1246.
- Yaldagard, M., Mortazavi, S. A., & Tabatabaie, F. (2008). Application of Ultrasonic Waves as a Priming Technique for Accelerating and Enhancing the Germination of Barley Seed : Optimization of Method by the Taguchi Approach. *Journal of the Institute of Brewing*, 114(1), 14–21.
- Zeileis, A., & Hothorn, T. (2002). Diagnostic Checking in Regression Relationships. *R News*, 2(3), 7–10.
- Zeileis, A., Kleiber, C., & Jackman, S. (2008). Regression Models for Count Data in R. *Journal of Statistical Software*, 27(8), 1–25.
- Zhang, H., Irving, L. J., McGill, C., Matthew, C., Zhou, D., & Kemp, P. (2010). The effects of salinity and osmotic stress on barley germination rate: sodium as an osmotic regulator. *Annals of Botany*, 106(6), 1027–35.
- Zhou, L.-M., Li, F.-M., Jin, S.-L., & Song, Y. (2009). How two ridges and the furrow mulched with plastic film affect soil water, soil temperature and yield of maize on the semiarid Loess Plateau of China. *Field Crops Research*, 113(1), 41–47.

References

Zhu, Q., & Benková, E. (2016). Seedlings' Strategy to Overcome a Soil Barrier. *Trends in Plant Science*, 21(10), 2–4.

APPENDICES

Appendix A PANICLE DRYING SUB-EXPERIMENT

The usual method of drying panicles in the Aberystwyth *Miscanthus* breeding program is to lay them out for approximately one week in a glasshouse. To sample methods around this, a small un-replicated test was conducted. For this test 25 seed panicles from Blankney were dried for 93 hours in five different ways: 1) In a warm and humid greenhouse, 2) at room temperature and low humidity, 3) at 30°C and low humidity (oven), 4) at 30°C and high humidity (germination cabinet), 5) at 50°C and low humidity (oven). The humidity conditions were classed as low unless noticeably high (the greenhouse) or measured to be high (the germination cabinet – 60% RH). After drying, the heads were stripped from the panicles by machine and the seeds threshed using a Westrup LA-H. Germination frequency was tested using a sub sample of ~50 cleaned seeds that was germinated on damp blue tissue paper at 25°C in Petri dishes. Germination was assessed at 7 and 15 days and percentage germination calculated. To determine the quality of the seed several hundreds of seed were examined for broken seed and a percentage of broken seed calculated. To determine the seed moisture content at time of sowing seed were weighed and dried for 72 hours at 65°C and re-weighed.

Table 1: Germination (7 and 15 day), moisture content, and breakage of seed shown for threshed cleaned seed, after drying for 72 h, in variety of temperatures and humidity settings. Rooms were more variable in temperature than the controlled environments; therefore, the temperature and humidity information is a guideline.

Dried	Dried Temp (~°C)	Dried Humidity	Mean Moisture Content (%)	Broken (%)	Germ 7 Days (%)	Germ 15 Days (%)
Oven high	50	Low	6.3	17.2	0	0
Room	20	Mid	10.89	.3	8.0	20.0
Oven low	30	Low	10.02	.7	10.0	16.0
Germ cabinet	25	High	7.88	.6	18.8	27.1
Greenhouse	30	High	11.82	.3	14.9	23.4

This test showed that seed breakage was only affected if they were dried so hot they were killed (50°C) (see Table 1). Germination varied but was best at seven and fifteen days with the seed that experienced the least temperature change, and worst with low humidity and low temperature, apart from the 50°C seed that were killed. This test was only an indication and normally the seed are stored in cold and dry conditions before germination.

Appendix B THRESHING OBSERVATIONS

Analysis of five seed lots threshed in the last few years was done by counting the number of broken seed in a sample of a few hundred seed. Table 2 shows that the seed breakage percentage in *Miscanthus* seed threshed using the Westrup (LA-H) was low (< 2%). This result is in line with the seed threshed and scored in Table 1 under all but the hottest drying methods.

Table 2: Seed breakage rates for seed threshed in different years using the Westrup threshing machine.

Seed Name	Year	Grown in	Stripped Using	Breakage (%)
MX300	2014	Wales	Head Machine	1%
SYN55	2013	Texas	Paddle	2%
GNT1	2014	Texas	Paddle	2%
GNT3	2014	Texas	Paddle	3%
Syn79	2012	Texas	Paddle	10%
MMX	2015	Blankney	Head Machine	17%
GNT33	2011	Texas	Paddle	19%

Appendix C STERILISATION PRE-TEST

Testing the effectiveness of the sterilisation method was performed on three replicates of approximately 65 MX300 seed, which were sprinkled on four layers of blue roll wet to capacity (40 mL SDW) in twelve Petri dishes. The seed were sterilised using the above method in 20%, 10% and 5% household bleach solution plus 0.1% Triton x-100 and compared with a control where the seeds were rinsed in only Triton x-100 0.1% solution. The seed were germinated at 25°C and levels of germination and mould recorded at days 7 and 15. Data of the effects of treatment on mould and germination were examined using a one-way ANOVA.

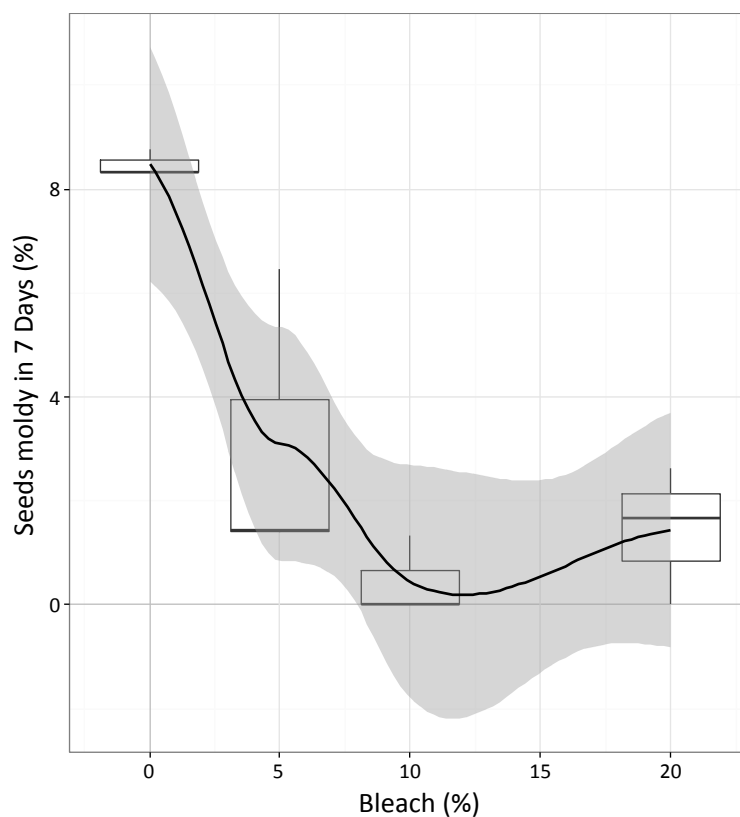


Figure 1: The effect of bleach concentration on mould in the first 7 days. Boxplots show the variability in the three replicates. Loess line with 95% confidence interval added.

The results (see Figure 1) indicate that the all levels of sterilisation had a significant (one-way ANOVA, $P < 0.01$) effect on mould at 7 days. A Tukey's HSD showed all bleach concentrations were significantly different from zero but not from each other; the result was simpler at 15 days ($P < 0.05$). There also was no significant effect on germination at either time point (one-way ANOVA, $P = 0.429$ & $P = 0.536$). Because mould decreased significantly between 5% bleach and 10% bleach but not between 10 and 20% bleach, 10% bleach was used for all following experiments using sterilisation, because 10% is the lowest concentration that is equally effective to 20%.

