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Seed development and germination of Miscanthus sinensis

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Seed development and germination of *Miscanthus sinensis*

by

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ABSTRACT

Seed development and germination research was conducted to understand the biology of *Miscanthus sinensis* (Andersson). A thermogradient table was used to determine whether *M. sinensis* germinated better under constant or alternating temperature conditions and which temperature treatments provided the best environment for maximum seed germination percentage for the species. It was determined that *M. sinensis* germinated best under alternating temperatures and achieved the highest germination with a combination of 22 and 16°C for 16 and 8 h. Results from the thermogradient table experiment were used to guide the design of the second experiment. The second experiment explored the effect of temperature combinations, light, and dormancy breaking techniques on seed germination and was used to determine a base for establishing a standard germination protocol for *M. sinensis*. Alternating temperatures commonly used by seed laboratories (15/25, 15/30, 20/30°C for 16/ 8 h) were compared to the best germination temperature combination from the first experiment (22/16°C for 16/8 h) obtained using the thermogradient table. In addition, seeds were subjected to either 8 h of light during the period corresponding to the higher germination temperature, or 24 h of dark. The influence of dormancy breaking techniques on seed germination was also explored. A 500 mg L^{-1} GA_3, 2000 mg L^{-1} KNO_3, and a 5°C prechill for 7 d were compared to an untreated control. The highest germination of freshly harvested seeds was recorded when germinated using 20/30°C for 16/8 h. There was no significant difference in the germination of one-year-old seed regardless of the germination temperature treatment. Across all seed lots and years there was no significant difference in the germination percentage between seeds germinated using light and those kept in the dark. The germination percentage of seeds treated with GA_3 and prechill treatments was

significantly higher than the germination percentage of seeds treated with KNO_3 and control treatments. Finally, an experiment was conducted to examine the water relations and seed development of *M. sinensis*. Plants were grown in the greenhouse and crossed to produce seed. Light microscopy was used to create a seed development timeline. Seeds of *M. sinensis* reached physiological maturity at 30 d after pollination and moisture content of 33%. These results give us a better understanding of the biology of *M. sinensis* seeds. In the future when *M. sinensis* becomes a more widely cultivated species, these results will help seed producers and farmers make informed decisions on seed production and quality.

CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation is organized using the journal paper format. It begins with chapter 1: A literature review followed by three journal papers. Chapter 2: "Alternating Temperatures Promote Seed Germination of *Miscanthus sinensis*" covers research completed using a thermogradient table. It has been submitted and accepted by the Journal of Seed Technology. Chapter 3: "Temperature and light requirement for *Miscanthus sinensis* germination" and Chapter 4: "Seed development and water relations of *Miscanthus sinensis*." Completing the dissertation is Chapter 5: General Conclusions.

Literature Review

The renewed interest in cellulose-based production of biofuel has prompted the reevaluation of many candidate plant species. In order for a plant species to be considered a viable candidate for biofuel production, it must meet several criteria. According to Sticklen (2008), an ideal plant species would be one that is a C4, perennial, has high water use efficiency and redistribution of nutrients into the below ground portions of the plant.

The plants from the genus *Miscanthus* have great potential because of their favorable agronomic traits and abundant biomass production (Clifton-Brown et al., 2008). These plants are perennial and when above ground harvest is delayed, they deposit nutrients into their rhizomes, thus reducing the amount of nutrients needed for growth in the following season (Beale and Long, 1997; Clifton-Brown et al., 2008). *Miscanthus* × *giganteus* Greef et Deu ex Hodkinson et Renvoize has received special attention as a widely adapted sterile hybrid of *Miscanthus,* capable of yielding large volumes of biomass. *Miscanthus* × *giganteus* is mostly

propagated by plant rhizomes and does not produce fertile flowers or seeds. Because of its vegetative method of propagation, large scale production of the species is limited because crop establishment is costly and requires specialized equipment (Atkinson, 2009; Clifton-Brown et al., 2008; Clifton-Brown and Lewandowski, 2000; Clifton-Brown and Lewandowski, 2002). Possible solutions for reducing the cost of establishment and promoting the wide adoption of this crop, are to increase the amount of land devoted to rhizome production or to use the seed-propagated species *Miscanthus sinensis* (Andersson) to plant new fields (Christian et al., 2005).

Reproductive Biology of the Miscanthus Plant

Miscanthus spp. are C4 perennial rhizomatous grasses (Clifton-Brown and Lewandowski, 2002; Ercoli et al., 1999) that are self-incompatible (Petersen et al., 2002; Quinn et al., 2010) and must cross-pollinate to produce seed, similar to many other C4 grasses. *M.* × *giganteus* is a cross between two species of *Miscanthus*: *M. sinensis*, which is diploid, and *Miscanthus sacchariflorus* (Maxim.) Franch., which is tetraploid (Scurlock, 1999). *Miscanthus sacchariflorus* is normally diploid. Because of the triploid nature of *M.* × *giganteus*, any seeds produced are sterile (Clifton-Brown and Lewandowski, 2002).

One agronomic advantage of *Miscanthus* production as a biomass crop is its nutrient relocation prior to harvest (Himken et al., 1997). When harvesting is delayed until the spring, nutrients are relocated to the rhizomes for growth in subsequent seasons.

There is concern about the invasive potential of *Miscanthus* (Barney and DiTomaso, 2008; Quinn et al., 2010; Raghu et al., 2006). A weed is defined as an unwanted plant species. Many of the factors that make it a good candidate as a biomass species also qualify it as a potential weed species. Its ability to thrive under low input systems is an example of

both a crop and a weed characteristic. Therefore, monitoring species movement is required in order to keep *Miscanthus* from becoming a problem. *Miscanthus sinensis* and other seeded *Miscanthus* species also pose the risk of seed escapes. Since *M. sinensis* is a wind-dispersed seed, it can very easily spread into areas where it is not wanted. This trait makes *M. sinensis* a possible candidate invasive species.

Grass Seed Morphology

The seeds of grasses are also called caryopses. A caryopsis is a single-seeded, dry, indehiscent, hard fruit in which the seed coat and the ovary wall fuse to form the pericarp (Rost et al., 1979). The length of time between the fertilization of the ovule and seed physiological maturity varies widely among grass species (Jones, 1985) ranging from 8 to 60 days from fertilization to maturity, during which time it will undergo enormous change as it develops into a mature seed. Following fertilization, seed fresh weight increases rapidly coupled with cell division and water uptake. At a certain point during seed development, the rate of cell division decreases and cell expansion begins (Esau, 1977; Rost et al., 1979). Once the connection with the plant is severed, the seed has reached its maximum fresh weight. The seed then dries down and enters a quiescent state until proper conditions for germination are met. Seeds of *M. sinensis* should follow a similar developmental pattern of other C4 grass species.

Miscanthus sinensis

Miscanthus sinensis (Andersson), which from this point on will be referred to as *M. sinensis*, is a seed bearing species of *Miscanthus*. Rhizomes of *M. sinensis* are shorter than those of *M.* × *giganteus* and, therefore, develop into plants that form in bunches. Due to its

growth habit, *M. sinensis* has a higher shoot density than *M.* × *giganteus* (Clifton-Brown et al., 2001).

In comparative studies with *M.* × *giganteus* and *M. sacchariflorus* at five locations across Europe, *M. sinensis* showed the most cold hardiness (Clifton-Brown et al., 2001). *Miscanthus sinensis* had stand losses in Sweden between 1 and 60% the first year compared with *M.* × *giganteus,* which suffered almost 100% mortality. Winter soil temperatures in Sweden reached a low of -5.4° C. This considered, it was determined that *M. sinensis* has an advantage in cooler growing areas when compared to *M.* × *giganteus*. Although research conducted in Illinois has shown that *M.* × *giganteus* was able to survive winter temperatures as low as -8° C (Heaton, 2006), the overall conclusion is that *M. sinensis* has shown it is more cold hardy than *M.* × *giganteus* and *M. sacchariflorus*. However, it does not produce as much biomass as *M.* × *giganteus* grown in in temperate areas.

Miscanthus sinensis hybrids show potential as higher yielding cultivars when compared to *M. sinensis* wild populations. *Miscanthus sinensis* hybrids have also shown similar yields to *M.* × *giganteus* in the year of establishment and the second growing season, but have not produced similar yields in subsequent seasons (Clifton-Brown and Lewandowski, 2002). Hybrids of *M. sinensis* are created when two populations are mated. This is in contrast to open pollinated varieties of *M. sinensis*.

Quality of biomass is another factor for comparison between *M. sinensis* and *M.* × *giganteus*. A few early senescing varieties of *M. sinensis* produced better biomass quality (ash, moisture, etc.) than *M.* × *giganteus* (Clifton-Brown and Lewandowski, 2002).

Miscanthus sinensis Seed

Miscanthus sinensis reproduces by orthodox seed (Stewart et al., 2009). Orthodox seeds are seeds which can be dried down after physiological maturity (Copeland and McDonald, 2004). This is the opposite of recalcitrant seeds which cannot be dried down after physiological maturity (Copeland and McDonald, 2004). Seeds of *M. sinensis* are very small, usually measuring from 1.9 to 2.7 mm in length and 0.74 to 1 mm in width and weighing between 0.5 and 1.4 mg per seed (Aso, 1976; Hayashi, 1979). Due to the light weight of its seeds, *M. sinensis* is anemochorous (Hayashi, 1979). Anemochorous seeds are seeds for which the main dispersal method is the wind. Hydrochory and zoochory are two other methods of seed dispersal in which water and animals, respectively, move the seed. Wind dispersal is an ecological adaptation of plants usually requiring the production of large amounts of lightweight seed. Hayashi (1979) reported *M. sinensis* produces between 962 and 1051 seeds per plant. It is also important to note that according to a review by Stewart et al. (2009), *M. sinensis* is self-incompatible (Petersen et al., 2002).

Little is known about the germination requirements for *M. sinensis* seed. Aso (1976) looked at dormancy and germination of *M. sinensis* during the late 1960's and early 1970's in Japan, and studied the effect of gibberellic acid on germination. The author found that seed size was not related to final seed germination percentage. Large seeds initiated germination earlier, but did not have a higher germination percentage than smaller seeds. Aso (1976) also found that seed germination percentage increased when gibberellin was added to the germination media at rates of 10-50 ppm. However, rates higher than 50 ppm injured the seeds.

M. sinensis is a C4 warm-season grass and, therefore, requires warmer temperatures for germination (Jones, 1985). Aso (1976) suggested that the temperature requirement for germination is between 20 and 30°C.

Germination Testing

In order to standardize germination testing in the United States, a group of seed scientists and seed analysts formed the Association of Official Seed Analysts (AOSA) in 1908 (Association of Official Seed Analysts, 2011). The International Seed Testing Association (ISTA) was formed in 1924 and is an international body which standardizes and serves the seed testing industry worldwide. By establishing an organization in which seed laboratories can be involved, seed testing can be standardized, and by standardizing seed testing procedures, seed testing laboratories operate under a more repeatable and reliable system. Seeds are tested for two physiological quality characteristics: viability testing and vigor testing. Seed viability testing is the determination of maximum germination potential of a seed lot under ideal growing conditions, while seed vigor testing evaluates how the seed lot performs under less than optimal conditions.

Standard germination test is a viability test used to determine the suitability of a seed lot for planting. These results are used for labeling seeds for sale and are printed on the seed tag to inform the buyer of the physiological quality of the seed lot. There are several seed vigor testing procedures and these tests are divided into categories according to the basis for seed vigor evaluation used. Seed vigor tests are divided into seedling growth and evaluation tests, stress tests and biochemical tests (Association of Official Seed Analysts, 1983).

Most of the commonly planted species have a set of protocols used to determine seed lot viability and vigor. Species without standardized protocols must be researched in order to

create a standard germination test. The research into developing a seed testing protocol for new species should include determining the requirements for temperature, light, and dormancy alleviation. Baskin and Baskin (2001) recommend any seeds used in germination studies should be harvested at physiological maturity or soon thereafter. Seeds allowed to dry down and stored for a period of time often have reduced dormancy or no dormancy at all.

