Variation in seed dormancy in *Echinochloa* and the development of a standard protocol for germination testing. II: Breaking dormancy in seeds unresponsive to light or dark conditions alone by using heat and ethanol pretreatment

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Summary

A recently established method for the germination of *Echinochloa* seeds recognised and accounted for variation in responses to light and darkness. This method used parallel light and dark tests and was successful in promoting germination in most seed lots. However, some samples exhibited deeper dormancy and were not fully responsive to either test. In the present study, we employed warm pretreatments where seeds were exposed to dilute aqueous ethanol solutions to attempt to break their dormancy. Based on tests of five *Echinochloa* accessions, we propose a new, follow-up protocol that can be used on samples unresponsive to the established method. The additional step involves a 3-day dark pretreatment at 35-37°C, imbibing the seeds in ~0.25M ethanol (aq). After this pretreatment, the seeds should be germinated for 14 days at 20/30°C (16 hours/ 8 hours) with half held in darkness and the other half exposed to an 8-hour light cycle.

Introduction

In a previous paper, we reported on the variation in germination response of dormant seeds of *Echinochloa* to light and dark treatments and surveyed the current state of knowledge on classes of dormancy in this genus, which includes both domesticated crops and weeds (Kovach *et al.*, 2010). Some seed lots responded to light, others to darkness, and the remainder were neutral in their response to light conditions. No patterns relating sensitivity to light with taxon or country of origin could be discerned. We also reported that some accessions did not germinate well regardless of light/dark conditions and that it would be useful for seed-testing laboratories to have an alternative, yet simple, method to break seed dormancy for such samples. In this paper we report on a simple and successful method to break seed dormancy in four of five *Echinochloa* seed lots that exhibited this

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deeper dormancy in previous tests. The method tested is a modification of one reported by Taylorson and Hendricks (1979), which was tested primarily on dormant seeds of *Panicum*, but also tested on a more limited basis on seeds of *Digitaria*, *Echinochloa*, *Eragrostis* and *Setaria*. Our modifications make the method simpler and less time-consuming – which is of benefit to seed-testing laboratories and germplasm centres.

The method employed by Taylorson and Hendricks (1979) treated seeds by placing them on wet filter paper in small Erlenmeyer flasks that also held very small containers containing ethanol. The flasks were then sealed with rubber serum stoppers. These were placed at 35°C in the dark for several days (three days being effective), after which the stoppers were removed and loosely covered with aluminum foil for an additional four days (also at 35°C) to allow for the gradual escape of ethanol vapour while maintaining moisture within the flasks. After this 7-day pretreatment, the experimental units were either exposed to red-light for five minutes or to continued darkness. They were then kept in the dark at 20°C for 16 hours alternating with 30°C for eight hours for an additional five days, after which germination was assessed. During their experiments, light was excluded by placing the flasks in opaque boxes. Taylorson and Hendricks (1979) noted two requirements for this method to work: warm temperatures (at least 30°C, preferably 35°C) and a reduction of the ethanol concentration to allow germination to proceed. Lastly, these authors also mentioned that direct application of ethanol in the imbibition solution gave similar results, but did not report any data for that approach. Their statement inspired our examination of this approach as a simplified method.

In addition to ethanol, Bewley and Black (1982) listed acetone, chloroform, ethyl ester and methanol as anesthetic substances that have been used to break seed dormancy. Cohn *et al.* (1989) tested a broad spectrum of 25 organic and hydroxy acids, esters, aldehydes, and alcohols to break dormancy in red rice (*Oryza sativa* L.). Highly lipophilic alcohols, such as butanol and propanol, were effective in breaking dormancy at lower concentrations than ethanol. Amritphale *et al.* (1993) reported on the successful use of acetone to prevent or release far-red light-induced, secondary dormancy in cucumber (*Cucumis sativus* L.) seeds. However, this and many of the other chemicals tested are more toxic than ethanol. This is an important factor in developing a dormancy-releasing protocol for seed laboratory use.

