

## Appendix 6: Proposal 6 Supporting Evidence:

2015 AOSA Elymus referee.ppt

June 2015

The original experiment employed a split-plot, nested design with temperature as the main plot and accession and seed source as a subplot for preliminary testing on germination viability (percentage viable seed). Constant temperatures of 15°C, 20°C and 25°C were the main parameters tested. Lights were used for 8 h on and 16 h lights off per the AOSA standards for use of lights (2014b). Chamber temperatures were monitored to be within 2°C of targeted parameter. Three germination chambers were used for this study; two water curtain germinators (Stults Scientific Engineering Corporation, Springfield, IL) and one dry chamber (Seedburo Equipment Company, Des Plaines, IL).

The experimental model had 8 runs. A single run for ELWA2 consisted of 18 germination boxes (50 seed per box) representing six different seed sources (Table 1. Experimental sample identification and seed source). The 18 boxes were randomly placed in each of the three chambers on the same two top shelves for each run. ELWA2 had six samples of varying age, quality and seed source within the experiment model (Table 1). There was an exception to this design, only five independent samples of ELMA7 were available for the study. A single run for ELMA7 consisted of 15 germination boxes (50 seed per box) representing five different seed sources. The 264 total samples (8 runs x 18 germination boxes x 3 chambers for ELWA2 and 8 runs x 15 germination boxes x 3 chambers for ELMA7) had 263 degrees of freedom. The location of germination boxes within the chambers were randomized for every run with the use of an online tool (<https://www.randomizer.org/>). A ninth run was established in the same methodology but without a light source to establish if darkness effected seed germination.

The procedure started with saturated standard blotter paper (Anchor Paper Company, St. Paul, MN) with distilled water. A single saturated blotter paper was placed in a 10.12 cm<sup>2</sup> germination box. For each sample, 50 seeds were counted and placed in 5 rows of 10 seed on top of the moist blotter paper. A total of 1.65 g of wettable powdered Captan 50% WP fungicide was sprinkled over the seed. The germination box was labeled with the sample number and seed identification (ELMA7 or ELWA2). Between each sample set-up, forceps were dipped in Waxie Green Neutral Cleaner<sup>®</sup> to reduce the spread of seed coat fungus. Moisture in germination boxes were monitored daily by the “thumb indent test” for the moisture content of the blotter paper (2014b, Section 6.3). Distilled water was added as necessary to maintain a moist blotter paper.

Seedlings were evaluated on day seven (7 d), fourteen (14 d), twenty-four (24 d) and thirty-five (35 d) for normal seedling, abnormal seedling and dead seed. Testing was continued for an additional 2 d if more than 10% of the seed had not germinated by 35 d. The seedling counts were recorded on an Excel spread sheet and are graphically displayed in Figure 1. Number of normal seedlings over time at temperatures 15°C, 20°C and 25°C. The single run of samples tested within darkness resulted in < 10%

viability for all species and no further replicates were tested without light. There was no comparative analysis performed between viability results with and without lights.

Results were analyzed using SAS 9.2 software (2013) for variation among temperature treatments, normal seedlings and abnormal seedlings. ANOVA ( $P < 0.05$ ) showed no statistical variation for the number of abnormal seedlings ( $P = 0.7839$ ) or dead seeds ( $P = 0.6793$ ) observed in this experiment for all species. There was no statistical difference between the number of normal seedlings on 35 d ( $P = 0.1005$ ) indicating that a final count on 35 d is not necessary. ELMA7 had no significant variation between all temperature treatments. The ANOVA analysis of viability provided evidence of no significant difference between temperatures 15°C and 20°C for ELWA2 ( $P = 0.0059$ ). There was no significant difference in all three temperature treatments for ELMA7 ( $P = 0.6651$ ). Statistical output for all species is presented in Figure 2. Statistical analysis of viability at three constant temperatures 15°C, 20°C and 25°C and Table 2. Analysis of Variation (ANOVA) model for ELMA7 and ELWA2.