Appendix D INITIAL FIJI MACRO

```
run("HSB Filter", "hue=1 hue_width=1 saturation=0.80 saturation_width=0.40 brightness=0  
brightness_width=1");  
run("8-bit");  
setThreshold(0, 38);  
run("Convert to Mask");  
run("Invert");  
run("Analyze Particles...", "size=0.86-Infinity circularity=0.3-1.00 show=Ellipses display  
exclude include summarize record");
```

Measurements of perimeter, area, and shade of each seed run on an 8-bit binary image using FIJI.

Appendix E SINGLE IMAGE THRESHOLDING FIJI SCRIPT

```

run("Set Scale...", "distance=732 known=40 pixel=1 unit=mm global");
makeRectangle(266, 142, 1068, 1026);
run("Crop");
run("Select All");
run("Copy");
// Remove background
run("Internal Clipboard");
run("HSB Filter", "hue=1 hue_width=0.20 saturation=0.55 saturation_width=0.35 brightness=1
brightness_width=1");
run("8-bit");
run("Invert");
setThreshold(223, 255);
setOption("BlackBackground", false);
run("Convert to Mask");
run("Invert");
run("Analyze Particles...", "size=0.86-Infinity circularity=0.3-1.00 show=Ellipses display
exclude include summarize record");
// Threshold only the germination
selectImage(1);
run("HSB Filter", "hue=1 hue_width=0.15 saturation=0.50 saturation_width=0.50
brightness=0.50 brightness_width=0.50");
run("8-bit");
run("Invert");
setThreshold(223, 255);
setOption("BlackBackground", false);
run("Convert to Mask");
run("Invert");
run("Analyze Particles...", "size=0.86-Infinity circularity=0.3-1.00 show=Ellipses display
exclude include summarize record");

```

This is the main part of the single image thresholding script.

Appendix F IMPROVED CAMERA SETUP



Figure 2: A stable camera setup, with four fluorescent lights, shining onto a copy stand with a setsquare stuck to it to keep square dishes in the same place. A DSLR camera was attached to the screw thread in the centre of the image to photograph the seed.

Appendix G MAIN FIJI GERMINATION DETECTION SCRIPT

```

//this is the header for the results
header = "Lot & Time\t Volume (mm^3)\t Surface (mm^2)\t Nb of obj. voxels\t Nb of surf.
voxels\t IntDen\t Mean\t StdDev\t Median\t Min\t Max\t X\t Y\t Z\t Mean dist. to surf.....
misso = 0;
miss= 0;
germlist = "G-";

list = getFileList(input);

run("Set Scale...", "distance=1452 known=50 pixel=1 unit=mm global");
run("HSB Stack");
makeRectangle(320, 380, 2652, 2132);
run("Crop");
// Threshold
run("Stack to Images");
selectWindow("Saturation");
setThreshold(0, 82);
run("Convert to Mask");
selectWindow("Brightness");
run("Close");
selectWindow("Hue");
setThreshold(0, 140);
run("Convert to Mask");
imageCalculator("Add", "Hue", "Saturation");
selectWindow("Saturation");
run("Close");

selectWindow("Hue");
run("Erode");
run("Dilate");

//get total no of seeds to check
run("Analyze Particles...", "size=1-Infinity circularity=0.00-1.00 show=Nothing clear
add");
roiManager("Show All with labels");
roiManager("Show All");

```

First setup and count seed.

Then go through all the dishes.

```

for (i = i; i<list.length; i++) {
    path = input+list[i];
    open(path);

    name = File.nameWithoutExtension;
    t = t + 1;
    makeRectangle(320, 380, 2652, 2132);
    run("Crop");
    saveAs("Tiff", output+"Seedlot"+Lotno+"_t"+t+"_Cropped");

    run("Select All");
    run("Copy");
    run("Internal Clipboard");
    selectImage("Clipboard");
    // Threshold
    run("RGB Stack");
    run("Stack to RGB");
    run("8-bit");
    selectWindow("Clipboard");
    run("Stack to Images");
    selectImage(1);
    run("Select None");
    run("HSB Stack");
    run("Stack to Images");
    selectWindow("Clipboard-1");
    run("Color Transformer", "colour=HSL");
    run("Stack to Images");
    selectWindow("S");
    selectWindow("L");
    selectWindow("H");
    setThreshold(0.36, 0.83);
    run("Convert to Mask");
    run("Invert");
    selectWindow("Clipboard-1");
    selectWindow("Saturation");// Saturation Threshold
    setThreshold(0, 100);
    run("Convert to Mask");
    selectWindow("Green");// Green Threshold
    setThreshold(185, 255);
    run("Convert to Mask");
    selectWindow("Red");// Red Threshold
    setThreshold(150, 255);
    run("Convert to Mask");
    selectWindow("Hue");// Hue Threshold
    setThreshold(0, 145);
    run("Convert to Mask");

```

Calculate a final image.

```
imageCalculator("OR", "Saturation", "Red");
imageCalculator("OR", "Green", "Saturation");
imageCalculator("AND", "Red", "H");
selectWindow("Red");
run("Dilate");
// remove reflections
imageCalculator("Subtract", "Green", "Red");
imageCalculator("OR", "Hue", "Green");

//hole filling
selectWindow("Hue");
run("Dilate");
run("Erode");
imageCalculator("AND", "Hue", "Clipboard (RGB)");
```

Go through each individual seed from each dish.

```

for(s = 0; s < LotROIIno; s++){
    open(...); // open previously saved seed image
    roiManager(...); // get areas of interest
    run("Crop");
    run("3D Objects Counter", "threshold=2 slice=0 min.=450 max.=43200 objects
statistics");

    selectWindow("Statistics for seed this threshold.tif");
    IJ.renameResults("Results");
    // Get the results form the object analysis
    selectWindow("Results");
    text = getInfo();
    lines = split(text, "\n");
    columns = split(lines[0], "\t");
    noL = lines.length;
    noL = noL - 1;
    row = "";

    if (noL == 0) {
        "Seed not found" // in all
    }

    if (noL > 0) {
        res0 = getResult(columns[0]);
        res1 = getResult(columns[1]);
        // ... ..
    }
    row = row + name + "\t";
    row = row + res1 + "\t";
    // ... ..
    //key variables Start deducted from start image
    mSA = FSA - SSA;
    mNoVo = FNoVo - SNoVo;
    mIntDen = FIntDen - SIntDen;
    mMean = FMean - SMean;
    mStDev = FStDev - SStDev;
    mMeed = FMeed - SMeed;
    mMax = FMax - SMax;
    mMeanDist = FMeanDist - SMeanDist;
    mSDDist = FSDDist - SSDDist;
    mMeedDist = FMeedDist - SMeedDist;
    mBW = FBW - SBW;
    mBH = FBH - SBH;
    MiunGY = 0;

```

Then check values for each variable deducted from the start image to count

germination.

```

    if (mSA > 1.4208) {
        MiunGY = MiunGY + 1;
    }
    if (mNoVo > 99) {
        MiunGY = MiunGY + 1;
    }
    if (mIntDen > 172082) {
        MiunGY = MiunGY + 1;
    }
    if (mMean > 39.0048) {
        MiunGY = MiunGY + 1;
    }
    if (mStDev > 10.7855) {
        MiunGY = MiunGY + 1;
    }
    if (mMeed > 45) {
        MiunGY = MiunGY + 1;
    }
    if (mMax > 36) {
        MiunGY = MiunGY + 1;
    }
    if (mMeanDist > 0.238) {
        MiunGY = MiunGY + 1;
    } // ... Several more below

    // This is repeated for the % change method
    // Results are saved to the table
}
// After all seeds are done the table was saved as a .csv file

} // End

```

When it is above the threshold, it is scored with an extra MiunGY or PestGY for percentage calculations.

This is a brief overview of the macro used to score germination, a full version available at <https://github.com/Miscanthus-Germination/FIJI-Germination-Detection>.