Once fundamental research is completed, a standard germination test protocol can be developed and implemented. Rules are added and labs across the world are able to use the same standards to test the viability and vigor of a species.

Germination Temperature

Temperature plays a key role in seed dormancy and germination. Every plant species has temperature requirements for germination. These requirements are a direct result of the indigenous environment of the species natural habitat. Many factors relating to temperature must be studied including the following: does the species require constant or alternating temperatures to break dormancy and initiate germination? Does the species have a requirement for alternating temperatures, what are the minimum and maximum temperature points and what is the amplitude (the difference between the high and low temperature) requirement?

It is widely recognized that some plant species need alternating temperatures to germinate. In many cases, soil temperature sends a signal to the seed to either germinate or remain dormant and soil temperatures fluctuate with the seasons. Through selection and adaptation to different environments, seeds have different requirements for germination. Thompson et al. (1977) screened 292 plant species to determine their need for alternating temperatures. Of those 292 species, 68 required alternating temperatures when germinated in

the light. Species requiring alternating temperatures also exhibited temperature amplitudes ranging from 1 to 9°C.

Alternating or different diurnal and nocturnal temperatures promoted a higher germination rate in goosegrass (*Elusine indica*), perennial ryegrass (*Lolium perenne* L.) as well as in the weed species common waterhemp (*Amaranthus tuberculatus* L.) and giant foxtail (*Setaria faberi* Herrm.) (Leon et al., 2004; Nishimoto and McCarty, 1997; Shen et al., 2008).

Temperature amplitude also plays a key role in temperature promotion of germination. Leon and Owen (2004) showed that common waterhemp and giant foxtail required an 18°C temperature amplitude for optimum germination, but in many cases a temperature amplitude as little as 1°C can promote germination over no amplitude at all (Thompson and Grime, 1983; Thompson et al., 1977).

In order to create alternating temperatures in the lab, a thermogradient table is used. The table is a very useful tool, often used in germination ecology studies, because it allows the researcher to create a gradient of multiple temperatures at one time (Larsen, 1965). Creating multiple temperatures alleviates the need for multiple germination chambers and reduces the time it takes to obtain results.

Seed Dormancy

Seed dormancy is defined as the inability for a viable seed to germinate when environmental conditions are favorable for germination. Seeds are first classified as either being in primary or secondary dormancy. Primary dormancy is a state of dormancy that is acquired directly after physiological maturity. Whereas, secondary dormancy is where seeds are non-dormant after physiological maturity, but acquire dormancy later on after

environmental conditions become less favorable. Within the categories of primary and secondary dormancy, there are an additional five classifications: physiological, morphological, morphophysiological, physical, and combinational (Baskin and Baskin, 2001). Physiological dormancy is a type of dormancy in which the embryo is inhibited from germinating. Most often physiological dormancy can be overcome by alternating temperatures which most likely leads to alleviation of metabolic inhibitors. Morphological dormancy is a type of dormancy in which the embryo is not fully developed at physiological maturity. Once the embryo reaches full development, the seed can germinate. Morphophysiological dormancy is a combination of physiological and morphological dormancy. Seeds with morphophysiological dormancy have both an immature embryo and a physiological limitation to germination. To overcome morphophysiological dormancy, seed usually need time and a chemical or environmental pretreatment in order to break dormancy. Physical dormancy is an inhibition such as water impermeability by the seed coat which is usually due to hardseededness. Hardseededness occurs when the seed coat has some sort of impermeable layer, most likely suberin, which impedes water imbibition (Bewley and Black, 1994). Physical dormancy is overcome by mechanical means, such as scarification. Combinational dormancy is a both physiological and physical dormancy. The seed not only has an impermeable seed coat, but also an underlying physiological dormancy mechanism.

Seeds can be tested for both viability and germination. Dormant seeds are recorded as viable, but when a germination test is conducted, do not germinate. At the end of the germination testing period, any seeds which are still intact and not germinated are subjected to a 2, 3, 5,-triphenyl tetrazolium chloride or TZ test. The TZ test is a seed viability assay, which is also called a quick germination test, and it is based on a biochemical reaction. The

colorless TZ compound changes into a bright red compound (formazan) as a result of the release of hydrogen molecules during cell respiration (Association of Official Seed Analysts, 2010; Copeland and McDonald, 2004). Consequently, live cells in the imbibed seed actively respiring stain red, while dead cells remain unstained (white). Dormant seed, which are live seeds, stain red even though they do not germinate when placed under ideal conditions for germination. By conducting a TZ test following a germination test, the scientist can get a clearer picture of the level of dormancy in the seed lot. For example, at the end of a standard germination test, if there are very few ungerminated seeds remaining, the seed lot either has a very low dormancy rate, or the dormancy has been alleviated by the test conditions. If a seed lot has a large amount of ungerminated seeds at the conclusion of the standard germination test that are viable, then the seed lot has a high level of dormancy, which was not alleviated by the testing procedures.

Effect of Light on Germination

All plant species vary in their light requirement for seed germination. Some seeds require light to germinate (Jones, 1985), while others do not. The effect of light on plants has a two-fold purpose, it not only supplies energy, but it also supplies information providing necessary signaling for stress response (Copeland and McDonald, 2004). Seeds only rely on light to supply information or signaling, because the energy is supplied by the seed reserves. The light can activate phytochrome photoreceptor in the seed, which translates this information or signaling into a response. The light requirement of a seed can vary during the period of time from shedding, through dormancy (if present), and ending at germination (Bewley and Black, 1994).

In this review, we will focus primarily on the effect of light on the induction of germination. The phytochrome protein photoreceptor is the main mechanism in which seeds receive light-induced signals (Bewley and Black, 1994; Hendricks and Borthwick, 1967; Toole, 1973). The conversion of the phytochrome red (Pr) protein to the phytochrome far red (Pfr) protein is light-induced and this signal initiates the biochemical process necessary for dormancy-breaking and germination. Phytochrome red, in the embryonic axis, absorbs light radiation at the 660 nm wavelength (Bewley and Black, 1994). Once the light is absorbed, a sequence of events takes place which alleviate dormancy.

Some species require light to germinate, others require the complete absence of light, and others are indifferent. Fulbright et al. (1983) found that 8 h of constant light inhibited the germination of the cool-season green needlegrass (*Stipa viridula* Trin.). Flenniken et al. (1987) found that seeds of brownseed paspalum (*Paspalum plicatulum* Michx.) germinated at a higher rate in the light. The annual weed species barnyardgrass also germinated better under light conditions over dark (Boyd and Van Acker, 2004), while the commonly cultivated crop species, wheat (*Triticum aestivum* L.), along with the weed species, foxtail barley (*Hordeum jubatum* L.), green foxtail [*Setaria viridis* (L.) Beauv.], and wild oat (*Avena fatua* L.), germinated equally well in the dark or in the light. Switchgrass (*Panicum virgatum* L.), sideoats grama [*Bouteloua curtipendula* (Michx.) Torr.], and annual ryegrass (*Lolium rigidum* Gaud.) germinated equally well in the dark, as they did when supplied with 8 h of light (Aiken and Springer, 1995; Cole et al., 1974; Gramshaw, 1972).

Growth Hormones and Germination

It is possible a seed will require the use of a plant hormone to break dormancy. Common plant hormones are gibberellins, abscisic acid, cytokinins, and auxins. In this

review, we will focus on the growth and seed germination promoting hormone gibberellic acid.

There is a delicate balance between the growth promoting hormones and the growth inhibiting hormones in each seed, a balance that can be affected by many environmental factors. This balance, occurring in every seed, leads to the alleviation (or maintenance) of dormancy and the initiation (or inhibition) of germination.

Gibberellic acid or GA_3 is commonly used by germination labs to promote the germination of species in which dormancy is an issue. Four varieties of switchgrass were tested for the effect of a 0.25mM GA_3 treatment (Zarnstorff et al., 1994). Three varieties showed no response to GA_3 and one variety, Blackwell 6096, showed an increase in germination from 59 to 65%. Willis et al. (1991) had mixed success with the use of a 500 mg L^{-1} GA_3 solution on seven forb species from Australia. The germination of *Helipterum albicans* (A. Cunn.) DC. and *Stylidium graminifolium* Swartz ex Willd. increased when exogenous application of GA_3 promoted germination; whereas, the addition of GA_3 had the opposite effect on *Vittadinia muelleri* N.T. Burbidge in which germination was inhibited. The germination percentage of five Australian native grass species improved when exogenous GA_3 was used in concentrations from 100 to 1000 mg L^{-1} (Mott, 1978). Germination also increased in eastern gamagrass seed, without the cupule, when GA_3 was used (Tian et al., 2003).

Other Methods to Promote Germination

Sometimes other methods are required to promote seed germination in the laboratory. Some seeds require a period of cold, moist conditions (stratification or prechill), (Copeland and McDonald, 2004). Cold and moist conditions simulate the conditions in nature that

occur with the changes of the seasons. A 5°C moist prechill for 14 d increased the final germination percentage of switchgrass seed over that of the control (Zarnstorff et al., 1994). Temperature of 2 to 5°C for 30 d increased the germination percentage of dormant green needlegrass seeds (Fulbright et al., 1983).

Another way in which seed laboratories promote germination is the use of potassium nitrate (KNO_3). The mechanism by which KNO_3 affects seed germination has not yet been conclusively determined (Copeland and McDonald, 2004). It is, however, known that KNO_3 works very well to stimulate germination of seeds that possess requirement for light. Germination of wild cherry (*Prunus avium* L.) seeds was increased when concentrations of 7,500 mg L^{-1} KNO_3 were used to stimulate germination (Çetinbaş and Koyuncu, 2006). A 0.002 M solution of KNO_3 was enough to increase the seed germination of four grass species: *Agrostis capillaris* L., *Holcus lanatus* L., *Poa trivialis* L., and *Festuca rubra* L. (Williams, 1983).

Seed Development

To effectively produce seed of good physiological quality for sale and planting, a good understanding of seed formation is required. Therefore, the majority of seed development research has been directed toward cultivated species. Knowledge of seed development guides seed production decisions, such as irrigation timing and harvest. By knowing the timeline of seed formation, seed physiologist, seed analysts, and seed producers can make informed decisions about the quality of seed being produced. Because of the large amount of information available about seed development, this review will focus on the period of development between anthesis and physiological maturity in monocotyledonous species.

Anthesis is the point when the stigmas are receptive to pollen (Esau, 1977) and physiological maturity is the point of maximum dry weight accumulation (Copeland and McDonald, 2004).

Seed development can be divided into three stages: histodifferentiation, reserve deposition, and maturation drying (Copeland and McDonald, 2004). During histodifferentiation, cells within the seeds are dividing and multiplying. During reserve deposition, the cells formed during histodifferentiation gain mass, and finally, after the seed has reached physiological maturity, it begins to dry down and enters a quiescent state.

Studies on the development of pearl millet (*Pennisetum glaucum* L.) showed that fertilization occurred at 3 to 5 h after pollination and the first zygotic division occurred 9 to 10 h after fertilization (Taylor and Vasil, 1995). Embryo maturation in pearl millet was observed at 11 d after pollination. In *Paspalum dilatatum* Poir., fertilization occurred approximately 8 to 12 h after pollination and embryo maturation occurred at 14 to 18 d after pollination (Bennett, 1944). When studying the development of *Sorghum bicolor* (L.) Moench, Paulson (1969) observed that fertilization occurred at 2 to 4 h after pollination and maturity was reached at 25 d after pollination.

Seed Water Relations

Between anthesis and physiological maturity, seeds rapidly gain weight due to the rapid uptake of water and nutrients (Copeland and McDonald, 2004). As physiological maturity approaches, seed water content decreases. Research conducted for developing seed water relations curves is very difficult to conduct (Egli and TeKrony, 1997; Gambín and Borras, 2005) because factors such as varietal differences, weather/environment, and seed position on the inflorescence can all lead to different results.

Frey et al. (1958) found that oat (*Avena sativa* L.) seed moisture content was between 43 and 48% moisture at physiological maturity. Depending on the seed lot, physiological maturity occurred between 13 and 24 d after anthesis. The moisture content of sorghum seeds at physiological maturity was between 23 and 31%, and was reached between 33 and 45 d after pollination (Kersting et al., 1961). Egli and TeKrony (1997) found that soybean, wheat, and maize reached physiological maturity at 34, 30, and 40 d after pollination, respectively. Smooth bromegrass (*Bromus inermis* L.) reached physiological maturity after 17-18 d and at a moisture content of 47% (Grabe, 1956).