Although Taylorson and Hendricks (1979) reported results that were positive and their method can certainly be utilised, the use of Erlenmeyer flasks, serum stoppers, the addition of small containers for the ethanol and the additional evaporation period do not fit well into seed-testing protocols. Later research by Taylorson (1982), where induced secondary dormancy in *Setaria faberi* R.A.W. Herrm. seeds by a high temperature (35°C) treatment was prevented by a direct application of ethanol, involved the imbibition of seeds in various concentrations of ethanol before conducting germination tests. Other researchers have also reported favourable results with various ethanol pretreatments to overcome seed dormancy, including Le Deunff (1983) for *Hordeum vulgare* L., Petruzzelli (1984) for *Pinus halepensis* Mill., Jeavons and Jarvis (1984) for *Corylus avellana* L., Cohn *et al.* (1989) for *Oryza sativa*, and Sreenivasulu and Amritphale (1999) for *Cucumis sativus*. Exposure to ethanol as a pretreatment is better suited to current existing seed-testing protocols, as it employs standard germination boxes without having to use additional small

containers to hold the ethanol, thus avoiding the extra care needed in handling the boxes to prevent spillage. To this end, we tested five accessions of *Echinochloa* to determine if they responded to ethanol, with and without light, and whether the pretreatment time could be reduced. We also compared the use of red and cool white fluorescent lighting, as cool white lighting is more widely available than are monochromatic light sources. The objective was to develop a straightforward protocol that would be effective and easier to use, thus facilitating its adoption by seed-testing laboratories and germplasm centres.

Materials and methods

Five *Echinochloa* accessions from the USDA-ARS North Central Regional Plant Introduction Station, Ames, Iowa were tested in these experiments (table 1). This limited sample was chosen expressly because these few accessions displayed levels of dormancy that could not be broken by following the germination protocol established by Kovach *et al.* (2010). These accessions were either originally donated from other germplasm centres or collected by the station curator and increased locally. Seed lots were stored at 5°C in glass jars, except for PI 664493, which was stored at -18°C in a thick, re-sealable plastic bag. The seeds were not threshed from their bracts.

Table 1. *Echinochloa* accessions used in the germination tests, showing the most recent germination-test results prior to our experiments.

Accession	Lot[1]	*Echinochloa* species	Country of origin	Previous test results[2]			Experiment[3]
				% Normal	% Dormant	Year tested	
PI 217911	1957	E. frumentacea	India	43	47	2007	B
PI 220518	2005	E. crus-galli	Afghanistan	0	100	2008	A, B
PI 383642	1990	E. crus-galli	Turkey	0	98	2005	A, B
PI 420160	1990	E. crus-galli	France	9	90	2005	B
PI 664493	2008	E. crus-galli	United States	11	75	2009	B

[1] The year in which the seed lot was regenerated.
[2] The proportions of abnormal seedlings and dead seeds are omitted from this table.
[3] A = lot used in two-accession test; B = lot used in five-accession test.

A. Two-accession germination test

Two accessions of *E. crus-galli* (L.) P. Beauv. were tested for germination response to red-light and ethanol pretreatments at 36°C (table 2). Fifty seeds in three replicates were placed on wetted germination blotter papers – blue-dyed paper on bottom (Anchor Paper Company, St. Paul, MN, USA) and white paper (Ahlstrom Corporation, Helsinki, FI) on top (in contact with seeds) in 13 × 13.5 × 3.5 cm plastic germination boxes. Blotter

Table 2. Summary of treatment conditions for the two-accession and five-accession germination tests. In all cases, N = 150 seeds (three replications of 50 seeds).

Experiment	Step	Comparison	Duration
Two-accession test:	Pretreatment at 36°C	0.5 M ethanol (aq) vs. a water control	3 days
	Germination at 20°C (16 hours)/ 30°C (8 hours)	One 8-hour red light period followed by darkness vs. complete darkness	8 days
Five-accession test:	Pretreatment at 37°C	0.25 M ethanol (aq) vs. a water control vs. a no pretreatment control	3 days
	Germination at 20°C (16 hours)/ 30°C (8 hours)	One 8-hour cool white light period followed by darkness vs. complete darkness	14 days

