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Temperature and Light Requirements for Miscanthus sinensis Laboratory Germination Test

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Comments
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TEMPERATURE AND LIGHT REQUIREMENTS FOR *MISCANTHUS SINENSIS* LABORATORY GERMINATION TEST

Erik J. Christian, A. Susana Goggi, and Kenneth J. Moore

Abstract

A seed germination protocol for *Miscanthus sinensis* (Andersson) is not available. Our objectives were to evaluate requirements for germination. Eight seed lots (four varieties and two production years) were germinated at four alternating temperatures: 15-25, 15-30, 20-30, and 16-22°C for 16-8 h, respectively; with or without light; and GA_3, KNO3, prechill, or water (control) (32 treatment combinations). Fresh seeds (2011) germinated best at 20-30°C, whereas seeds from 2010 were not affected by temperature. This difference may suggest the presence of dormancy, as seed germination was enhanced by prechill and GA_3. Light was not a significant factor. These results provide a basis for developing a standard germination protocol.

Introduction

Miscanthus sinensis (Andersson), known as Chinese silver grass, Japanese silver grass, zebra grass or eulalia, which will be referred to as *M. sinensis*, is a C4 perennial grass adapted to biofuel production. The species is not well understood and there are many problems related to cultivation. One of the issues is how to determine seed viability. The Rules for Testing Seeds (Association of Official Seed Analysts, 2011) describe standard germination test protocols for most widely cultivated species. However, neither seed testing laboratories nor seed producers have a standardized germination test protocol for *M. sinensis*.

Some species require alternating temperatures to enhance germination. 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 natural environment (Baskin and Baskin, 2001). The first step in identifying a germination temperature is to determine whether the species requires constant or alternating temperatures. Christian and Goggi (2012) used a thermogradient table to determine the optimum germination temperature for two-year-old *M. sinensis* seed. They reported that alternating temperatures promoted seed germination in *M. sinensis*, and that germination percentage was highest when seeds were germinated at 16 °C for 16 h and 22 °C for 8 h. The authors also reported that germination percentage was not significantly different for several other alternating temperature combinations used, as long as the temperature differential between high and low ranged from 6 to 30°C in 24 hours. The lowest germination percentages were recorded when seeds were germinated at constant 10°C or constant 40°C. Some species require a period of cold temperatures i.e., stratification, which is exposing imbibed seeds to low temperature (5-10°C) for a period of time (e,g., 7d) before germination to break dormancy (Copeland and McDonald, 2001). The use of a prechill also has positive effects on the germination of other perennial warm-season grasses (Olszewski and Folin, 2009; Watkinson and Pill, 1998; Zarnstorff et al., 1994).

The effect of light on seed germination has three main components: light quantity, duration, and quality. Seeds of different crops manifest three distinctive germination requirements regarding light: seeds that require light for germination, those that require the

absence of light, and seeds that are indifferent to light (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 used. GA_3 is a naturally occurring hormone in seeds which is associated with germination induction (Copeland and McDonald, 2001). Aso (1976) reported that using solution of GA_3 from 0.1 to 100 mg L^{-1} increased germination percentage of *M. sinensis*. Additional dormancy breaking methods might include using germination-promoting compounds such as KNO_3. KNO_3 is a salt used in a water-based solution for breaking seed dormancy and promoting germination in seed testing laboratories.

Our objective was to determine the combinations of temperature, light, and dormancy breaking treatments to achieve optimum germination of *M. sinensis* across varieties and seed lot produced in different years.

Materials and Methods

Seed Source

Four varieties and two years of production per variety (2010 and 2011) of *M. sinensis* seed were obtained from Mendel BioEnergy Seeds (Hayward, CA) in December 2011 total of eight seed lots. Seed lots were stored in a cold room at 10°C and 50% relative humidity for approximately 2 mo until used in these experiments. The seed was cleaned and 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 *M. sinensis* was evaluated using the tetrazolium chloride test. Two replicates of 50 seeds per seed lot were imbibed, cut and stained, and evaluated separately. 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 outlined in the Tetrazolium Testing Handbook (Association of Official Seed Analysts, 2010).

Seed Germination Determination

The germination treatments included four alternating temperatures, two light combinations, and three dormancy breaking treatments (GA_3, KNO_3, Pre-chill treatment), in addition to check control where water is used, for a total of 32 seed germination treatment combinations (Table 1). The entire experiment was replicated twice (two blocks).