The objectives of this research are to add to the understanding of seed biology of *M. sinensis* by developing a standard germination test protocol and creating a seed development timeline for this species. The main factors (temperature, light, and pretreatment) that make-up a standard germination protocol will be researched in order to determine the proper combination which maximizes *M. sinensis* germination. A standard germination test protocol will be advantageous to seed laboratories and seed companies which deal with *M. sinensis*. Seed development of *M. sinensis* will be explored using light microscopy. Seed water content and dry weight will be paired along with images obtained from light microscopy. These two sets of information should give researchers and seed producers a better idea of the crucial times in the development of *M. sinensis* seed.

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CHAPTER 2. ALTERNATING TEMPERATURES PROMOTE SEED GERMINATION OF *MISCANTHUS SINENSIS*

A paper submitted to Seed Technology

Erik J. Christian and A. Susana Goggi

Abstract

Miscanthus sinensis can propagate through seed. However, there is little information regarding seed germination requirements. The objective of this study was to determine the optimal germination temperature requirements for the species. Seed germination was studied using a two-way thermogradient table without light. The temperature gradient in the table had 30 cells with alternating day/night temperatures and 6 cells with constant temperatures. The constant temperature cells were 10, 16, 22, 28, 34, and 40 °C, and the alternating temperature cells were a combination of these same temperatures. Fifteen of the alternating temperatures were higher for 16 h and lower for 8 h. The other 15 alternating temperatures were higher for 8 h and lower for 16 h. Seeds exposed to alternating temperatures showed a higher germination percentage value than those at constant temperatures. The highest germination percentage was recorded in cells with a temperature combination of 16 °C for 16 h and 22 °C for 8 h. These results are important to seed analysts developing a standard germination protocol for *Miscanthus sinensis* seed.

Introduction

Renewed interest in cellulose-based production of biofuel has prompted the reevaluation of many candidate plant species. Plants of the genus *Miscanthus* have strong potential for use in

biofuel production because of their favorable agronomic traits and abundant biomass (Clifton-Brown et al., 2008). *Miscanthus* × *giganteus* J. M. Greef & Deuter ex Hodk. & Renvoize has received special attention because it is a widely adapted sterile hybrid of *Miscanthus* capable of yielding a large volume of biomass. *Miscanthus* × *giganteus* is propagated by plant rhizomes or tissue culture and does not produce fertile flowers or seeds. This vegetative method of propagation constrains large-scale production of this hybrid. Crop establishment is costly and requires specialized equipment (Atkinson, 2009; Clifton-Brown et al., 2008; Clifton-Brown and Lewandowski, 2000; Clifton-Brown and Lewandowski, 2002). Possible solutions for reducing the cost of establishing this crop are to increase rhizome production or to use the seed-propagated species *Miscanthus sinensis* Andersson to plant new fields (Christian et al., 2005).

Seeds of *M. sinensis* are small, usually measuring from 1.9 to 2.7 mm in length and 0.74 to 1 mm in width, and weighing between 0.5 and 1.4 mg (Aso, 1976; Hayashi, 1979). Due to the weight of its seeds, *M. sinensis* is dispersed by wind (Hayashi, 1979). Wind dispersal is an ecological adaptation of plants requiring the production of large amounts of light-weight seed. Hayashi (1979) reported *M. sinensis* produces between 962 and 1051 seeds per plant.

Little is known about the germination requirements of *M. sinensis* seed. Aso (1976) examined dormancy and germination of *M. sinensis*, as well as the effect of gibberellic acid on germination. He found that final seed germination percentage was not related to seed size and although large seeds initiated germination earlier, they did not have a greater germination percentage than smaller seeds.

All plant species vary in their light and temperature requirements for seed germination. Some seeds require light to germinate (Baskin and Baskin, 2001; Bewley and Black, 1994; Copeland and McDonald, 2004; Jones, 1985), whereas others do not. In many cases, soil

temperature signals the seed to germinate. Soil temperature fluctuates with the seasons, sending signals to seeds to either germinate or remain dormant (Baskin and Baskin, 2001). Through selection and adaptation to specific environments, plants have specific requirements for seed germination. *Miscanthus sinensis* is a C4, warm-season grass and, therefore, requires a relatively warm temperature for germination (Jones, 1985). Aso (1976) suggested that the temperature requirement for germination of *M. sinensis* was between 20 and 30 °C. Currently, no rules exist to test *M. sinensis* germination.

The objective of this research was to evaluate germination-temperature regimens to use in the development of a germination protocol for *M. sinensis*. While previous studies of *M. sinensis* germination focused on wild collections (Aso, 1976), this study focused on lines in development as commercial varieties. Understanding the temperature requirements for germination of improved varieties of *M. sinensis* will aid in the development of a standard germination protocol.

Materials and Methods

Four seed lots of *M. sinensis* seeds were obtained from Mendel BioEnergy Seeds, Hayward, CA. Seeds were produced in 2008 and the seed lots represented a randomly selected group typical of those received in a seed laboratory for testing. No other information on seed origin, viability, or quality of the seed lots used was available. Seeds were stored in a cold room at 10 °C and 50% RH until tested in 2010. Seed lots were conditioned to remove broken seeds before testing. Only seeds from the pure seed component following a purity analysis were used in this experiment.

A thermogradient table was used to evaluate the effect of temperature on germination. The table was built in-house and consisted of an aluminum plate measuring 75 × 75 × 3.5 cm with four channels running along the edges. Cooled and heated water was pumped through the

channels by two circulating water baths. Cooled water circulated through two perpendicular channels and heated water circulated through the other two creating a temperature gradient across the table. The flow of water was set for 16 h in one direction and 8 h in the opposite direction to simulate the fluctuations in temperature between day and night. This two-way thermogradient table created 36 areas with different temperatures, 30 cells with alternating temperatures, and 6 cells with constant temperatures. Fifteen of the alternating temperatures were higher for 16 h and lower for 8 h. The other 15 alternating temperatures were higher for 8 h and lower for 16 h. The remaining six temperatures were constant throughout the 24-hour cycle. These constant temperatures were 10, 16, 22, 28, 34 and 40 °C. Table 1 lists the different temperature regimens used in this study. The mean temperature for each alternating temperature regimen was calculated as a weighed mean using the formula $[(T_{16} \times 16) + (T_8 \times 8)] \times 24^{-1}$, where T_{16} was the temperature during 16 h and T_8 the temperature for 8 h (Table 1). The amplitude was calculated as the difference between high and low temperature ($T_{max} - T_{min}$).

The thermogradient table was covered to allow seeds to germinate in the absence of light. Seeds were only briefly exposed to normal low intensity fluorescent light, while being evaluated every 48 h. Twenty-five seeds of each seed lot were planted on moistened blotter paper (Stults Scientific Engineering Corporation, Springfield, IL) inside plastic boxes measuring 11 × 11 × 3.5 cm. The seeds were watered when planted, then watered as needed for the duration of the experiment.

Every 48 h the boxes were removed from the thermogradient table to be evaluated under a dissecting scope (Fisher Scientific Company LLC, Pittsburgh, PA). Seed germination was recorded when the radicle had emerged 1 mm from the seed. Germinated seedlings were removed from the boxes to avoid secondary seedling infection from seed pathogens in case they

were present in the seed lot. The experiment was terminated at 14 d and any ungerminated seeds were subjected to a firmness test to assess viability (Borza et al., 2007). The firmness test consisted of pressing the seeds with forceps. Firm seeds were classified as viable, while seeds that collapsed under the pressure of the forceps were classified as dead. The entire experiment was repeated three times.

Each plastic box containing seeds was considered an experimental unit. The experimental design was a randomized complete block, with three replications over time. The experiment was blocked by time due to the size-restriction imposed by the thermogradient table. The main effects were temperature and seed lot and the interactions between block × temperature and temperature × seed lot were tested. The three-way interaction was used as the error term. Temperature was considered as a fixed effect, while seed lot and blocks were random. Germination data were analyzed using the generalized linear model statement (GLM) of SAS release 9.2 (SAS Institute, 2009) after testing and verifying that data was normally distributed and that error variances were homogenous. Germination means were compared using Tukey's test at the 5% probability level. Also, germination mean single degree of freedom contrasts were calculated between constant temperature and alternating temperature regimens.

Results

Based on ANOVA results, the main effect of seed lot and the interactions between temperature and seed lot were not significant (data not shown). Consequently, we discuss the results of temperature and seed lot independently.

The single degree of freedom contrasts calculated between the mean germination percentage for all seed lots showed that mean germination percentage was significantly greater

($p < 0.0001$) at alternating temperatures than constant temperatures, with an overall mean of 51.4% for alternating temperatures compared to 40.9% for constant temperatures.

The numerically highest mean germination percentage (64.3%) was observed at the alternating temperature regimen of 16 h at 22 °C and 8 h at 16 °C (Table 1). However, this germination percentage was not statistically different from the mean germination percentage recorded at the next best 26 temperature regimens. The germination percentage of seeds at constant temperature of 10 °C was significantly lower than all other temperature regimens and was the lowest of all constant temperature regimens used in the experiment at 10.3% (Table 1). The germination percentage at constant temperatures of 16, 22, and 28 °C were not significantly different from each other. Germination percentage of seeds germinated using the constant temperature regimens were not among the top five values. When treatments were ranked based on germination percentage, three of the top five alternating temperature regimens were 16 h warm, with temperature ranging from 22 to 28 °C, and 8 h cool temperatures ranging from 10 to 16 °C (Table 1). The top five ranking treatments were observed when mean temperatures ranged from 14 to 22 °C and had an amplitude of 6 to 18 °C. Two of the top-ranked five temperature regimens where highest germination percentage were recorded, had lower temperature for 16 h and higher temperature for 8 h (Table 1). This regimen is the standard temperature and time combination normally used in commercial laboratories. The temperature during the 16 h period was 10 °C and 22 °C during the 8 h time period. The mean temperature was 14 °C and the amplitude was 12 °C.

Discussion

In order to develop a standard germination protocol for a species several factors must be considered, the main one being the optimum germination temperature. Seed testing laboratories

use temperature regimens which simulate natural conditions in order to obtain the maximum germination from a seed lot. Both alternating and constant temperatures are used, depending on the species and their inherent temperature requirements. The temperature regimen that maximized *M. sinensis* seed germination in our study was a combination of 22 °C for 16 h and 16 °C for 8 h. The weighted mean temperature of the top-ranking alternating temperature regimens was 18 °C and the amplitude was 10 °C (data not shown). The mean germination percentage for seeds germinated in temperature regimens with an amplitude of 6 °C was 9% higher than the mean germination percentage of seeds germinated at a constant temperature. Previous studies had shown that for some species even an amplitude of 1 or 2 °C was enough to promote germination over an amplitude of 0 °C (Baskin and Baskin, 2001; Bewley and Black, 1994). In six of the nine treatments resulting in the highest germination values, the higher temperature was applied for 16 h and the lower for 8 h. These alternating temperature combinations were the reverse of those prescribed in the AOSA rules for testing seeds (Association of Official Seed Analysts, 2011) for almost all species requiring alternate temperatures, where the lower temperatures are prescribed for 16 h and the higher temperature for 8 h. Consequently, it would be impractical to use some of these temperature regimens when developing a standard germination test protocol for use in seed analysis, since the equipment in seed testing laboratories is usually set for the opposite temperature/duration cycle.

Clifton-Brown et al. (2011) compared the germination of *M. sinensis* to switchgrass (*Panicum virgatum* L.), reed canary grass (*Phalaris arundinacea* L.), maize (*Zea mays* L.), and perennial ryegrass (*Lolium perenne* L.) to determine the thermal requirements for germination of *M. sinensis* and the geographical areas best suited for establishment using seeds. The authors showed that *M. sinensis* required a minimum germination temperature between 9.7 and 11.6 °C

to reach 50% germination of viable seeds. They also showed that seed germination of *M. sinensis* required warmer minimum germination temperatures when compared to other C4 grasses. Aso (1976) reported that constant temperatures between 25 and 30 °C resulted in the greatest seed germination. However, the author did not compare the effect of constant temperatures to alternating temperature regimens on seed germination. Our results showed that the constant 22 °C temperature regimen had the greatest germination percentage of all constant temperature used, but was significantly lower than seeds germinated at the 22/16 °C temperature regimen (Table 1).