Appendix H *k*-NEAREST NEIGHBOUR R SCRIPT

R code for testing *k*-NN on the results from FIJI Analysis (with or without image histogram data) by default uses PCA. It uses the ‘class’ package (Ripley, 2015) to run the *k*-NN and the ‘pROC’ package (Robin et al., 2011) to plot the ROC curve. It also saves a file of the least certain values. This was produced with reference to The DataCamp Team (2015) machine learning tutorial.

```

library("class") # (Ripley, 2015)
library("pROC") # (Robin et al., 2011)
set.seed(SEED) # is 1234
# import the Human ground truth (or amended ground truth) and FIJI data
Germinfo <- read.csv(paste(PATH, GroundTruth_FileName, sep = ""))
GermData <- read.csv(paste(PATH, FIJI_FileName, sep = ""))
GermData <- cbind2(Germinfo, GermData)
# Use a PCA custom PCA function to normalise the data and produce 21 pcs
GermData_norm <- PCA_Data(GermData, ret_PC = 21)
trainSet = 1
testSet = 2
SubSample <- sample(2, nrow(GermData), replace=TRUE, prob=c(0.5, 0.5))
GermData_training <- GermData_norm[SubSample == trainSet, 5:ncol(GermData_norm)]
GermData_testing <- GermData_norm[SubSample==testSet, 5:ncol(GermData_norm)]
trainingLabels <- GermData[SubSample == trainSet, State_of_Seed] # 3 for germ
testingLabels <- GermData[SubSample==testSet, State_of_Seed] # 3 for germ
# k = 7
GermDataRun <- knn(train = GermData_training, test = GermData_testing, cl = trainingLabels,
k=K, prob = T)
CrossTable(x = testingLabels, y = GermDataRun, prop.chisq=FALSE)
# output the least confident set to check the human scoring
test <- attr(GermDataRun,"prob")
test <- cbind.data.frame(GermData[SubSample==testSet,1:4], as.factor(GermDataRun), test)
colnames(test)[5] <- "Predict"
colnames(test)[State_of_Seed] <- "Real"
test <- test[test$Predict != test$Real,]
test <- test[test$test == min(test$test),]
write.csv(test, paste(PATH, "predict_First.csv", sep = ""))
# Produce a ROC curve
levels(GermData.testLabels) <- c("1","0")
levels(GermData_pred) <- c("1","0")
Rok <- roc(response = as.numeric(as.character(GermData.testLabels)), predictor =
as.numeric(as.character(GermData_pred)))
plot.roc(Rok, print.auc = TRUE, auc.polygon = TRUE)
par(new = TRUE)
print(auc(Rok))
# Switch test and train randomisation and re-run
trainSet = 2
testSet = 1
...

```

Appendix I IMAGEJ FIELD BIOMASS

The FIJI Code for a green pixel counts to estimate field biomass. Two simple functions were used to find the length of a selection and to scale the green pixel area to mm².

```
function Dist(setlength) {
    getLine(x1, y1, x2, y2, linewidth);
    if (x1==-1)
        exit("This requires a straight line selection");
    getPixelSize(unit, pw, ph);
    x1*=pw; y1*=ph; x2*=pw; y2*=ph;
    dx = x2-x1; dy = y2-y1;
    length = sqrt(dx*dx+dy*dy);
    length = length/setlength;
    return length;
}
function TotArea(scail) {
    if (nResults > 0) {
        for (a=0; a<nResults(); a++) {
            total_area=total_area + getResult("Area",a);
        }
        total_area = total_area / scail;
    } else {
        total_area = 0;
    }
    return total_area;
}
```

A simplified version of the main FIJI script is below.


```

run("Set Scale...", "distance=0 known=0 pixel=1 unit=pixel global");
header = "plot,Stems,PlantNo,Sen(0-10),P/Np,Film,Sowing,Area(mm)\n";
list1 = getFileList(input);
x = 0;
for (r = 0; r<list1.length; r++) {
    path = input+list1[r];
    open(path);
    x = x + 1;
    setTool("rectangle");
    waitForUser("put the box over the Plot");
    run("Crop");
    row = plotinfo(x);
    setTool("line");
    waitForUser("make a 102mm line");
    M = Dist(102);
    run("Color Transformer", "colour=HSL");
    run("Stack to Images");
    selectWindow("S");
    setAutoThreshold("Li");
    run("Convert to Mask");
    selectWindow("L");
    setAutoThreshold("Mean");
    run("Convert to Mask");
    run("Invert");
    selectWindow("H");
    run("Duplicate...", "title=j");
    setAutoThreshold("IsoData");
    run("Convert to Mask");
    run("Invert");

    imageCalculator("Add", "H","S");
    run("Make Binary");
    setTool("rectangle");
    waitForUser("put the box over the Plot");
    run("Crop");
    run("Analyze Particles...", "size=0-Infinity circularity=0.00-1.00 show=Nothing
clear");
    res = TotArea(M);
    // Save the total areas
    // Subscript to close all images
}

```

Appendix J SCREEN CAP OF MANUAL METHOD

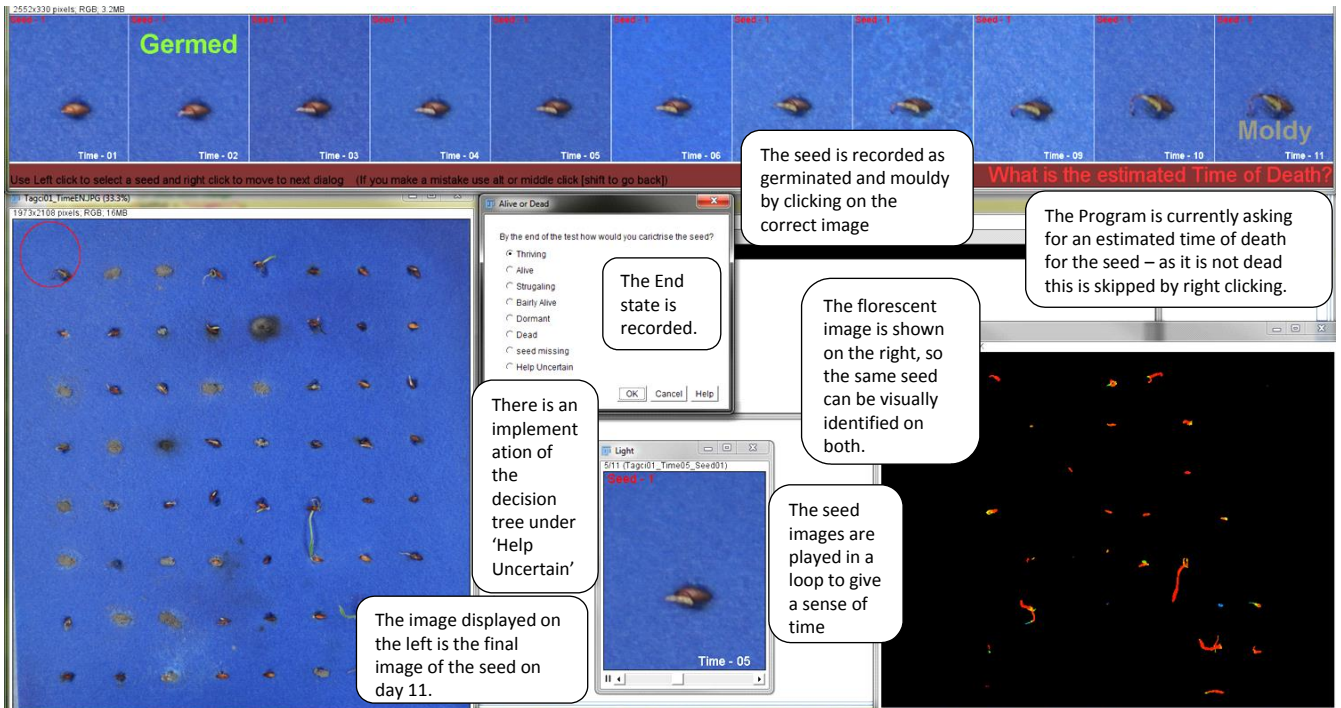


Figure 3: A labelled screen capture of the method used to identify germination from a series of photos of an individual seed manually.

