Alternating Temperatures Promote Seed Germination of *Miscanthus sinensis*

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 (Clifton-Brown and Lewandowski, 2000; Clifton-Brown and Lewandowski, 2002; Clifton-Brown et al., 2008; Atkinson, 2009). 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.

Department of Agronomy, Iowa State University, Seed Science Center, Ames, IA 50011. *Corresponding author (echrstn@iastate.edu). Received 12 June 2012.

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 (Jones, 1985; Bewley and Black, 1994; Baskin and Baskin, 2001; Copeland and McDonald, 2004), 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 used seed lots 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

TABLE 1. Mean germination of *Miscanthus sinensis* seeds subjected to 36 treatments of temperature combinations for 16 and 8 h, simulating diurnal fluctuations. Four seed lots were used with three replications. Germination was recorded every 48 h for 14 d.

Temperature				Germination	Mean of firm seeds at the end of the test
16 h	8 h	Mean†	Amplitude‡		
(°C)				(%)	(%)
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 abc	1
40	16	32	24	56.7 abc	0
10	16	12	6	56.7 abc	3
34	10	26	24	56.3 abc	1
28	16	24	12	56.3 abc	0
22	28	24	6	55.0 abcd	0
22	22	22	0	54.3 abcde	0
10	28	16	18	54.3 abcde	1
10	34	18	24	53.7 abcdef	0
40	28	36	12	53.3 abcdef	0
40	22	34	18	53.3 abcdefg	0
34	28	32	6	53.3 abcdefg	0
16	16	16	0	53.3 abcdefg	1
34	16	28	18	52.7 abcdefg	1
28	22	26	6	52.0 abcdefg	0
22	34	28	26	52.0 abcdefg	0
34	22	30	12	51.7 abcdefg	0
16	34	22	18	51.3 abcdefg	0
16	28	20	12	51.3 abcdefg	0
16	40	24	24	50.0 abcdefg	0
28	28	28	0	49.3 abcdefg	0
40	10	30	30	49.0 abcdefgh	1
34	34	34	0	44.7 bcdefgh	0
28	34	30	6	44.3 bcdefgh	0
10	40	20	30	44.3 cdefgh	0
40	34	38	6	43.7 cdefgh	0
34	40	36	6	38.3 defgh	1
22	40	28	18	38.3 efgh	0
28	40	32	12	37.7 fgh	0
16	10	14	6	36.0 gh	5
40	40	40	0	33.7 h	1
10	10	10	0	10.3 i	13
		Overall mean		49.6	

†Temperature (T) mean is the weighted mean of 16 h and 8 h periods at alternating temperatures using the formula $[(T_{16} \times 16) + (T_8 \times 8)] \times 24^{-1}$.

‡Temperature amplitude calculated as $T_{max} - T_{min}$.

§Means with the same letter do not significantly differ ($p \leq 0.05$), according to Tukey's test.

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 Inc., Cary, NC) 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.

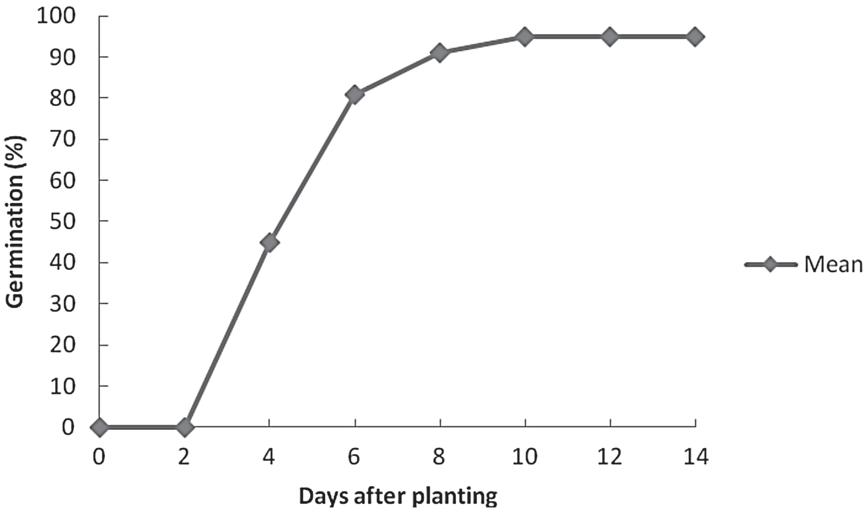
The temperature regimen that maximized *M. sinensis* seed germination in our study was a combination of 22 °C for 16 h and 16 °C for 8 h. The weighted mean temperature of the top-ranking alternating temperature regimens was 18 °C and the amplitude was 10 °C (data not shown). The mean germination percentage 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 (Bewley and Black, 1994; Baskin and Baskin, 2001). 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 (AOSA, 2009) 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 because at the time of this study 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

Figure 1. Cumulative germination of *Miscanthus sinensis* seeds exposed to a temperature regimen of 22/16 °C for 16/8 h. After planting, germinated seeds were removed every 48 h until the experiment was terminated at 14 d, and remaining ungerminated seeds were tested for viability.



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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