Average germination of the four seed lots was relatively low with a mean of 49.6% over all temperatures (Table 1). Baskin and Baskin (2001) recommend that any seeds used in germination studies should be those freshly harvested at physiological maturity or soon thereafter. Seeds used in this study were produced in 2008 and were not fresh seed because at the time of this study as fresh seed was unavailable. Fresh *Miscanthus* seeds could have dormancy. However, seeds that are allowed to dry down and are stored for a period of time, often have reduced dormancy or no dormancy at all (Baskin and Baskin, 2001). However, according to previous work, *Miscanthus* does not exhibit dormancy (Matumura and Yukimura, 1975) and our results with one exception, indicated that the number of firm seeds was insignificant. Moreover, the rate of germination of seeds at the alternating temperature of 22/16 °C for 16/8 h, showed that most viable seeds germinated within the first 10 d, indicating that the seed lot achieved maximum germination and that seed vigor was good (Fig. 1). Within the first six days of our study, 81% of the viable seeds had germinated at this alternating temperature (Fig. 1). The remaining 18% of viable seeds germinated within the next four days. Aso (1976) showed that *M. sinensis* seeds reached their maximum germination in 3 4, 5, and 9 d, when

germinated at constant temperatures of 30, 25, 20, and 15 °C, respectively. After 14 d, only one firm seed was found in the test. The firmness of this seed was confirmed using the published methodology by Borza et al. (2007). Borza et al. (2007) compared the results from the firmness test to those of the tetrazolium chloride test and demonstrated that there were no significant differences between these two tests. Consequently, this test was chosen due to the large number of ungerminated seeds at some cooler temperature regimens. Clifton-Brown et al. (2011) showed similar results where seeds subjected to alternating temperatures with means of 26.5 and 18.4 °C, achieved maximum germination within 5 and 10 d, respectively. When developing a standard germination test protocol, it will be important to use alternating temperatures and to corroborate the absence of seed dormancy in *M. sinensis*.

Results from our study indicated that progress has been made towards the development of a standard germination protocol for *M. sinensis* in the absence of light. The alternating temperatures of 22/16 °C in the dark produced the greatest germination. However, future studies should include fresh seeds to evaluate possible dormancy in *Miscanthus* seed and, if possible, test a higher number of seeds per sample. These conditions should be compared to temperature regimens currently used by germination laboratories to assess use-feasibility. Constant temperatures should not be considered when developing a seed germination test protocol for this species. It also has been shown that light has an effect on *M. sinensis* germination (Hsu, 1989) and the possibility of using light during the hours of exposure to higher temperatures should be explored in the development of a standard germination protocol.

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Table 1. Germination percentages for the 36 temperature treatments for 16 and 8 h, simulating diurnal fluctuations. Germination was recorded every 48 h for 14 d, and summed across all measurements to reach a total germination. Four varieties were used with three replications.

Temperature (°C)					
16h	8h	Mean[†]	Amplitude[‡]	Germination (%)	Mean of firm seeds at the end of the test (%)
22	16	20	6	64.3 a[§]	1
22	10	18	12	60.3 ab	1
10	22	14	12	60.3 ab	1
16	22	18	6	57.7 abc	0
28	10	22	18	57.3 abcd	1
40	16	32	24	56.7 abcde	0
10	16	12	6	56.7 abcde	3
34	10	26	24	56.3 abcde	1
28	16	24	12	56.3 abcde	0
22	28	24	6	55.0 bcde	0
22	22	22	0	54.3 bcde	0
10	28	16	18	54.3 bcde	1
10	34	18	24	53.7 bcde	0
40	28	36	12	53.3 bcde	0

Table 1. (Continued)

Temperature (°C)					
16h	8h	Mean[†]	Amplitude[‡]	Germination (%)	Mean of firm seeds at the end of the test (%)
40	22	34	18	53.3 bcde	0
34	28	32	6	53.3 bcde	0
16	16	16	0	53.3 bcde	1
34	16	28	18	52.7 bcdef	1
28	22	26	6	52.0 cdefg	0
22	34	28	26	52.0 cdefg	0
34	22	30	12	51.7 cdefgh	0
16	34	22	18	51.3 cdefgh	0
16	28	20	12	51.3 cdefgh	0
16	40	24	24	50.0 cdefgh	0
28	28	28	0	49.3 defgh	0
40	10	30	30	49.0 efgh	1
34	34	34	0	44.7 fghi	0
28	34	30	6	44.3 ghi	0
10	40	20	30	44.3 ghi	0
40	34	38	6	43.7 hij	0
34	40	36	6	38.3 ijk	1

Table 1. (Continued)

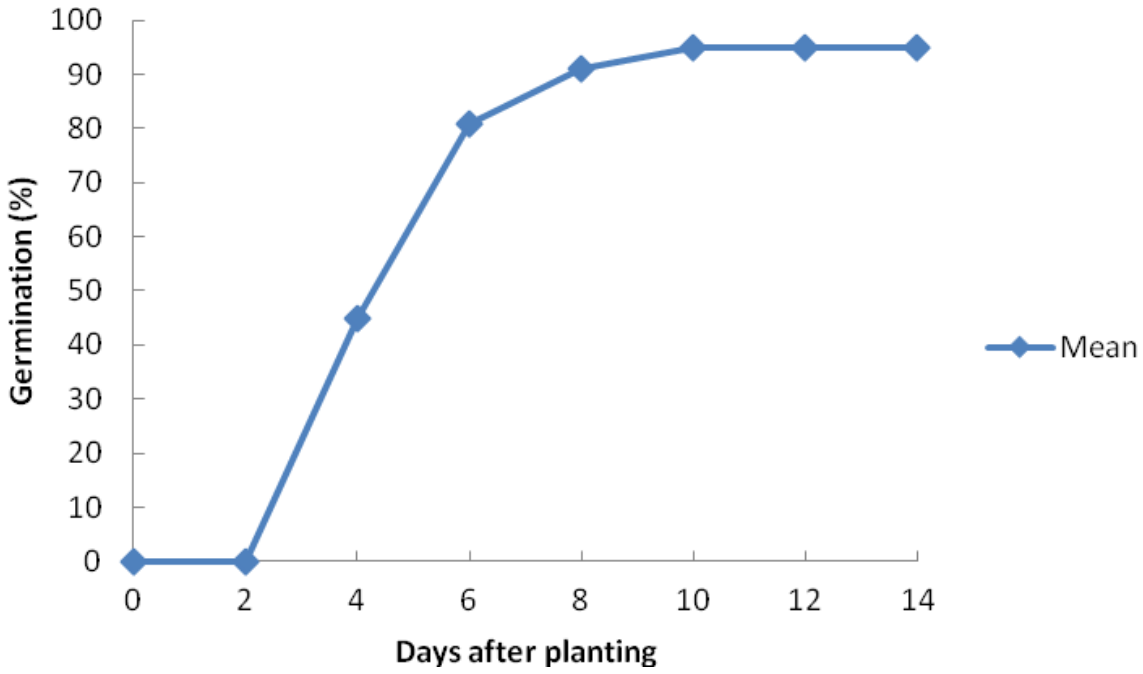
Temperature (°C)					
16h	8h	Mean[†]	Amplitude[‡]	Germination (%)	Mean of firm seeds at the end of the test (%)
22	40	28	18	38.3 ijk	0
28	40	32	12	37.7 ijk	0
16	10	14	6	36.0 jk	5
40	40	40	0	33.7 k	1
10	10	10	0	10.3 l	13
		Overall mean		49.6	

[†]Temperature mean is the weighted mean of 16 hours and 8 hours using the formula $[(T16 \times 16) + (T8 \times 8)] \times 24^{-1}$

[‡] Temperature amplitude was calculated using the formula $T_{max} - T_{min}$

[§]Means with the same letter are not different according to Fisher's LSD test at the $P \leq 0.05$ level.

Figure 1. Accumulated germination percentage of the viable seed of the 22/16°C for 16/8 h temperature regimen. After planting, germinated seeds were removed every 2 d until the experiment was terminated at 14 d and any ungerminated seeds were tested for viability



CHAPTER 3. TEMPERATURE AND LIGHT REQUIREMENTS FOR *MISCANTHUS SINENSIS* GERMINATION

A paper to be submitted to Crop Science

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Abstract

Miscanthus sinensis is an important species for biomass production. However, a standardized seed germination protocol is not available. Our research objectives were to determine the temperature, light, and dormancy breaking requirements for optimal seed germination of Miscanthus. Miscanthus seeds from four varieties and from two production years were planted on top of blotters and germinated at four different alternating temperature regimens: 15/25, 15/30, 20/30, and 22/16°C for 16/8 h. Seeds harvested in 2011 (fresh seeds) germinated best when subjected to 20/30°C for 16/8 h, whereas germination of seeds harvested in 2010 were not affected by a specific temperature regimen. The difference in germination requirements between fresh and year-old seed may allude to the presence of dormancy. Light or the absence of light was not a significant factor in seed germination. Seed germination was enhanced when moist seeds were prechilled at 5°C for 7 d or germinated on media moistened with 500 mg L^{-1} GA_3 solution. These results highlight the importance of germination temperature and a dormancy breaking pretreatment when developing a standard germination protocol for *M. sinensis*.

Introduction

Interest in the C4 perennial grass *Miscanthus sinensis* (Andersson), which will be referred to as *M. sinensis***,** for biofuel production has been increasing. The species is not well

understood and there are many problems related to cultivation. One of the issues is how to determine seed viability. In the Rules for Testing Seeds (Association of Official Seed Analysts, 2011), a standard germination test is available to determine seed lot viability in the most widely cultivated crop and forage species. Currently, neither seed testing laboratories nor seed producers have a standardized germination test for *M. sinensis*. Factors to be considered when developing a standardized germination test are temperature, light, and any techniques needed for breaking dormancy. Seed dormancy is defined as the inability for a viable seed to germinate when environmental conditions are favorable for germination.

Some species require alternating temperatures to overcome dormancy and to germinate. When seeds are in the soil bank, alternating temperature conditions occur in the spring as soils begin to warm-up. Temperature cycle characteristics of minimum, maximum, and amplitude all play a role in signaling the seed to germinate. Each species has a unique germination temperature requirement, which often resembles conditions in its indigenous environment. The first step in determining a germination temperature is to determine whether the species requires constant or alternating temperatures. Bass (1959) used alternating temperatures of 15/30°C and 15/25°C for a period of 15 h at the highest temperature and 9 h at the lowest temperature to determine the optimum germination temperature for highland bentgrass (*Agrostis castellana* Boiss. & Reut.). The highest germination was recorded when seeds were subjected to 15/25°C and light during the 15°C portion of the alternating temperatures. Fifty-five different constant and alternating temperature treatments were explored for determining the optimum germination temperature of robust needlegrass (*Achnatherum robustum* [Vasey] Barkw.) (Young et al., 2003). The

authors determined that alternating 15/20°C for 16/8 h in the dark was the optimum temperature combination for germination of this species.

Some species require a period of cold temperatures and moist stratification before germination. This requirement is called prechill or stratification. Seeds are planted in moistened substrata and subjected to a temperature below the minimum for germination (Copeland and McDonald, 2004). The germination percentage of green needlegrass (*Stipa viridula* Trin.) seeds increased when subjected to a prechill of 2-4°C for one month prior to planting (Fulbright et al., 1983). On the other hand, a prechill for 5 d at 5°C produced a lower germination in seeds of Texas needlegrass (*Stipa leucotricha* Trin. and Rupr.) (Andersen, 1965). A 21-d prechill at 4.4°C promoted a higher germination of green needlegrass when seeds were placed on top of moistened blotters (Niffenegger and Schneiter, 1963). Seeds of big bluestem (*Andropogon gerardii* Vitman), indiangrass [*Sorghastrum nutans* (L.) Nash], and switchgrass (*Panicum virgatum* L.) also showed increased germination when subjected to a 5°C prechill for 14 d (Olszewski and Folin, 2009; Watkinson and Pill, 1998; Zarnstorff et al., 1994).