Seeds were planted on top of two blotters (Stults Scientific Engineering Corporation, Springfield, IL) placed inside 16 × 27 × 4 cm plastic boxes (Melmat, Huntington Beach,

CA). Seeds were planted using a vacuum planter (E.L. Erickson Products, Brookings, SD). Seed lots were randomized within boxes and 100 seeds each from two different seed lots were planted per box.

Seeds were placed in four germination chambers (Hoffman Manufacturing, Inc., Jefferson, OR) at four different alternating temperatures: 15-25, 15-30, 20-30 and 16-22°C for 16-8 h, respectively (Christian and Goggi, 2012).

Three breaking dormancy treatments, and a check control treatment were used. In the control treatment, blotters were moistened with 70 ml of tap water and boxes were placed directly in the germination chamber (no-pretreatment check control). In the pre-chilling treatment, blotters were moistened with 70 ml of tap water and boxes were placed in a prechill chamber at 5°C for 7 d before moving the boxes to the germination chambers. In the GA_3 treatment, 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 chambers. In the KNO_3 pretreatment, blotters were moistened with a 2000 mg L^{-1} KNO_3 solution (Association of Official Seed Analysts, 2011), and seeds were planted on the KNO_3-moistened blotters, and boxes were placed in the germination chambers. After the dormancy breaking treatments, seeds were germinated at the various alternating temperature combinations mentioned above for a total of 21d.

Two identical sets of boxes for each treatment were prepared, one set of boxes were subjected to 8 h light and 16 h dark; while the other set of boxes was subjected to complete darkness for the entire experiment by wrapped boxes in 0.10 mm-thick black plastic.

Seedling Evaluation

Boxes were evaluated every 7 d for a total of 21 d. Seeds and seedlings were classified into normal, abnormal seedlings, and dead seeds 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 (decayed) 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. The main effects were seed lot (four varieties x two years of production), germination temperatures, dormancy breaking treatment, and light. There were eight seed lots; two levels of light (8 h light – 16 h dark and complete dark); four germination temperatures (15-25, 15-30 20-30, and 16-22°C for 16 h-8 h, respectively; and four breaking dormancy treatments: GA_3, KNO_3, 5°C prechill, and check control. 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. The interactions among year of production, germination temperature, and seed lot were significant ($P \leq 0.05$) (Table 2). Consequently, the corresponding interaction means were calculated (Table 3). 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 2).

The temperature regimes used in this study had no significant effect on the germination of seed harvested in 2010, regardless of seed lot (Table 3). The germination percentage for all 2010 seed lots and for all germination temperatures ranged from 52 to 63% (Table 3). Seed viability of all seed lots harvested in 2010 was also higher than seed harvested in 2011 as determined by the tetrazolium tests (Table 3; Figure 1). The seed viability values ranged from 76 to 96% for seed harvest in 2010 and from 46 to 60% for seed harvested in 2011 (Table 3).

The germination percentage of seed harvested in 2011 was significantly affected by temperature regimes and by the difference among seed lots used in this experiment. The two extreme germination percentage values for seed lots harvested in 2011 were recorded for seed lot 2 (29%) at the alternating germination temperature of 20-30°C for 16-8 h, respectively and seed lot 3 (9%) at the alternating germination temperature of 16-22°C for 16-8 h, respectively. Although these two germination percentage values were significantly different from each other, each value alone was not significantly different from the germination percentages recorded at other temperatures for the same seed lot or from other seed lots (Table 3). Overall, the 16-22°C alternating temperature resulted in the highest

number of dormant seeds at the end of the germination tests. The least number of dormant seeds at the end of the germination tests in 2010 was recorded for the alternating temperature treatments of 15-30°C and 20-30°C (Table 3).

Both dormancy breaking treatments GA_3 and prechill at 5°C significantly increased the germination of *M. sinensis* to 42 and 41%, respectively, compared to KNO_3 or the check control, regardless of year of production, temperature, light, and seed lot (Table 4).

Discussion

The initial seed viability values by TZ were higher than the actual seed germination percentages, regardless of the temperature, light or dormancy-breaking treatment used (Table 3). However, the regression coefficients of determination between seed viability (TZ) and germination values were very high (R^2 ranged from 0.7343 to 0.8763) (Figure 1). Other authors have reported that the TZ test can overestimate seed viability compared to standard germination tests (Association of Official Seed Analysts, 2010; Zorrilla et al., 1994).