Appendix K SEEDLING END STATE DECISION TREE

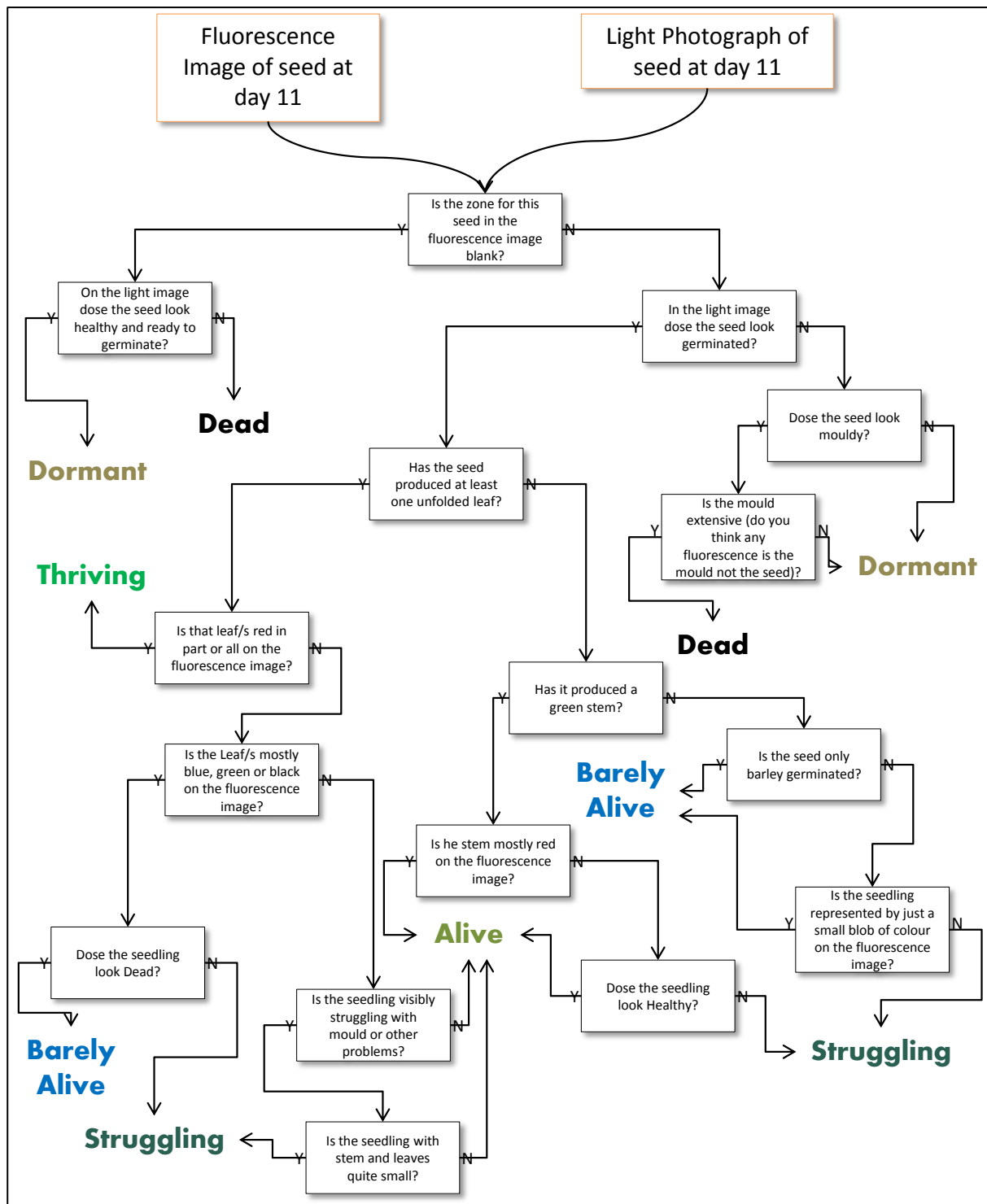


Figure 4: The decision tree used to determine seed end states.

Appendix L TAGUCHI RESULTS TABLES

Detailed inputs and outputs for the Taguchi experiment. The raw Taguchi analysis was performed by Dr Sreenivas Rao Ravella of Aberystwyth University.

Table 3: The results for all 16 Taguchi experiments, each of the eight metrics that went from the experiment into the Taguchi analysis are along the top with the numbers calculated below; weightings are shown at the bottom.

Experiment Number	Germination Index	Stem: Root	Fv/Fm Area	Fv/Fm Median	Percentage Germ 7d	Germ Rate (1/T50)	Stem Elongation	Root Elongation
1	1.688	3.64	338.6	0.795	29.688	0.0313	12.2	3.35
2	3.047	2.52	375.4	0.739	54.688	0.0294	8.14	3.24
3	2.344	1.96	133.0	0.776	39.063	0.0357	5.87	3.00
4	0.563	2.73	32.36	0.683	12.500	0.0187	5.00	1.83
5	2.484	2.69	227.3	0.774	46.875	0.0243	6.14	2.29
6	2.344	3.81	345.6	0.797	40.625	0.0311	11.1	2.91
7	0.422	1.40	6.2	0.271	7.8125	0.0272	1.40	1.00
8	2.828	1.79	207.0	0.764	48.438	0.0332	6.45	3.61
9	2.281	1.04	10.0	0.680	39.063	0.0320	1.36	1.31
10	0.266	1.00	0.5	0.261	4.6875	0.0282	1.00	1.00
11	2.094	2.94	225.0	0.775	34.375	0.0427	9.65	3.28
12	2.594	2.54	161.8	0.694	46.875	0.0302	6.50	2.56
13	0.266	1.00	1.0	0.201	4.6875	0.0307	1.00	1.00
14	1.188	0.88	10.9	0.443	21.875	0.0268	1.46	1.67
15	2.172	1.05	60.1	0.735	39.063	0.0272	2.85	2.70
16	2.672	1.51	55.7	0.744	46.875	0.0313	2.52	1.67
Weightings	0.25	0.2	0.15	0.15	0.075	0.075	0.05	0.05

Table 4: A table showing the percentage effects on each metric from each factor, from the Taguchi analysis. Metrics may not total to 100% due to rounding.

Experimental Factor	Germination Index	Stem: Root	Fv/Fm Area	Fv/Fm Median	Percentage Germ 7d	Germ Rate (1/T50)	Stem Elongation	Root Elongation
Abscisic Acid	3.1	36.3	33.6	15.1	10.9	16.1	36.1	21.5
Gibberellic Acid	4.4	3.9	5.4	8.1	10.2	13.7	0.2	4.9
Auxin	4.3	13.9	2.3	1.2	9.6	7.2	8.7	11.9
Brassinosteroid	5.5	4.2	8.8	9.5	19.4	22.9	5.8	11.3
Water Stress	35.6	38.1	44.3	35.9	24.2	2.7	38.0	28.0
Light Reduction	14.1	1.0	4.9	19.3	3.0	37.4	10.4	13.1
Priming	33.1	2.5	0.7	10.8	22.6	0.0	0.8	9.2

Table 5: The optimal dosage for each treatment in the Taguchi experiment for each metric recorded.