The effect of light on seed germination has two main components: light quantity and quality. Intensity of light signals day length and light quality signals depth of seed burial. There are three preferences regarding light: seeds that require light for germination, those that require the absence of light or darkness, and seeds that are indifferent to light. Seeds of brownseed paspalum (*Paspalum plicatulum* Michx) exhibited a higher germination in light than in darkness (Flenniken and Fulbright, 1987). Whereas, seeds of green needlegrass germinated better in darkness under alternating temperatures (Fulbright et al., 1983). Sideoats grama [*Bouteloua curtipendula* (Michx) Torr.], switchgrass, and annual ryegrass

(*Lolium rigidum* Gaud.) germinate in both light and dark conditions (Aiken and Springer, 1995; Cole et al., 1974; Gramshaw, 1972).

When light and/or temperature are not sufficient to break seed dormancy of a species, other methods are employed. For example a germination-promoting hormone such as GA_3 may be added. Additional dormancy breaking methods might include subjecting seeds to a cold moist stratification, or a germination-promoting compound such as KNO_3.

Buffered GA_3 solutions increased the germination of eastern gamagrass [*Tripsacum dactyloides* (L.) L.] seed when the cupules were removed (Tian et al., 2003). A treatment of 500 mg L^{-1} GA_3 increased desert beardtongue (*Penstemon parryi* Gray) germination (Raeber and Lee, 1991). The combination of 1000 mg L^{-1} GA_3 with a 5°C prechill for 14 d increased the seed germination of indiangrass [*Sorghastrum nutans* (L.) Nash.] in the lab (Watkinson and Pill, 1998). Soaking seeds of fall panicum (*Panicum dichotomiflorum* Michx.) in GA_3 solution 24 h prior to planting was insufficient to break dormancy and improve seed germination (Brecke and Duke, 1980). Aso (1976) studied the effect of GA_3 on *M. sinensis* germination and determined that concentrations of GA_3 from 0.1 to 100 mg L^{-1} increased seed germination percentage when compared to the untreated control.

KNO_3 is a salt used in a water-based solution for breaking seed dormancy and promoting germination in seed testing laboratories. A small increase in seed germination of wild field collected downy brome (*Bromus tectorum* L.) was observed when seeds were treated with a solution of 0.1 mmol KNO_3 in combination with 0.1 mmol GA_3 (Evans and Young, 1975). A 0.2% solution of KNO_3 had no effect on the germination of Virginia pepperweed (*Lepidium virginicum* L.) germinated in the dark (Toole et al., 1955), but increased the germination of little barley (*Hordeum pusillum* Nutt.) when applied in

combination with alternating temperatures (Fischer et al., 1982). A 0.2 % KNO_3 treatment increased seed germination percentage of both hulled and unhulled bermudagrass [*Cyndon dactylon* (L.) Pers] seed (Ahring and Todd, 1978).

Due to a current lack of data that could lead to the development of a standardized seed germination testing protocol for *M. sinensis*, it is our objective to determine the combinations of temperature, light, and dormancy breaking treatments that lead to the highest germination of *M. sinensis* across seed varieties and seed lot ages.

Materials and Methods

Seed Source

Four seed lots of *M. sinensis* seed were obtained from Mendel BioEnergy Seeds, Hayward, CA in December 2011 and stored in a cold room at 10°C and 50% relative humidity for approximately 2 mo until used in these experiments. Each seed lot had two harvest dates, November 2010 and November 2011, for a total of eight seed lots. The seed was received cleaned, without the lemma and palea. Seeds were sorted by hand in the laboratory to remove any broken or damaged seeds, as well as any foreign matter.

Seed Viability Determination

The initial seed viability of all varieties of Miscanthus was evaluated using the tetrazolium chloride test. Fifty seeds were imbibed between two blotters (Stults Scientific Engineering Corporation, Springfield, IL) moistened with tap water for 14 h at 25°C. Seeds were then bisected longitudinally through the embryo. One half of the seed was placed into a 1000 mg L^{-1} 2,3,5-triphenyl tetrazolium chloride (TZ) solution for 5 h at 35°C, while the other half was discarded. Seeds were then evaluated using the guidelines for the *Poaceae*

group outlined in the Association of Official Seed Analysts/Society of Commercial Seed Technologists TZ Testing Handbook (Association of Official Seed Analysts, 2010). Two replications of 50 seeds per seed lot were evaluated.

Seed Germination Determination

Four different pretreatments were used for all varieties of Miscanthus. Seeds were planted in 16 × 27 × 4 cm plastic boxes (Melmat, Huntington Beach, CA) on top of two blotters (Stults Scientific Engineering Corporation, Springfield, IL) using a vacuum planter (E.L. Erickson Products, Brookings, SD).

In the first pretreatment, blotters were moistened with 70 ml of tap water and placed directly in the germination chamber. In the second pretreatment, blotters were moistened with 70 ml of tap water and boxes were placed in a pre-chill chamber at 5°C for 7 d before moving the boxes to the germination chamber. In the third pretreatment, blotters were moistened with a 500 mg L^{-1} GA_3 solution (Association of Official Seed Analysts, 2011), seeds were planted on the GA_3-moistened blotters, and boxes were placed directly in the germination chamber. Moreover, in the fourth pretreatment, blotters were moistened with a 2000 mg L^{-1} KNO_3 solution (Association of Official Seed Analysts, 2011), the seeds were planted on the KNO_3-moistened blotters, and boxes were placed in the germination chamber. After the initial pretreatments at planting, all boxes were moistened with tap water as needed for the duration of the seed germination period. In each pretreatment, seed varieties were randomized within boxes and 100 seeds from two varieties were planted in each box.

Two sets of identical boxes for each pretreatment were prepared, one set of boxes were subjected to a light treatment of 16 h light and 8 h dark, to simulate day/night differences, while the other half were wrapped in 0.10 mm-thick black plastic and subjected

to complete darkness for the entire experiment. There was a 1°C temperature difference between the inside the boxes wrapped in black plastic and those that were not. Based on the results of a preliminary seed germination temperature study in *M. sinensis* conducted using a thermogradient-table (Christian and Goggi, 2012), seeds were placed in four germination chambers (Hoffman Manufacturing, Inc., Jefferson, OR) at four different alternating temperatures: 15/25, 15/30, 20/30 and 22/16°C for 16/8 h, respectively.

Consequently, the experiment consisted of a total of eight temperature and light combinations and four pretreatments (GA_3, KNO_3, Prechill, and control) for a total of 32 seed germination treatment combinations (Table 2). The entire experiment was replicated twice over time using the same germination chambers.

Seedling Evaluation

Boxes were evaluated every 7 d. Normal, abnormal, and dead seeds were removed in accordance with the Association of Official Seed Analysts Rules for Testing Seeds (Association of Official Seed Analysts, 2011). Boxes in the dark treatment were evaluated under a green light to avoid even minimal exposure to light (Withrow, 1957). At the end of 21 d, all seeds were checked for firmness. All non-firm seeds were counted as dead and viability of all firm seeds were determined using the tetrazolium test, as previously described in Materials and Methods, Seed Viability Determination section.

Statistical Analysis

The experiment was designed as a randomized complete block with two blocks. Data were analyzed using proc glm in SAS (SAS Institute, 2009) after testing the data set for normality and homogenicity of the error variance. Year of production, seed lot, and block

were considered random effects. Main effect and interaction means were compared using the Tukey's means comparison test.

Results

The normality of the data set and homogenicity of the error variances were confirmed so the data did not require transformation. Table 3 shows the tests of significance for all main effects and their interaction. The interaction among year of production, germination temperature, and seed lot was significant ($P \leq 0.05$). Consequently, interaction means were calculated and presented in Table 4. The effect of dormancy breaking pretreatment on seed germination also was significant ($P \leq 0.001$). The presence or absence of light did not have a significant effect on seed germination of *M. sinensis* (Table 3).

The temperature regimes used in this experiment had no significant effect on the germination of seed harvested in 2010 (Table 4). The germination percentage for all 2010 varieties and for all germination temperatures ranged from 52 to 63% (Table 4). Seed viability of varieties harvested in 2010 was also higher as determined by the germination percentage and the initial seed viability (TZ) values, compared to seed harvested in 2011. The seed viability values ranged from 76 to 96% for seed harvest in 2010 and 46 to 60% for seed harvested in 2011.

The germination percentage of seed harvested in 2011 was significantly affected by germination temperature and the different varieties were affected differently. The germination percentage of seed from Seed lot 2 harvested in 2011 was highest (29%) at the alternating germination temperature of 20/30°C for 16/8 h, while germination of Seed lot 3 was lowest (9%) at the alternating germination temperature of 22/16°C for 16/8 h. Overall,

the 22/16°C alternating temperature showed the highest number of viable dormant seeds following the experiment. The least number of dormant seeds at the end of the experiment in 2010 was recorded for the alternating temperature treatments of 15/30°C and 20/30°C.

Pretreating the seed germination media with 500 mg L^{-1} GA_3 and a 5°C prechill for 7 d significantly increased the germination of *M. sinensis* to 42 and 41% respectively over that of the control (36%) (Table 5).

Discussion

This research focused on the effects of temperature, light, and dormancy-breaking pretreatment on seed germination of *M. sinensis*. An initial TZ test was conducted on the seed lots to determine seed viability. The initial seed viability values (Table 4) were higher than the actual seed germination percentages, regardless of the temperature, light or dormancy-breaking pretreatment used. We speculated that this discrepancy could be related to the presence of seedborne pathogens in the seed, which lowered germination rate. Seedborne pathogens are pathogens associated externally or internally with the seed (Neergaard, 1977). These seedborne pathogens can attack the seeds and destroy the embryo in the germination test. Because the seed imbibition period used for conducting the TZ test is very short (24 h), seedborne pathogens do not have enough time to attack and destroy the seed and, consequently, they are not detectable. However, the germination test occurs over several days, and seedborne pathogens have a longer time to develop and infect the seed. Other authors have reported that the TZ test can overestimate seed germination (Association of Official Seed Analysts, 2010; Zorrilla et al., 1994).

The standard germination test evaluates seed germination under optimum temperature conditions. The goal of the test is to determine the highest final germination percentage of the seed lot (Association of Official Seed Analysts, 2011). A prior study demonstrated that the seed germination percentage of two-year-old *M. sinensis* was highest when seeds were germinated using alternating temperatures in a thermogradient table, when compared to constant temperature regimens (Christian and Goggi, 2012). Several alternating temperature regimes were used in this study to determine the optimal temperature and/or temperature combinations for germination. The highest germination percentage of freshly harvested seed (2011) was recorded at an alternating temperature of 20/30°C for 16/8 h. However, the germination percentages at 20/30°C were not significantly different from those of 15/25 and 15/30°C for most seed lots. The germination percentage of seeds produced in 2010 was not significantly different for all alternating temperatures used in this experiment. Alternating temperatures are known to break dormancy in many species (Leon et al., 2004; Nishimoto and McCarty, 1997; Shen et al., 2008).

The amplitude between diurnal and nocturnal temperatures signals the initiation of many metabolic pathways associated with seed germination (Thompson and Grime, 1983; Thompson et al., 1977). However, seeds kept in cold storage for long periods of time can lose their inherent dormancy, a process known as after-ripening. This process of after-ripening most likely contributed to the lack of response to temperature in the two-year-old seed. This finding was corroborated by the low number of dormant seed present after completion of the germination test (Table 4).