papers were wetted with either 25 ml distilled water (water treatment) or with 25 ml 0.5M ethanol (aq) (ethanol treatment). After three days, seeds were transferred to new blotters wetted with 25 ml tap water and additional tap water thereafter as needed. Transfers to the new blotter papers were done under green 'safe' lighting generated by using a green LED lighting system (SolarOasis, Reno, NV, USA, Part No: Pro540, 525 nm – Custom) that was also covered with green and blue-green acetate sheets (Roscolene #874 and #877, Rosco Laboratories Inc., Stamford, CT, USA) because the bandwidth of the uncovered green LEDs was too wide. To maintain moisture and restrict ethanol gas evaporation and cross contamination with other experimental units, boxes were paired according to treatment (e.g., EtOH-treated with EtOH-treated, H_2O-treated with H_2O-treated, accession 1 – replicate 1 with accession 2 – replicate 1) and placed in re-sealable plastic bags (Elkay Plastics, Commerce, CA, USA, Part No: F21012). The plastic-bag-enclosed units were then placed on the bed of a Seed Processing Holland BV thermogradient table (Model 5008.00.00.A5, Enkhuizen, NL) set for a constant temperature (36°C was the actual table top temperature attained) and dark conditions for three days, four days shorter than reported by Taylorson and Hendricks (1979), more closely resembling the two-day pretreatment employed by Le Deunff (1983). After this 3-day pretreatment, the thermogradient table was set at 20°C for 16 hours alternating with 30°C for eight hours, for eight days, when the test ended. During the first eight hours at 30°C, half of the experimental units received red light achieved by using a red LED lighting system (SolarOasis, Reno, NV, USA, Part No: Pro540, 660 nm – Custom; 80-85% of emission between 650 – 670 nm, balance between 640 – 650 nm and 670 – 680 nm). The remaining experimental units were kept in dark conditions both during this initial eight hours at 30°C and continuously thereafter. Light and dark conditions were produced by using clear and opaque covers on the thermogradient table with a light block placed between the two halves by using a roll of carpet padding (cut to the exact width of the table) and black plastic. Seeds were considered to be germinated when coleoptiles and radicles emerged and appeared normal, as per ISTA (2010) and AOSA (2009) standards.

B. Five-accession germination test

Five *Echinochloa* accessions (two of which were also used in *A. Two-accession germination test*; table 1) were tested for germination response to fluorescent-light, ethanol and heat treatments (table 2). Since the high [0.5 M] concentration of ethanol that we used in our first experiment may have masked a response of the seeds to light, we reduced it in this test to 0.25 M.

Fifty seeds in three replicates were placed on wetted blue and white germination blotter papers in plastic boxes in re-sealable plastic bags, as described above. The plastic-bag-enclosed units were then placed inside three Hoffman Manufacturing Company (Albany, OR, USA) SG-8 model germinators, retrofitted with Omron E5CJ controllers, at 37°C and dark for three days, with each germinator housing a replication.

After this 3-day pretreatment, the germinators were set for 20°C for 16 hours alternating with 30°C for eight hours, for 14 days. There were two reasons for extending the germination period for this second test: 1) the light source used was cool white fluorescent light, which we thought might be less effective than a more monochromatic red light; 2) the concentration of ethanol used was half that used in the first experiment. We wanted to allow for complete expression of dormancy release under these possibly less promotive conditions. During the first eight hours at 30°C, half of the experimental units received eight hours of cool-white fluorescent lighting, after which they were excluded from light. The remaining experimental units were kept in dark conditions both during this initial eight hours at 30°C and continuously thereafter. Each germinator had six 15-watt fluorescent tube lights illuminating a space approximately 61 × 56 × 61 cm (H × W × D), at an intensity of 555 lx (measured at the site of seed placement). Dark conditions were achieved by holding the samples in thick, black plastic bags. Ethanol treatments consisted of wetting the blotter papers with 25 ml of 0.25 M ethanol (aq) solution. Water treatments consisted of wetting the blotter papers with 25 ml distilled water. For both treatments, after three days, new blotter papers were used and wetted with 25 ml tap water and additional tap water thereafter as needed. Seed transfer to the new blotter papers was done in the laboratory with the overhead lights turned off. Indirect, dim light from high windows was just sufficient to facilitate seed transfer.