Experiment Number	Germination Index	Stem: Root	Fv/Fm Area	Fv/Fm Median	Percentage Germ 7d	Germ Rate (1/T50)	Stem Elongation	Root Elongation	Units
Abscisic Acid	0.2	0.02	0.02	0.02	2	2	0.02	0.02	mg L ⁻¹
Gibberellic Acid	15	15	0.15	15	0.15	1.5	0.15	1.5	mg L ⁻¹
Auxin	0.05	0.5	0.5	5	0.005	0.5	0.5	0.005	mg L ⁻¹
Brassinosteroid	0.75	0.02	0.75	7.5	1.5	0.75	0.75	0.75	mg L ⁻¹
Light Reduction	80	80	80	80	80	80	80	80	PPFD
Water Stress	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	-MPa
Priming	no	yes	no	no	no	yes	yes	no	

Appendix M SOIL TEMPERATURE MONITORING

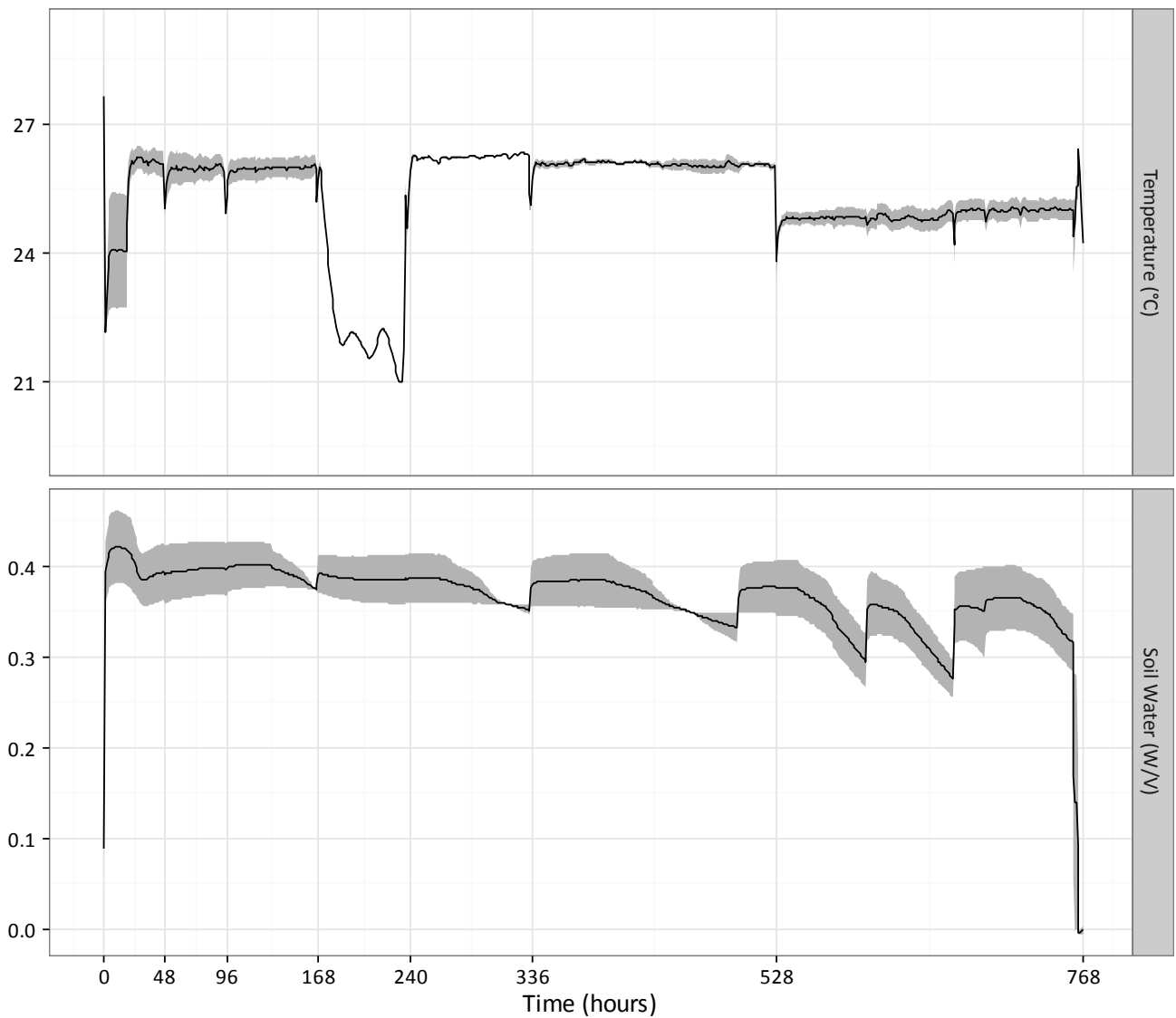


Figure 5: Mean soil water and temperature measured by two reflectometers and two type T thermocouples within a controlled environment in different trays of soil. A power cut is visible at 170 hours. The grey zone represents the standard error.

Appendix N MULCH FILM CE EXPERIMENT

A small experiment was conducted in a controlled environment to test the effect of mulch film. This was done to test how hot the soil surface got under film, how much protection from the cold film gave to the soil and if the transitions from day to night temperatures were slower under film.

The experiment was set up with two seed trays of soil from the 'Direct Sowing Agronomy Trial' and 'Multi Genotype Direct Sowing Trial' Aberystwyth field location. Each tray was filled with sieved (3 mm) soil, and two type T (single point calibrated) thermocouples were added, along with one CS616 water content reflectometer, connected to a Campbell Scientific CR1000. This logged every minute and averaged the readings every 5 minutes. The controlled environment attempted two day night cycles, both 6 hours night and 18 hours day, the first was 8°C and 22°C the second was 5°C and 25°C.

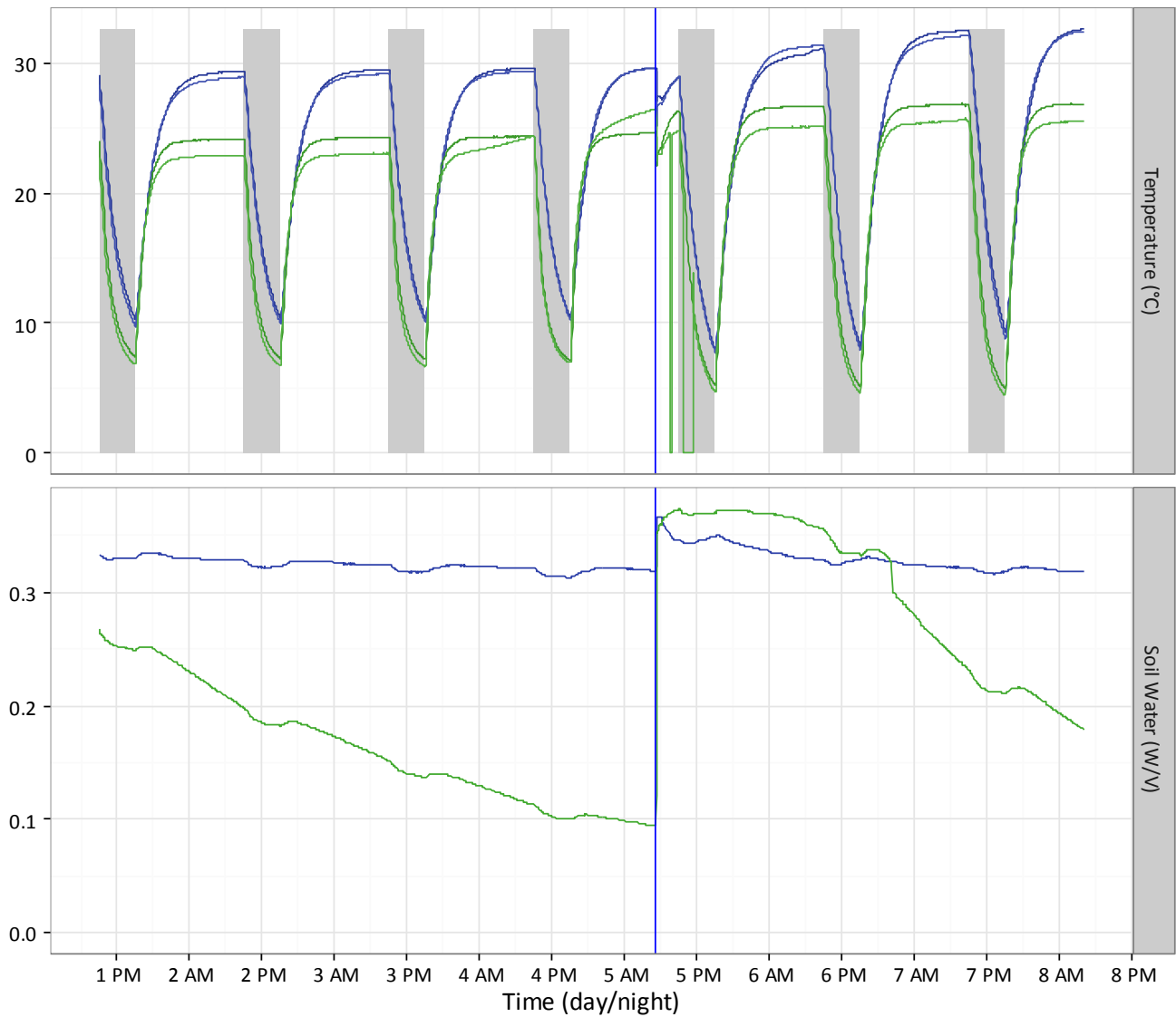


Figure 6: Film (blue) and not film (green) monitoring at the soil surface for temperature and moisture over eight days of the experiment. The blue vertical line shows where the soil was watered and the temperature settings were changed (8 down to 5°C at night, and 22 up to 25°C for the day).