The requirement for alternating temperatures is most often associated with physiological dormancy (Baskin and Baskin, 2001). Physiological dormancy is a type of

dormancy in which seeds, and specifically seed embryos, require environmental conditions to alleviate metabolic inhibitors and eventually dormancy. Other authors have also found that alternating temperatures promote seed germination in other grass species. Seeds of perennial ryegrass (*Lolium perenne* L.) exhibited the highest germination when subjected to alternating temperatures of 15/25, 20/15, 20/30, and 25/30°C for 16/8 h (Shen et al., 2008). Bermudagrass germination neared 100% when seeds were germinated at temperatures of 10/38°C for 18/6 h (Morinaga, 1926). Morinaga also found that alternating temperatures of 15/32°C for 18/6 h, promoted the highest germination of Canada bluegrass (*Poa compressa* L.) seeds. Similarly, Nishimoto and McCarty (1997) found that alternating temperatures of 20/35°C for 16/8 h, resulted in the highest germination of goosegrass (*Eleusine indica* L.) seeds. As our research and others have shown, alternating temperatures have a positive effect on seed germination and are commonly used in seed testing. As a result, alternating temperatures should be a part of the standard germination protocol for *M. sinensis*.

The level of seed dormancy in fresh *M. sinensis* seed was unknown; consequently, we used various dormancy breaking techniques on *M. sinensis* seed. Seed dormancy is the mechanism in which a seed postpones germination until a time when environmental conditions are favorable. We used several common dormancy-breaking techniques, such as adding GA_3 and KNO_3 to the germination substrate, and exposing seed to a prechill. GA_3 is a commonly occurring hormone in seeds which is associated with germination induction (Copeland and McDonald, 2004). KNO_3 is a nutrient-containing compound, which also has a positive effect on germination. Prechill or stratification simulates overwintering environmental conditions by subjecting the seeds to cool, wet conditions for a prescribed length of time. We limited our techniques to those commonly used in seed testing

laboratories so our seed testing protocol could be easily standardized and adapted into a standard germination protocol. Our results indicated that two commonly used methods of breaking seed dormancy, GA_3 and exposing the seed to a prechill increased the final germination percentage of *M. sinensis*.

Aso (1976) investigated the effect of GA_3 on the germination of *M. sinensis*. He found that GA_3 rates of 1 to 100 mg L^{-1} increased Miscanthus seed germination. Similarly, the germination of eastern gamagrass seeds with their cupules removed, increased from 25 to 47% when buffered GA_3 was applied (Tian et al., 2003). The addition of GA_3 was not effective when seed were germinated with the cupule, because the cupule encloses the seed and restricts the GA_3 from reaching the embryo.

The use of a prechill also has positive effects on the germination of C4 grasses. Hsu et al. (1985) found that seeds of big bluestem, caucasian bluestem [*Bothriochloa caucasica* (Trin.) C.E. Hubb.], indiangrass, switchgrass, and crabgrass [*Digitaria sanguinalis* (L.) Scop.] prechilled at 4°C for 14 d had a higher final germination percentage after 22 d. Seeds of dormant varieties of needlegrass had higher germination percentage after a prechill at 2 to 4°C prechill for 30 d (Fulbright et al., 1983). Future research should explore the use of different rates of GA_3 on the germination of *M. sinensis* seed, in addition to prechill.

Light had no effect on the germination of *M. sinensis*. Our result contradicts findings by Hsu (1989), who determined that light increased *M. sinensis* germination. Hsu (1989) found that light exposure for even 5 min was enough to induce germination. Seed age is an important factor affecting seed dormancy as is seed response to light during germination. Seeds used in our experiment were stored in a climate controlled, 10°C and 50% relative humidity room for a short period after harvest. Hsu (1989) did not report the age of the seed

lots used. Also, it is not uncommon for seeds of different varieties and different geographic regions to have different light requirements. When Aiken and Springer (1995) studied the light requirements for other C4 grasses found that light or the absence of light had no effect on switchgrass germination. Although the findings of our research and that of Hsu are contradictory, we have determined that the *M. sinensis* varieties used in our research do not require light stimulus during germination.

Results from our research with the thermogradient table (Christian and Goggi, 2012) and this research, strongly suggest that alternating temperatures are necessary to obtain maximum germination percentage in *M. sinensis* seed. Our results also suggest that GA_3 and prechill should be used on fresh seed, and that some *M. sinensis* genotypes do not require light for germination. The concentrations of the GA_3 solution and prechill temperatures and durations used in our study are those most commonly used in seed laboratories. These results suggest that our seed germination protocol can be used to develop a standard germination protocol for *M. sinensis* for efficiently and effectively testing seeds of *M. sinensis* for planting and sale. Possible areas of future research include the effect on germination of different prechill treatments as well as, different concentration levels of GA_3.

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Table 2. Temperature, light, and pretreatment combinations

Factor	Levels	Treatments			
Temperature	4	15/25°C	15/30°C	20/30°C	22/16°C
Light	2	Light		Dark	
Pretreatment	4	GA_3	KNO_3	5°C Prechill	Control

Table 3. Analysis of variance for the effects of year of production (Y), seed lot (SL), block (B (Y × V)), germination temperature (T), presence or absence of light (L), and pretreatment (P) and their interactions on the germination percentage of *Miscanthus sinensis*.

SV	df	SS	MS	F	P
Y	1	167440	167440	2385.56	0.0001
SL	3	8857	2952	42.06	0.0001
Y × SL	3	2864	955	13.60	0.0001
B (Y × SL)	8	1601	200	2.85	0.0001
T	3	383	128	1.82	NS
Y × T	3	82	27	0.39	NS
T × SL	9	693	77	1.10	NS
Y × T × SL	9	1209	134	1.91	0.05
L	1	73	73	1.04	NS
Y × L	1	189	189	2.69	NS
SL × L	3	73	24	0.35	NS
Y × SL × L	3	322	107	1.53	NS
T × L	3	13	4	0.06	NS
Y × T × L	3	50	17	0.24	NS
T × SL × L	9	206	23	0.33	NS
Y × T × SL × L	9	255	28	0.40	NS
P	3	6131	2044	29.12	0.0001
Y × P	3	51	17	0.24	NS

Table 3. (continued)

SV	df	SS	MS	F	P
SL × P	9	508	56	0.81	NS
Y × SL × P	9	536	60	0.85	NS
T × P	9	712	79	1.13	NS
Y × T × P	9	632	70	1.00	NS
T × SL × P	27	1969	73	1.04	NS
Y × T × SL × P	27	1522	56	0.80	NS
L × P	3	222	74	1.05	NS
Y × L × P	3	149	50	0.71	NS
SL × L × P	9	173	19	0.27	NS
Y × SL × L × P	9	366	41	0.58	NS
T × L × P	9	1059	118	1.68	NS
Y × T × L × P	9	514	57	0.81	NS
T × SL × L × P	27	876	32	0.46	NS
Error	275	19301	70		
Total	511	219034			

Table 4. Interaction means among year of production, germination temperature, and seed lot of *Miscanthus sinensis* seed. Initial seed viability values for each seed lot as determined by using the tetrazolium (TZ) test are shown for reference. Normal seedlings (Normal) and dormant seed (Dormant) values are cumulative 21 d counts.

		15/25		15/30		20/30		22/16	
2010	Initial TZ	Normal	Dormant	Normal	Dormant	Normal	Dormant	Normal	Dormant
	----------Count out of 100 seeds----------								
Seed lot 1	84	55 a[†]	2	56 a	1	57 a	1	54 a	2
Seed lot 2	83	59 a	0	61 a	0	61 a	0	57 a	0
Seed lot 3	76	52 a	0	53 a	0	56 a	0	53 a	0
Seed lot 4	96	63 a	0	55 a	0	52 a	0	55 a	0
2011									
Seed lot 1	46	20 bcdefg	2	24 bcde	2	27 bcd	2	24 bcde	4
Seed lot 2	49	28 b	1	27 bc	1	29 b	2	26 bcd	3
Seed lot 3	49	14 efg	0	10 fg	0	10 fg	0	9 g	0
Seed lot 4	60	17 cdefg	4	21 bcdef	3	20 bcdefg	3	15 defg	5

[†]Values with the same letter are not different according to Tukey's test at the $P \leq 0.05$ level. Comparisons can be made across all rows and columns.

Table 5. Pretreatment count means across all years, temperatures, and varieties.

Pretreatment	
GA_3	42 a[†]
KNO_3	34 b
5°C Prechill	41 a
Control	36 b

[†]Values with the same letter are not different according to Tukey's test at the $P \leq 0.05$.

CHAPTER 4. SEED DEVELOPMENT AND WATER RELATIONS OF *MISCANTHUS SINENSIS*

A paper to be submitted to Seed Technology

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Abstract

Very little is known about the seed development of *Miscanthus sinensis* (Andersson). The objective of this research was to establish a seed development timeline for *M. sinensis*, develop curves for seed moisture content and seed weight progression, and to determine the number of days from anthesis to physiological maturity. Plants were crossed in the greenhouse and fertilized ovules were sampled at regular intervals during development. Seeds were collected, weighed, and dried down to characterize a dry weight, fresh weight, and moisture content timeline of the developing seeds. Seeds reached a maximum moisture content of 74% at 5 d post anthesis (dpa) and maximum dry weight (physiological maturity) at 30 dpa with a moisture content of 33%. Sampled seeds also were dissected and placed in a fixative solution to photograph the embryo structures at different stages of seed development. The fixed seeds were embedded in resin, sectioned, and observed using a light microscope. Seed embryo structure development became visible at 8 dpa and at 29 dpa, all embryo structures were visible, with the exception of the first two leaves of the shoot. At 34 dpa, the seed embryo had reached maturity. The seed development timeline of *M. sinensis* developed through this research will help seed producers, farmers, and seed companies make informed decisions regarding the production and harvest of high quality *M. sinensis* seed.

Introduction

The importance of *Miscanthus sinensis* (Andersson), which will be referred to as *M. sinensis* throughout this manuscript, as a species for biomass production has increased in recent years. As a result, agronomists, seed producers and farmers have an interest in producing seed of good quality. Currently, there is very little known about the seed physiology of *M. sinensis* and an understanding of its seed development and water relations would be beneficial to the production of good quality seeds.

Seed development from anthesis to physiological maturity can be divided into three stages: cell division and differentiation, cell elongation and reserve deposition, and maturation drying (Bewley and Black, 1994). During cell division and differentiation, seed fresh weight and water content increase rapidly. Once the seed has reached the cell elongation and reserve deposition stage, seed water content reaches a plateau and dry weight accumulation increases rapidly as cells elongate and reserves are deposited. During the final stage of development seeds reach physiological maturity (point of maximum dry weight accumulation) and begin to dry down.

The number of days between seed formation and physiological maturity, and the moisture content of the seed when it reaches this stage, vary among species. Seed moisture content of grain sorghum (*Sorghum bicolor* L.) at physiological maturity ranged between 23 and 31% (Kersting et al., 1961), and it was reached 33 to 45 days post pollination (dpp). Frey et al. (1958) found that seeds of the cultivated cereal, oat (*Avena sativa* L.), reached physiological maturity between 13 and 24 dpa and seeds were at a moisture content between 43 and 48%. Smooth bromegrass (*Bromus inermis* Leyss.) reached physiological maturity between 17 and 18 dpa at a moisture content of around 47% (Grabe, 1956).

The seed development, and more specifically embryo development, timeline is based on cell division in early development. Later in the process, development can be defined at the structural level. Paulson (1969) found that ovule fertilization in sorghum occurred between 2 and 4 h post pollination (hpp). Based on embryo development, seed physiological maturity was reached 25 dpa. In the perennial grass *Paspalum dilitatum* (Poir.) fertilization occurred between 8 and 12 hpp (Bennett, 1944) and seeds were fully matured after 14 dpp. Pearl millet [*Pennisetum glaucum* (L.) R. Br.] ovules were fertilized between 3 and 5 hpp (Taylor and Vasil, 1995) and embryos reached maturity at approximately 11 dpp. Analyzing the development of seeds at the cellular and structural level can help us better understand and identify the crucial points in seed development in which physiological quality is affected.

The objective of this research was to establish a seed development timeline for *M. sinensis*, develop curves for seed moisture content and weight progression, and to determine the number of days from anthesis to physiological maturity.

Materials and Methods

In order to establish a seed development timeline for *Miscanthus sinensis*, a greenhouse experiment was designed to determine seed dry weight, moisture content and number of days to maximum dry weight.