After the 3-day pretreatment was completed, germination experiments for the control units were then started. Half of the controls received light at the same intensity noted above, and the others were placed in black plastic bags; all received 20°C for 16 hours alternating with 30°C for eight hours, for 14 days when the test ended. Controls received 25 ml tap water and additional tap water thereafter as needed. Blotter papers were never changed in the controls.

Statistical analysis

Germination data for each accession were analysed by ANOVA using the General Linear Model procedure in Statistical Analysis Software, version 9.2 (SAS®, 2008). Germination values were evaluated based on the Least Significant Difference and Duncan's Multiple Range tests.

Results

A. Two-accession germination test
Both accessions germinated to a much higher percentage when treated with 0.5 M ethanol as compared with the water-treated samples (figure 1). Seeds of PI 220518 showed no statistical difference in its response to red-light or dark treatments, whether treated with ethanol or water, but the response to ethanol was obvious (figure 1A), where more than 80% of seeds exposed to ethanol germinated while less than 25% germinated for the water treatment. The response to ethanol and red-light for PI 383642 was even more dramatic (figure 1B). The response to ethanol and red light was nearly 90%, greatly exceeding the response to water and red light. Even under dark conditions, the germination response for this accession was more than 60% for the ethanol treatment, but only about 2% for the water treatment. The seeds of PI 383642 also responded well to red light at 88% germination, which was significantly higher than the dark-treated seeds at 65%.
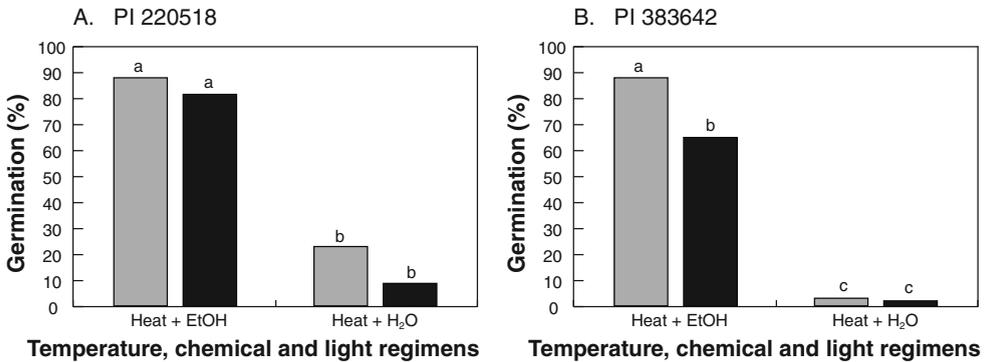

Figure 1. Response of *Echinochloa* accessions to 0.5 M ethanol pretreatment under initial red-light (grey bars) and dark (black bars) germination conditions in the two-accession germination test. Pretreatments: Heat + EtOH = three days at 36°C in dark, 0.5 M ethanol (aq); Heat + H_2O = three days at 36°C in dark, distilled water, initially, tap water thereafter. Means with the same letter are not statistically different (α = 0.05).

B. Five-accession germination test
The results of the five-accession germination test were quite varied (figure 2). The seeds of four accessions (PI 220518, 383642, 420160 and 664493) responded positively to at least one of the factors tested: heat, ethanol or light. Seeds of the fifth accession (PI 217911), our only sample of *E. frumentacea* Link, were highly non-dormant (figure 2A). Notably, the use of ethanol tended to suppress germination in this non-dormant seed lot.