The data was analysed excluding day five, which was the transition period, with the first temperatures one to four days and the second six to eight days. It is clear in Figure 6, that the uncovered container loses water much more quickly (-4.9% to -0.5% per day), particularly during the first temperature range.

Table 6: Temperature information averaged across the times and sensors to show the general effects of film at both controlled environment settings.

Treatment	Settings day/night	Minimum temperature	Maximum temperature	Mean temperature	Median temperature
Control	8 – 22°C	6.7°C	24.4°C	18.9°C	22.8°C
Film	8 – 22°C	9.7°C	29.6°C	23.3°C	26.5°C
Control	5 – 25°C	4.5°C	26.9°C	21.7°C	25.1°C
Film	5 – 25°C	7.9°C	32.6°C	26.3°C	30.1°C

In both controlled environment settings film fell by $\sim 3^{\circ}\text{C}$ less at night and peaked more than 5°C higher (Table 6). On average, the differences were more subtle but film was still 4.48°C higher, the difference in medians was that film was 4.31°C higher (Table 6).

Appendix O METEOROLOGICAL DATA

Monthly meteorological data for both sites in the ‘Direct Sowing Agronomy Trial’ and the Aberystwyth site in the ‘Multi Genotype Direct Sowing Trial’ from Chapter 6 is in Figure 7 below.

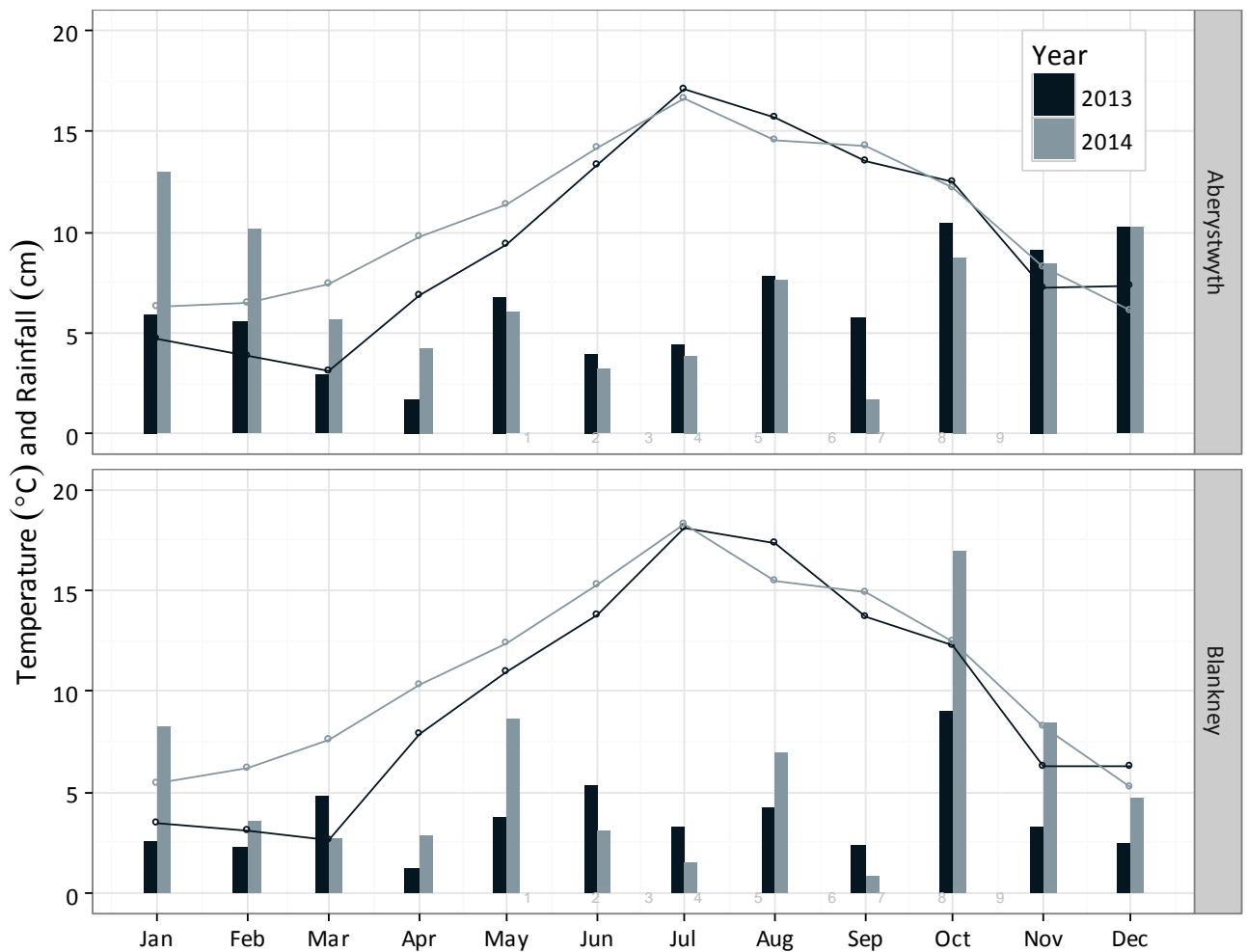


Figure 7: Lines show mean monthly air temperature (°C) near the Aberystwyth and Blankney field sites, bars represent total rainfall (cm). Both are coloured with the grey showing the second year data and the black the first year data. Faint sowing numbers at the time in the first year each was sown, are shown along the bottom in light grey.

Data collected from weather station by Mr Ashman of Aberystwyth University.

Appendix P MODEL MAIN CODE

The method of the seed class used to calculate if the seed has germinated.

```
# Test if seed has germinated with the current accumulated thermal time and water level
def seed_germ(self, day_temp, day_water):
    # increase thermal time up till germination
    self.tt += (day_temp - self.base_germ)
    self.is_seed_lost()
    # Calculate if the seed is dead or lost in the soil
    if self.tt >= self.tt_req and self.dead == False and day_water >= self.base_wat:
        self.germ = True
```

The method of the seed class used to calculate growth.

```
def seed_growth(self, Temp, wat, setup, clod_bed, crusty):
    # only increment thermal time if seed can grow
    if not self.stuck_clod and not self.stuck_crust:
        # tt since germination (used for weibull eq)
        self.ttd += (Temp - self.base_elong)
    # test if the seedling has any ransom to have died
    self.is_seedling_dead(wat)
    if self.dead == False:
        # germinated and alive one more day
        self.ds_germ += 1
        if self.ds_germ > 1:
            growth = self.growth(setup, Temp)
            if not self.emerge:
                # Dose the growing seed hit a clod
                hit = Fun.growth_pos_check(clod_bed, [self.pos[0], self.pos[1],
self.pos[2] + self.hieght + growth])
                if hit != 0 and not self.circumvented:
                    # can it circumvent the clod it is under
                    self.clod_circumvent(setup, clod_bed, hit)
                # has the seedling hit a crusty surface
                self.crust_stuck(setup.sow_depth, growth, crusty)
            # if notning is stoping it - let it grow
            if not self.stuck_clod and not self.stuck_crust:
                # Increment growth
                self.hieght += growth
```

The method of class seed used to determine if a clod was passable.