Seeds from four varieties of *M. sinensis* were obtained from Mendel BioEnergy Seeds, Hayward, CA. Seeds were received cleaned and without lemma and palea attached. The seed was sorted by hand before using in these experiments to remove any broken or damaged seeds as well as any foreign material.

In January 2011, seeds of all four varieties were planted on blotter paper (Stults Scientific Engineering Corporation, Springfield, IL). Seeds were watered as needed and germination was monitored to remove any abnormal and non-germinating seeds. Seeds were germinated on the blotter paper approximately 12 days till seedlings were established; when they were transferred to jiffy pots (Jiffy Products, Shippagan, Canada) filled with potting soil and watered as needed. The potting soil consisted of a ratio of one part peat to one part sand to two parts field soil.

After 15 additional days, the 80 remaining seedlings were transplanted into one gallon pots containing potting soil and again watered as needed. Osmocote 14-14-14 complete fertilizer (Scotts-Sierra Horticultural Products, Marysville, OH) and urea 46-0-0 were used as needed.

Flowering began in July 2011. Due to the self-incompatibility of *M. sinensis*, two plants were needed for each cross. When anthers and stigmas began to extrude, two flowering inflorescences were bagged together under a paper bag until all florets had completely flowered.

To determine dry weight, at each sampling time, 50 florets were taken from branches in the middle third of the panicle. The first sample was taken at the time of anthesis and subsequent 50 seed samples were taken every five dpa.

Using a dissecting microscope (Fisher Scientific Company, LLC, Pittsburgh, PA), and forceps, the florets were lined up on a glass microscope slide and affixed by placing a small amount of heated dental wax on the pedicel. The glumes, lemma and palea were removed. The ovary was separated from the receptacle. Finally, the stigmas were removed,

but the styles were retained. Excised ovaries were then placed into an aluminum weighing dish and stored in a closed container to reduce moisture loss until weighed.

Fifty ovaries were collected using this method and an initial fresh weight (fw) was recorded. Once weighed, the ovaries were placed into a 65°C oven for 1 h and an initial dry weight (dw) was recorded. Ovaries were returned to the oven and weighed every hour until weight became constant. The final weight was recorded as the dry weight. To determine the seed moisture content, the following formula was used: [(fw-dw)/fw] x 100.

To construct a timeline of seed development, florets were collected at pre-anthesis, 4 hours post-anthesis (hpa), 24 hpa, 2 dpa, 7 dpa, 8 dpa, 25 dpa, and 34 dpa. In order to control pollination, seeds were only exposed to pollen for 4 h. After exposure, panicles were bagged to preclude cross pollination from additional pollen. Due to the self-incompatible nature of *M. sinensis* (Stewart et al., 2009), it is unlikely that spikelets within bagged panicles would self-fertilize.

Ten ovaries were collected at each sampling time. Seeds were then dissected in the same manner as the seeds collected for the dry weight determination. Once dissected, ovaries were placed into a fixative solution of 2% paraformaldehyde/2% glutaraldehyde in a 0.1M cacodylate buffer at pH 7.2 and stored for future processing.

After fixation, ovaries were washed in a 0.1M cacodylate buffer and subjected to a graded ethanol dehydration series beginning with 50% ethanol and ending with 100% pure ethanol. Ovaries were infiltrated with ethanol and LR White resin (London Resin Co., Reading, Berkshire, England). Finally, ovaries immersed in pure resin were poured into pans and heated in the oven at 55°C for 24 h. Once cured, the embedded ovaries were cut out from the resin and fixed to a gelatin capsule for sectioning. Resin embedded ovaries were

sectioned using a Reichert Ultracut S ultramicrotome (Leica, Wetslar, Germany). Ovaries were cut into 1 µm thick sections using a glass knife. Sections were placed on superfrost plus microscope slides (Electron Microscopy Services, Hatfield, PA). Individual consecutive sections were placed in order into deionized water puddles on the slide. Slides were dried and sections were stained with toluidine blue O stain (Electron Microscopy Services, Hatfield, PA) for five seconds, and then rinsed with water. A cover slip was added using Permount (Fisher Scientific Company, LLC, Pittsburgh, PA). Slides were viewed using an Olympus BX40 microscope (Leeds Precision Instruments, Inc., Minneapolis, MN). Images were captured using AxioVision 4.8.2 software (Carol Zeiss MicroImaging GmbH, Germany).

Results

The 20 *M. sinensis* plants of each seed lot began flowering in July 2011, seven months after they were planted. One successful cross was made two months later. Plants continued flowering on an ongoing basis, but did not produce viable seed when crossed. Seeds were collected from multiple crosses. The crosses were set up such that a plant of one seed lot was the male pollen parent and one plant of another seed lot as the female. Samples were collected from the panicle branches of the female parent. In order to check for viable seed production, ovaries were sampled for fw, dw, and microscopy. It was not possible to determine whether ovaries had been fertilized until approximately 10d. These data were collected from approximately750 crosses in one plant.

Data were recorded and seeds were collected during the single successful cross pollination. The seed moisture content at anthesis was 67% and seeds quickly reached their

highest moisture content of 74% after five dpa (figure 2). At anthesis, the egg cell and synergids along with the antipodals were visible (figure 3a).

At 8 dpa the embryo was expanding and the coleoptile was becoming visible (figure 2b). Seed moisture content at 10 dpa was 66% and seed water weight reached its maximum at 0.39 mg seed^{-1} (figure 2). Seed fresh weight and dry weight also continued to increase rapidly.

After 29 dpa, seed structures were visible (figure 4a) and the radicle and coleorhiza were distinguishable but the first leaves were not yet formed. Physiological maturity is defined as the point of maximum dry weight accumulation and was reached at 30 dpa and a seed moisture content of 33% and dry matter was 0.81 mg seed^{-1}.

After 34 dpa all of the embryo structures were formed (figure 4b) and the first leaves (plumule) and shoot apical meristem were fully formed. Seed moisture decreased to 26% and seed dry weight was constant at 0.75 mg seed^{-1}. Fresh weight declined as the moisture content of the seed decreased.

After 40 dpa the decline in seed moisture content (14%) and fresh weight (0.90 mg seed^{-1}) slowed to reach a constant; while seed dry weight (0.77 mg seed^{-1}) reached a plateau after physiological maturity (figure 2). After 50 dpa the seed reached 7% moisture content for safe storage and all seed and embryo structures were fully developed.

Discussion

M. sinensis followed a pattern of seed development and maturation common to other grass species. Throughout seed development, seed water content initially increases rapidly to very high moisture content levels and later decreases until the seed reaches a safe moisture

content for storage (Bewley and Black, 1994; Copeland and McDonald, 2004). Fresh weight and water weight rapidly increase as cells within the seed are dividing and, after this initial period of rapid cell division slows, cell elongation commences. Seed dry weight rapidly increases as cells elongate and finally reaches a plateau at physiological maturity. At this stage, seed fresh weight begins to decline and moisture content decreases as seeds dry down for storage.

M. sinensis did not readily cross pollinate. Due to the self-incompatible nature of *M. sinensis* (Stewart et al., 2009), the timeline was difficult to construct. The flowers of *M. sinensis* were very small and were difficult to hand cross-pollinate, which made it difficult to identify the exact time in which pollination occurred. Fortunately, our successful cross resulted in enough seed to provide solid seed development data to better understand its timeline and compare it to other grass species.

Seed water weight and moisture content of *M. sinensis* followed a similar accumulation pattern as other grass species. Seed dry weight in *M. sinensis* rapidly increased until reaching its maximum (physiological maturity) at 30 dpa (figure 2). Although physiological maturity was reached more rapidly (between 17 and 18 dpa) in the cool season species smooth bromegrass (Grabe, 1956), most grass species reach physiological maturity between 8 and 60 dpa (Jones, 1985). Four varieties of oat had a wide range of maturities, ranging anywhere from 13 to 24 dpa (Frey et al., 1958). Physiological maturity was reached after 28 dpp in both cool season species perennial (*Lolium perenne* L.) and Italian ryegrass (Hyde et al., 1959). *Miscanthus sinensis* seed physiological maturity was reached at a similar dpa as other warm season grasses.

Seed moisture content peaked very early in the development of *M. sinensis* seeds at a moisture content of 74% at 5 dpa. Other grass species also reached the maximum moisture content very early in seed development. Smooth bromegrass reached its maximum moisture content around 6 dpa at approximately 62% (Grabe, 1956). Oats seeds reached their maximum moisture content level between 60 and 65% at 4 dpa (Frey et al., 1958). Grain sorghum reached a maximum moisture content of 85 to 90% between pollination and 4 dpp (Kersting et al., 1961). After this period, seed moisture content rapidly decreased as seeds developed and finally reached a plateau after maturation. When seeds of smooth bromegrass reached physiological maturity, seeds had moisture content of47% (Grabe, 1956). The moisture content of oat seeds at maturity was 47% (Frey et al., 1958). *Miscanthus sinensis* seeds matured at a lower (33%) moisture content than that of smooth bromegrass and oat. However, seeds of Italian ryegrass (*Lolium multiflorum* Lam.), matured at a similar (38%) moisture content to *M. sinensis* (Hyde et al., 1959).

The structural development of the *M. sinensis* embryo followed closely the changes in seed water weight and moisture content as the seed matured. Seed structures were easily differentiated at 8 dpa in *M. sinensis* (Figure 3a). The coleoptile was visible along with the developing embryo axis. Embryo development was much more rapid in seeds of *P. dilitatum*, in which structures were evident after 5 to 6 dpp (Bennett, 1944). Pearl millet embryo structures were visible after 5 dpp (Taylor and Vasil, 1995). Mature embryos of pearl millet and *P. dilitatum* were formed after 11 and 14 dpp, respectively. *Miscanthus sinensis* embryo development was completed at 34 dpa. The development timelines of pearl millet, *P. dilitatum*, and *M. sinensis* were very similar, but the length of each phase of development varied from species to species.

Future research should explore additional phases of this timeline including: determining the point at which embryos are developed enough to germinate and produce a normal seedling when removed from the seed and the developmental point when seeds acquire desiccation tolerance. Additional varieties of *M. sinensis* should be added in future experiments to better distinguish the differences in seed development across this species. The seed development timeline that resulted from our research will provide a solid base for the understanding of *M. sinensis* seed development.