The highest germination percentage of freshly harvested seed (2011) was recorded at an alternating temperature of 20/30°C for 16-8 h, respectively (Table 3); however, this germination percentage was not significantly different from those obtained at 15-25 and 15-30°C for most seed lots. The germination percentage of seeds produced in 2010 was not significantly different for all temperatures used. Alternating temperatures are known to enhance germination in many species (Leon et al., 2004; Nishimoto and McCarty, 1997; Shen et al., 2008). Christian and Goggi (2012) found that alternating temperatures promoted seed germination in *M. sinensis*. 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). The 2010 seed lots had few dormant seeds (Table 3). These seed lots were two-years old and would have likely undergone after-ripening in storage. After-ripening is a physiological process by which dry seed naturally lose dormancy overtime (Copeland and McDonald, 2004).

The level of seed dormancy in freshly harvested *M. sinensis* seed was unknown; consequently, we used various dormancy breaking techniques commonly used in seed testing laboratories to determine which would promote germination. We used GA_3, and KNO_3 solutions to moisten the germination substrate, or prechill. Our results indicated that two commonly used methods, GA_3 and prechill treatment, 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 0.1 to 100 mg L^{-1} increased *M. sinensis* seed germination. 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 even 5 min of light exposure was enough to induce germination. Seed age is an important factor in seed response to light. Seeds used in our experiment were stored in a climate controlled chamber, 10°C and 50% relative humidity 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. Although the findings of our research and those of Hsu are contradictory, we have determined that the *M. sinensis* varieties used in our research do not require light to stimulate germination.

Results from our research with the thermogradient table (Christian and Goggi, 2012) and this research, confirm that alternating temperatures and GA_3 are necessary to obtain maximum germination in *M. sinensis* seed. Our results also indicate that prechill is useful for breaking dormancy and should be used. It also showed that the varieties of *M. sinensis* used in this study were insensitive to light. Consequently, a standard germination protocol for effectively testing *M. sinensis* seeds should include alternating temperatures, and the use of GA_3 or prechill to break dormancy.

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Figure Caption

Figure 1 – Regression between seed viability as estimated by the tetrazolim (TZ) test and germination percentage of 2010 and 2011 seed lots and germinated at 15-25, 15-30, 20-30, and 16-22°C with 16-8 h light, respectively. Data were averaged overall dormancy-breaking treatments.

Table 1. Temperature, light, and dormancy-breaking treatment combinations used to evaluate optimum germination requirements for *M. sinensis* seed. .

Factor	Levels	Treatments			
Temperature	4	15-25°C	15-30°C	20-30°C	16-22°C
Light	2	8 h-Light		Complete Dark	
Dormancy-breaking treatment	4	GA_3	KNO_3	5°C Prechill	Control

Table 2. Analysis of variance for the effects of temperature (T), light (L), dormancy-breaking treatment (D), seed lot (SL), year of production (Y), block (B), 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 × SL	9	1209	134	1.91	*0.05*
L	1	73	73	1.04	*NS*
D	3	6131	2044	29.12	*0.0001*
Error	275	19301	70		
Total	511	219034			

[†]All other interactions were not-significant and are not presented in this table.

Table 3. Percentage of normal seedlings (Normal), dormant seed (Dormant) for all seed dormancy-breaking treatments after 21 d germination period, and initial viability by tetrazolium (TZ) test of four *Miscanthus sinensis* varieties in 2010 and 2011, germinated at four temperatures.

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

[†] Means followed by the same letter are not significantly different according to Tukey's test at $P \leq 0.05$. Comparisons can be made across all values within the column.

[‡] Means followed by the same letter are not significantly different according to the Tukey's test at $P \leq 0.05$. Comparisons can be made across all rows and columns.

Table 4. Mean germination percent of *Miscanthus sinensis* seeds treated with different dormancy-breaking methods. Germination means are averaged over varieties, years, and temperatures.

Dormancy-breaking treatment	Mean[†] of germination
	-------%----------
GA_3	42 a
KNO_3	34 b
5°C Prechill	41 a
Control	36 b

[†]Means within the column followed by the same letter are not significantly different according to the Tukey's test at $P \leq 0.05$.