Appendices

```
def clod_passable(self, setup):
    if 30 < self.clod.rotation[0] > 170:
        self.clod_impassable = False
    if (self.clod.pos[0] - setup.min_hit/4 < self.pos[0] > self.clod.pos[0] +
        setup.min_hit/4) and (self.clod.pos[1] - setup.min_hit/4 < self.pos[1] > self.clod.pos[1]
        + setup.min_hit/4):
        self.clod_impassable = False
```

A digital copy of the full working model is available at

<https://github.com/Miscanthus-Germination/Miscanthus-model>.

Appendix Q ALL THERMAL TIME GERMINATION GRAPHS

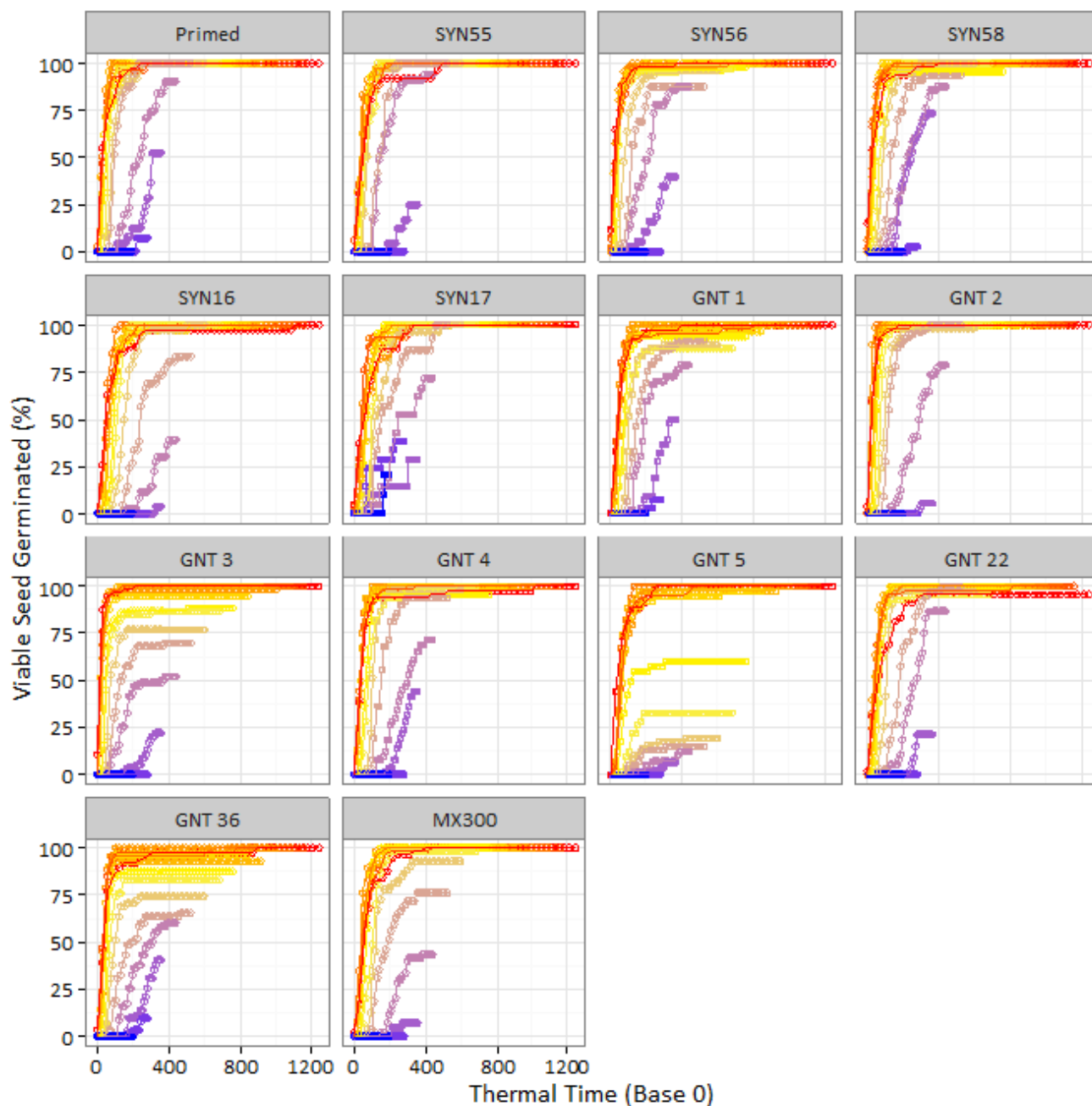


Figure 8: All seed lots' germination with thermal time set to 0°C, used to calculate base temperature in chapter 6.

Appendix R THERMAL GRADIENT TEMPERATURE GRAPHS

Table 7: The measured temperature of each cell on the thermal gradient as measured by the CA5233 Chauvin Arnoux. 1°C below the required temperature is shown in strong blue and cells 1°C above the required temperature is shown in strong red. Correct temperature ("Temp") and average values for all the rows are shown at the bottom.

R1	4.9	7.1	9.2	11.1	13.2	15.1	17.1	19	21.1	22.8	24.7	26.5	28.9	30.9
R2	4.7	6.7	8.9	10.8	12.7	14.8	16.8	18.9	20.8	22.8	25	27	28.9	31.2
R3	5.2	6.9	9	11	13.1	15.4	17.4	19.3	21.4	23.4	25.3	27.2	29.2	31
R4	5	6.9	8.9	11.1	13.3	15.2	17.3	19.2	21.1	23.1	25	26.8	28.7	30.9
R5	5	7.1	9.1	10.8	12.9	15.1	16.9	19	21.1	23.3	25.1	27.1	28.9	30.8
R6	4.5	6.5	8.5	10.7	12.6	14.6	16.6	18.8	21	22.8	24.7	26.5	28.5	30.9
R7	4.6	6.8	9.1	11.2	13.3	15.3	17.3	19.2	21.1	23.1	24.9	26.9	28.6	31.1
R8	4.6	6.5	8.5	10.7	12.6	14.6	16.7	18.7	20.7	22.6	24.9	26.7	28.4	30.5
R9	4.6	6.9	9	11.1	13.1	15	16.9	18.8	20.6	22.5	24.8	26.7	29.1	30.8
R10	4.8	6.7	8.7	10.8	12.9	14.8	16.8	19	20.6	22.7	24.9	26.6	29.1	30.9
R11	4.8	6.8	8.8	10.9	12.8	14.8	16.7	18.8	20.9	22.7	25.1	26.9	29	31
R12	5	7	9	11	13	15	16.9	18.9	20.9	22.8	25.3	26.9	29.2	31.2
R13	5.2	7.4	9.4	11.3	13.2	15.3	17.1	18.8	21	23.3	25.2	27	29.1	31.4
R14	6	8	9.5	11.4	13.3	15.1	17.2	19.2	21.3	23.1	25.1	27.2	29.3	30.4
Temp	5	7	9	11	13	15	17	19	21	23	25	27	29	31
mean	4.9	7	9	11	13.0	15.0	17	19	21	22.9	25.0	27	28.9	30.9
median	5	6.9	9	11	13.1	15.1	16.9	19	21	22.8	25	26.9	29	30.9

As Table 7 shows, there is consistency on the thermal gradient; the thermocouples attached to the plate were only used to monitor the temperature for consistency (Figure 9).