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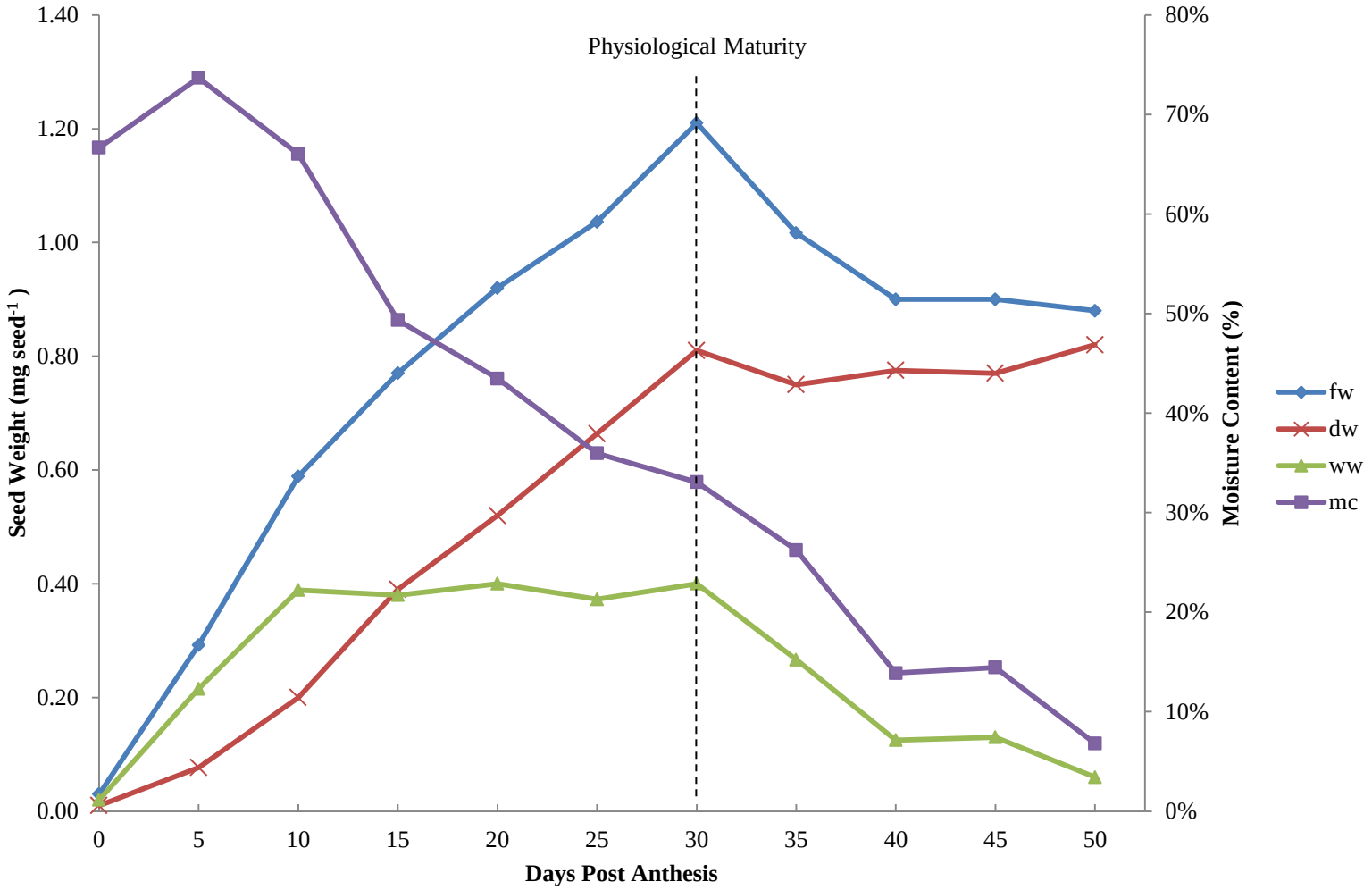
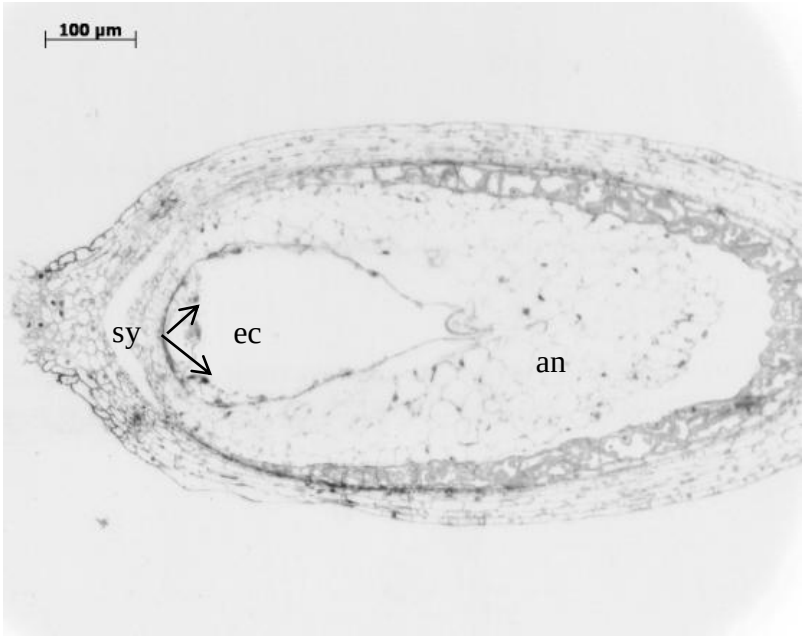
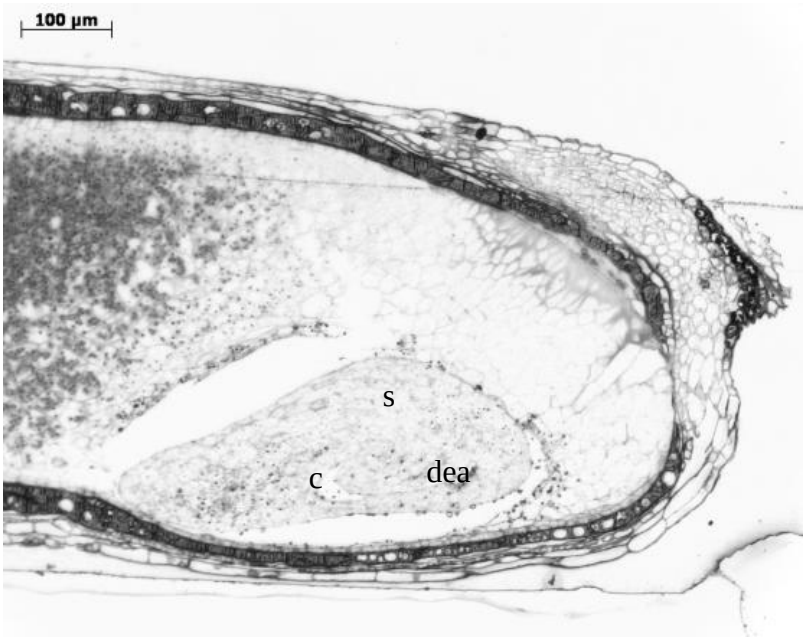


Figure 2. Seed water status from anthesis through physiological maturity. Sampling was conducted every five days post anthesis and 50 seeds were collected at each sample point. Seed fresh weight (fw) and dry weight (dw) were measured, while water weight (ww) and moisture content (mc) were calculated with the formula (fw-dw) and [(fw-dw)/fw] x 100, respectively.

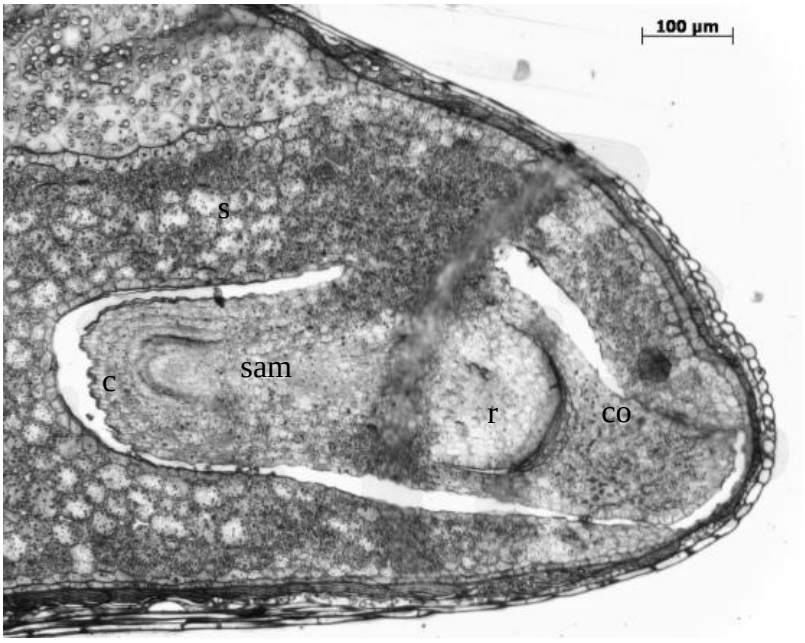


A.



B.

Figure 3a. *M. sinensis* seed pre-anthesis, showing an egg cell (ec) along with two synergids (sy). The base of the seed is to the left of the picture. Also, the antipodals (an) are becoming visible on the right end of the embryo sac and the polar nuclei are not visible yet. 3b. The developing embryo at 8 days post anthesis, showing some structural development. Scutellum (s), coleoptiles (c), developing embryo axis (dea). The base of the seed is to the right of the picture.



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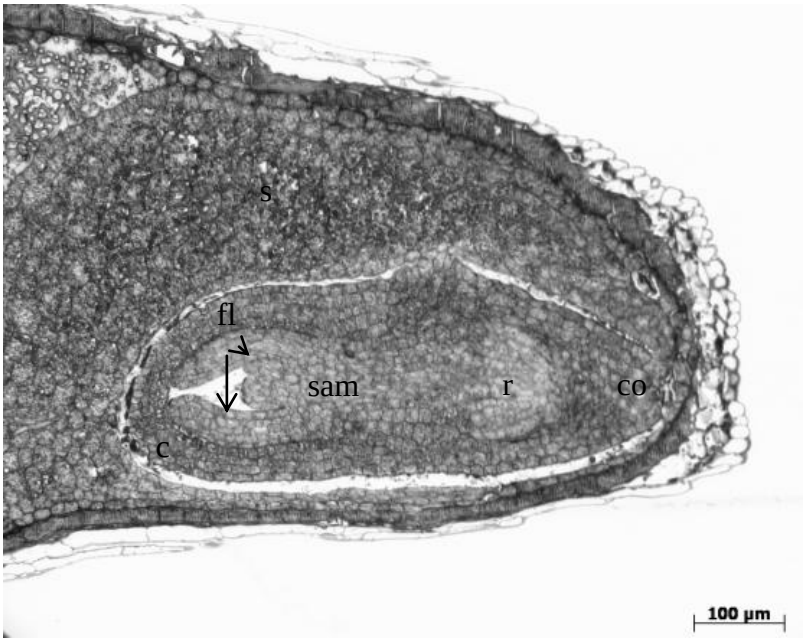

B.

Figure 4a. Embryo development at 29 days post anthesis. Both, the radicle (r) and shoot apical meristem (sam) are visible. The first leaves (fl) are not yet formed. 4b. The mature embryo showing the scutellum, coleoptile, first leaves, shoot meristem, radicle, and the coleorhiza (co). All structures have been formed and are visible. In both pictures the base of the seed is to the right.

CHAPTER 5. GENERAL CONCLUSIONS

This research was initiated to understand the biology of seed development and germination of *M. sinensis*. Experiments were designed to determine optimal germination conditions that could be used in the development of a standard germination protocol. Seed development also was explored using light microscopy and a timeline of seed development was established.

A wide range of constant and alternating temperatures was explored to determine the optimal temperature for best germination results. It was determined that alternating temperatures promote a higher final germination percentage when compared to constant temperatures. In addition, by studying a wide range of temperatures, we were better able to determine which alternating temperature combinations to study in further detail. This temperature combination included 22/16°C for 16/8 h, at which the highest germination percentage of all alternating temperatures was recorded.

Building on the initial results, we compared the temperature regimen of 22/16°C for 16/8 h to alternating temperatures commonly used seed laboratory germinators of 15/25, 20/30, and 15/30°C for 16/8 h. No difference was seen in the germination of 2010 (two-year-old) seed, but seed produced in 2011 (one-year-old) exhibited differences when exposed to different alternating temperature treatments. The temperature treatment of 20/30°C promoted the highest final germination percentage in 2011 seed lots.

Along with temperature, light and dormancy breaking treatments were studied. It was determined that final Miscanthus germination percentages were not statistically different between the light and dark treatments. Also, it was determined that the hormone GA_3 and a 5°C prechill for 7 d had the greatest positive effect on the final germination percentage.

When all germination results were combined, it was concluded that a 20/30°C germination regimen for 21 d was optimal for fresh seed. The use of a 5°C prechill for 7 d along with a 500 mg L^{-1} GA_3 treatment was also beneficial.

Seed development was studied to create a seed development timeline. Seeds were dissected at various times between anthesis and physiological maturity and examined using a light microscopy. A timeline was constructed that showed embryo development and seed weight changes. Seed fresh weight, dry weight, and moisture content were plotted. It was determined that *M. sinensis* seeds reached physiological maturity at 30 d, with a seed moisture content around 33%.

Possible areas of future *M. sinensis* research include the effect on germination of different prechill treatments, as well as, different concentration levels of GA_3. In addition, further research should be done to determine the point during seed development when *M. sinensis* seeds acquire desiccation tolerance and the point when the embryo is germinable, even if physiological maturity is not reached.

In conclusion, we have just begun to understand the seed development of *M. sinensis*. Our results also advance our knowledge of its germination ecology and help establish parameters for the development of a standard germination protocol. As we look forward, we can continue to expand upon this research and understand *M. sinensis* seed production and its use as a future biomass crop.

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