PI 664493, which was the only sample that had been stored at -18°C, responded to cool-white fluorescent light treatment alone (figure 2E), whether as a single 8-hour treatment or a daily 8-hour treatment in the alternating temperature regimen. The heat or ethanol treatments had no significant effect on that accession. The seeds of PI 220518 and PI 420160 responded best to the combined treatment of heat and ethanol, regardless of light or dark conditions (figure 2B,D), but the heat and light treatments without ethanol

had no effect on germination response. This result suggests that ethanol prevented the induction of secondary dormancy by warm temperatures for these two accessions, consistent with the findings of Taylorson (1982) for *Setaria*. Finally, PI 383642 only displayed high levels of germination (more than 80%) in response to the combination of ethanol and light (figure 2C). ANOVA confirmed highly significant effects for both ethanol and light, and also for their interaction.
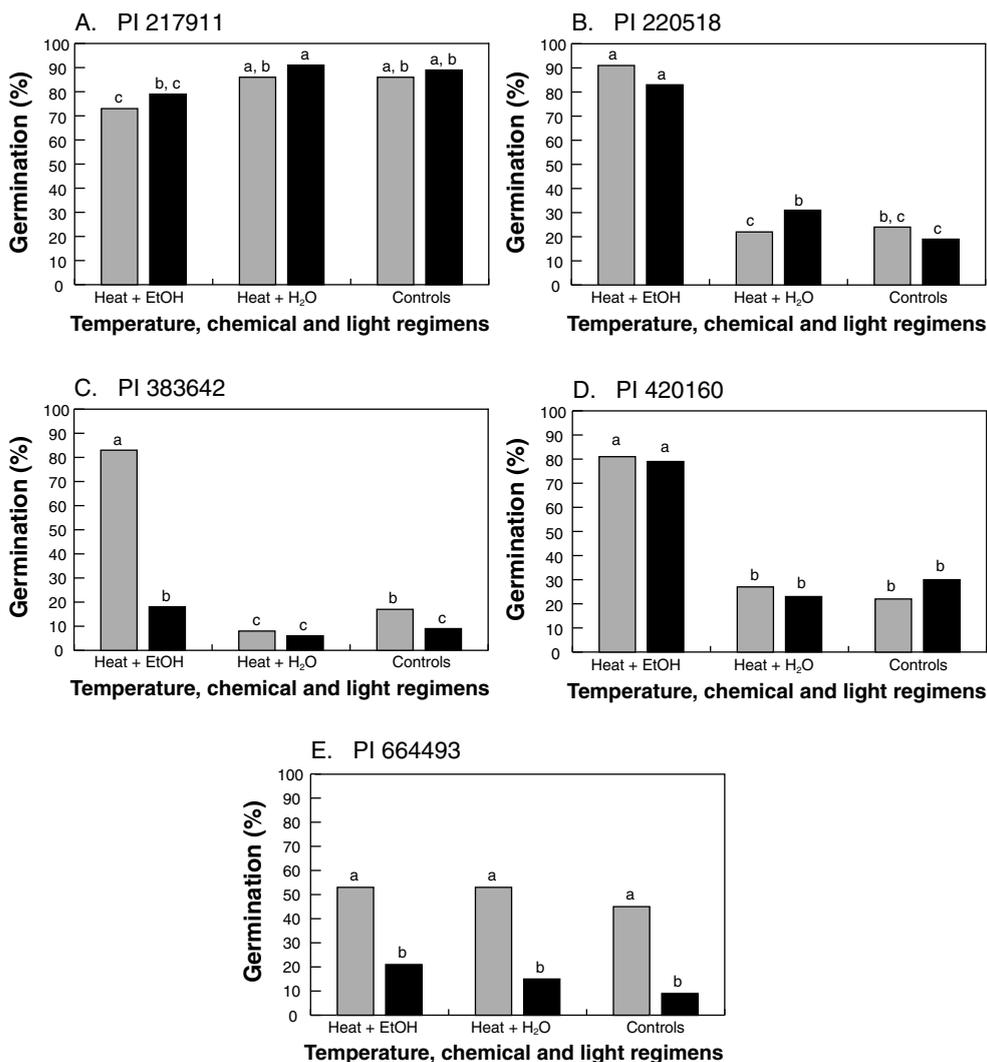

Figure 2. Response of *Echinochloa* accessions to 0.25 M ethanol pretreatment under initial light (grey bars) and dark (black bars) germination conditions in the five-accession germination test. Pretreatments: Heat + EtOH = three days at 37°C in dark, 0.25 M ethanol (aq); Heat + H$_2$O = three days at 37°C in dark, distilled water initially, tap water thereafter; Controls = no pretreatment. Means with the same letter are not statistically different (α = 0.05).