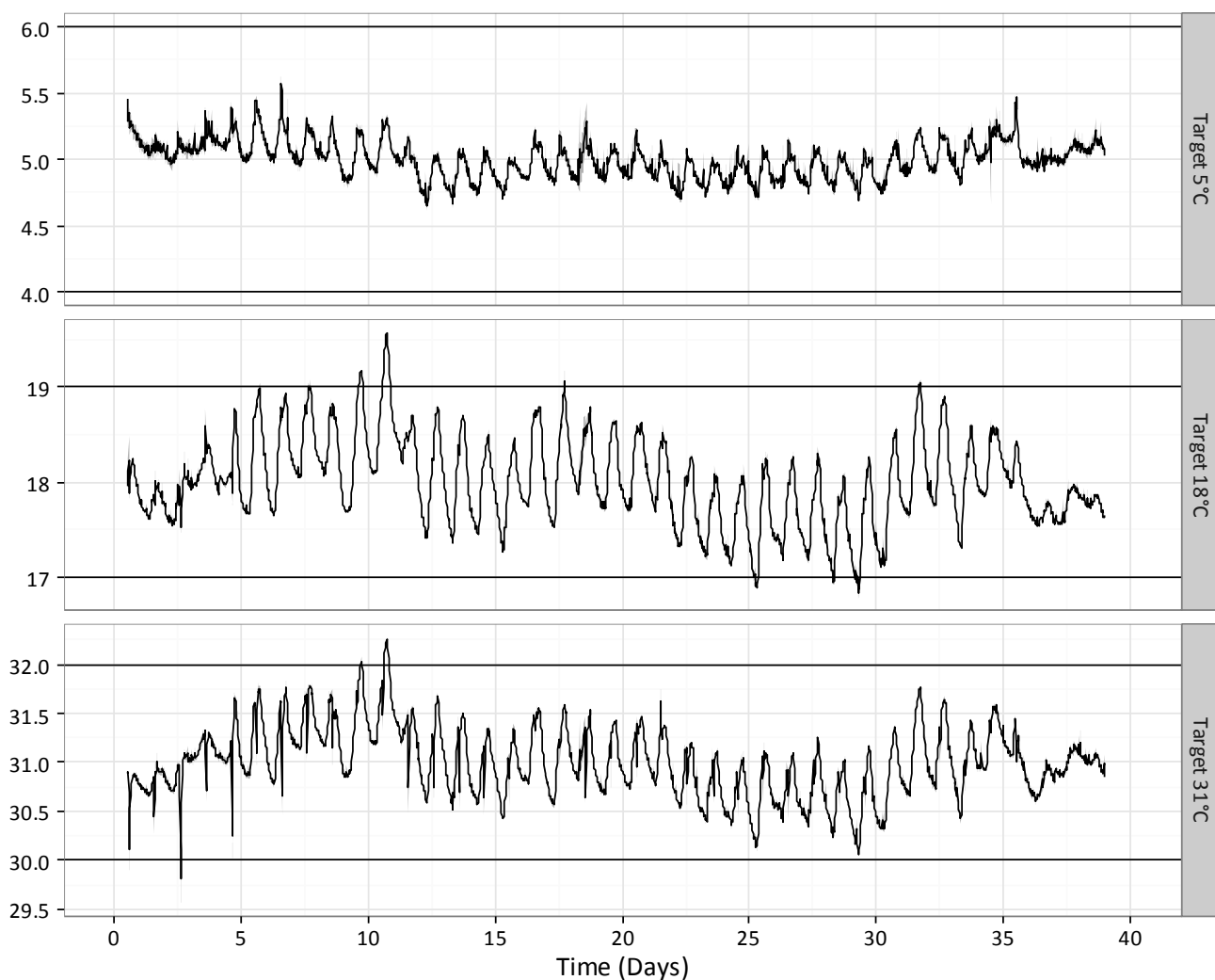


Figure 9: Thermal gradient plate thermocouple values from 'Thermal Gradient for Seed Germination' experiment (chapter 6), adjusted for systematic errors, and plotted with standard errors in grey. The black lines highlight a 1°C drift.

There are some diurnal temperature fluctuations as seen in Figure 9; this was due to the room the thermal gradient plate was situated within at the time of the experiment.

Appendix S TG TEMPERATURE GRAPHS FOR ELONGATION

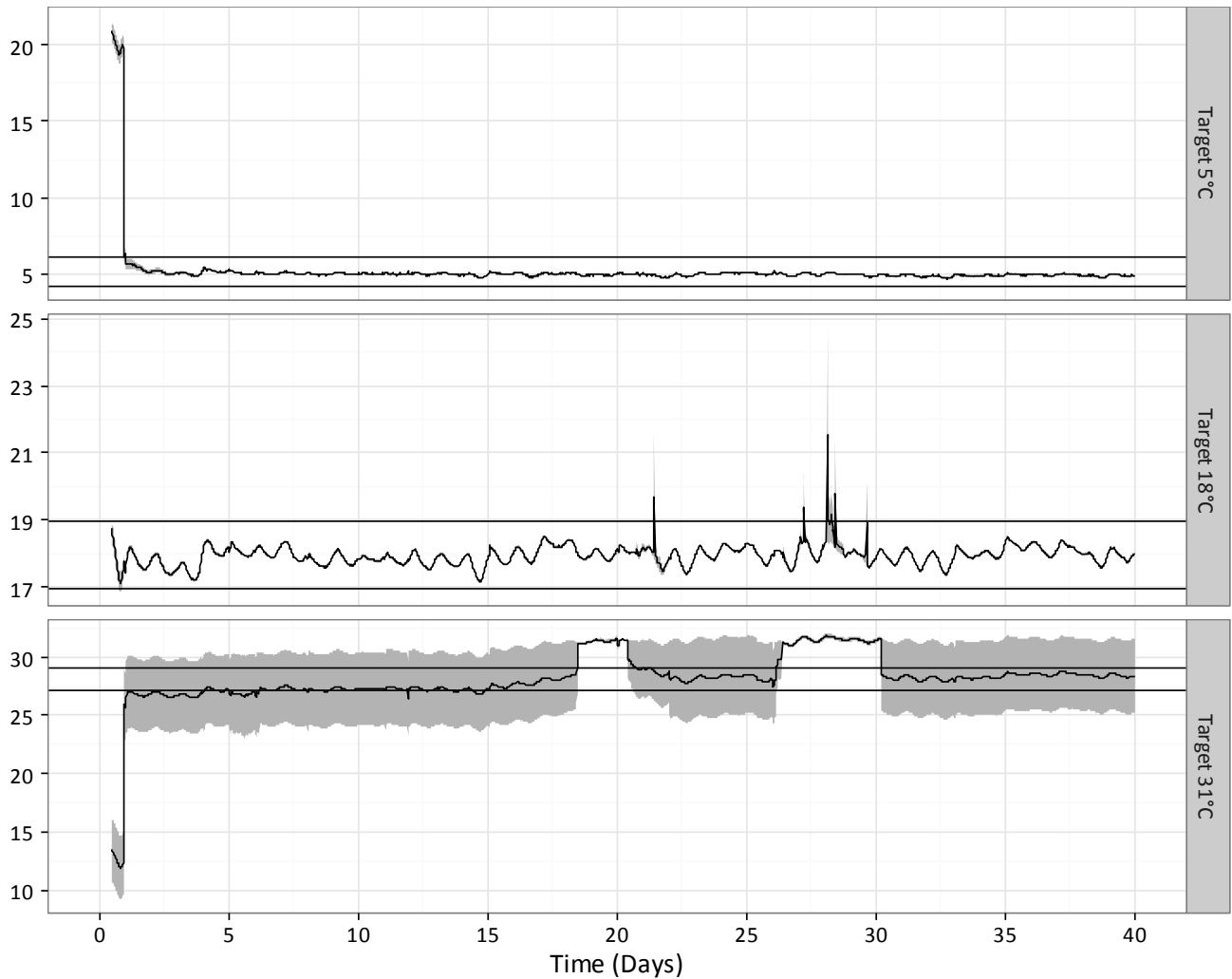


Figure 10: The temperature graphs for the 'Thermal Gradient for Seedling Stem Elongation' experiment, with standard error in grey and black lines highlighting when the readings are 1°C out.