Discussion

These varied results challenge the development of a simple follow-up procedure for more deeply dormant *Echinochloa* seeds that do not respond to the parallel test of light and dark treatments recommended previously (Kovach *et al.*, 2010). Elevated concentrations of ethanol might eliminate the need for light or dark conditions in some species, but may be detrimental in others – as seen in *Panicum* (Taylorson and Hendricks, 1979). Still, ethanol pretreatments at a somewhat elevated temperature (at least 30°C) were beneficial for three dormant accessions (figure 2B-D). Notably, results from our first experiment for PI 383642 compared favourably with previously reported results (Kovach *et al.*, 2010), when seeds of this same accession were subjected to seven weeks at 10 – 15°C before breaking dormancy. Thus, the use of ethanol substantially shortened the time required to achieve similar results. In our second experiment, one result was unexpected. A germination test conducted on this lot of PI 217911 in 2007 indicated a significant level of seed dormancy (almost 50%; table 1). However, such dormancy was not observed in our current study; this change may have resulted from gradual after-ripening in storage (reviewed by Trapp *et al.*, 2012).

In addition, there are two factors that varied between our experiments (red vs. cool white fluorescent lighting and ethanol concentration), for which we did not conduct replicated testing, that deserve discussion. Fortunately, there were two accessions tested in common between the two experiments, PI 220518 and 383642, allowing us make general comparisons. In the case of light, no obvious differences can be detected in either accession. Germination levels were nearly identical under both lighting conditions (comparison of grey bars in figures 1 and 2B,C). This supports the use of cool white lighting, which is widely available. In the case of ethanol concentration, there was only one clear distinction. PI 383642 when pretreated with 0.5 M ethanol displayed more than 60% germination in darkness (figure 1B), but, when 0.25 M ethanol was used, less than 20% of the seeds germinated under dark conditions, suggesting that the higher concentration partially obviated the need for light, providing one example in support of our initial rationale for a lower concentration.

We propose that for deeply dormant accessions of *Echinochloa*, a new side-by-side approach is needed for follow-up germination tests (as noted below). Alternatively, findings by Cohn *et al.* (1989) suggest that parallel research on the use of more highly lipophilic alcohols, such as butanol and propanol, may be effective in breaking dormancy at lower concentrations than ethanol.

In the first germination test, most germination occurred within five days (only a few more seeds germinated between days 5 and 8). In the five-accession germination test, where both the ethanol concentration was reduced and cool-white fluorescent light was used instead of moderately-narrowband red light, germinations proceeded slightly slower for three (PI 217911, 383642 and 664493) of the five accessions tested. Therefore, the following recommendation employs a 14-day germination count.

For *Echinochloa* species, we recommend the following two-step approach for seed germination testing.

Based on the findings of Kovach *et al.* (2010), first, during the initial germination test, two replicates should be conducted with the seeds placed on top of a germination blotter and exposed to cool-white fluorescent light during the warm cycle of a 20/30°C (16/8 hours) temperature regimen. Two additional replicates should be excluded from light either by placing seeds between and near the centre of two dark, opaque blotter papers or on top of blotter paper but with germination boxes carefully wrapped to exclude light.

However, any seed lots that do not respond to either the initial light or dark treatments should be given a follow-up test. In the follow-up test, seeds of all four replicates should be placed on top of blotter paper. The imbibition liquid should be comprised of approximately 0.25 M ethanol (achieved by adding 15 ml of 95% ethanol to 985 ml water). All replicates should be maintained at 35-37°C for three days in the dark (pretreatment). The seeds should then be transferred under dim light to new blotter papers wetted with tap water. Two replicates should be kept dark (employing techniques described previously), and two should be exposed to at least one 8-hour light treatment during the warm cycle of a 20/30°C (16 hours / 8 hours) temperature regimen. All replicates should receive this 20/30°C (16 hours / 8 hours) temperature regimen for the conduct of a 14-day germination test, with counts recorded at 4, 7 and 14 days after the start of the test.

Acknowledgements

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