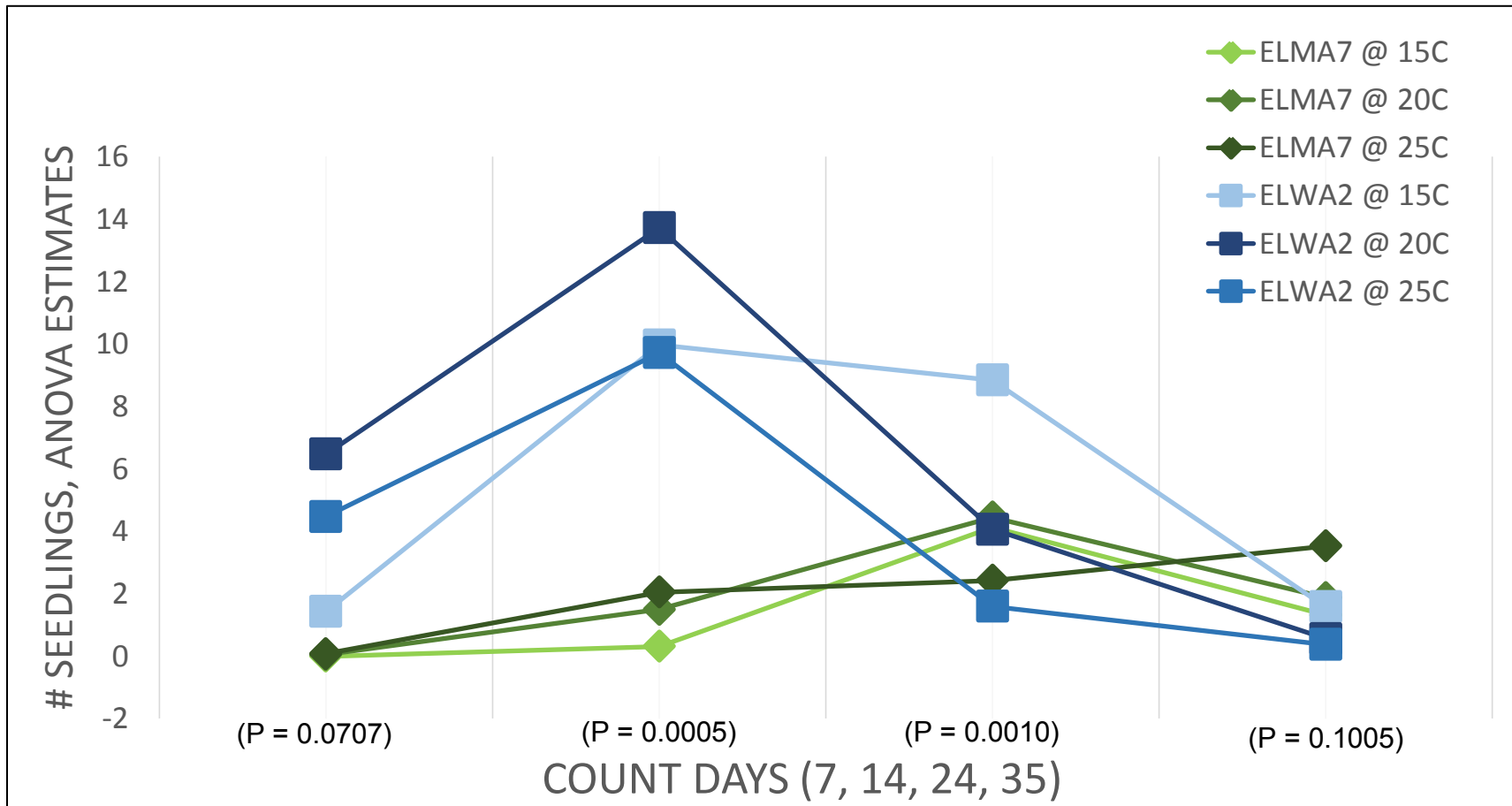
Based upon these findings, an AOSA referee project proceeded at a constant temperature of 20°C for a test period of 21 d. The constant temperature of 20°C was selected as a common chamber temperature available for seed testing laboratories. The establishment of a final count of either 14 or 21 days was assessed for statistical significance to determine the appropriate final count day.

Table 1. Experimental sample identification and seed source

<b>TAXON SYMBOL</b>	<b>ID #</b>	<b>CULTIVAR OR NAME</b>	<b>SOURCE</b>	<b>YEAR</b>
ELMA7	1	PI 531633	Western Regional Plant Introduction Station, Pullman Washington	1991
ELMA7	2	sfd-09-11pmc12	Alaska Plant Material Center, Palmer, Alaska	2009
ELMA7	3	sfd-09-11pmc12 *	Alaska Plant Material Center, Palmer, Alaska	2009
ELMA7	4	sfd-07-07pmc115	Alaska Plant Material Center, Palmer, Alaska	2007
ELMA7	5	sgo-05-05PMC138	Alaska Plant Material Center, Palmer, Alaska	2005
ELMA7	6	sfd-11-11pmc148	Alaska Plant Material Center, Palmer, Alaska	2011
ELWA2	7	PI 440921, 'SECAR'	Western Regional Plant Introduction Station, Pullman Washington	1982
ELWA2	8	PI 506266	Western Regional Plant Introduction Station, Pullman Washington	1988
ELWA2	9	'DISCOVERY'	United States Department of Agriculture – Forest and Range Research Laboratory, Logan Utah	2009
ELWA2	10	'DISCOVERY'	United States Department of Agriculture – Forest and Range Research Laboratory, Logan Utah	2011
ELWA2	11	'SECAR'	United States Department of Agriculture – Forest and Range Research Laboratory, Logan Utah	1995
ELWA2	12	'SECAR'	United States Department of Agriculture – Forest and Range Research Laboratory, Logan Utah	2003

\* duplicate was not counted in the statistical analysis.

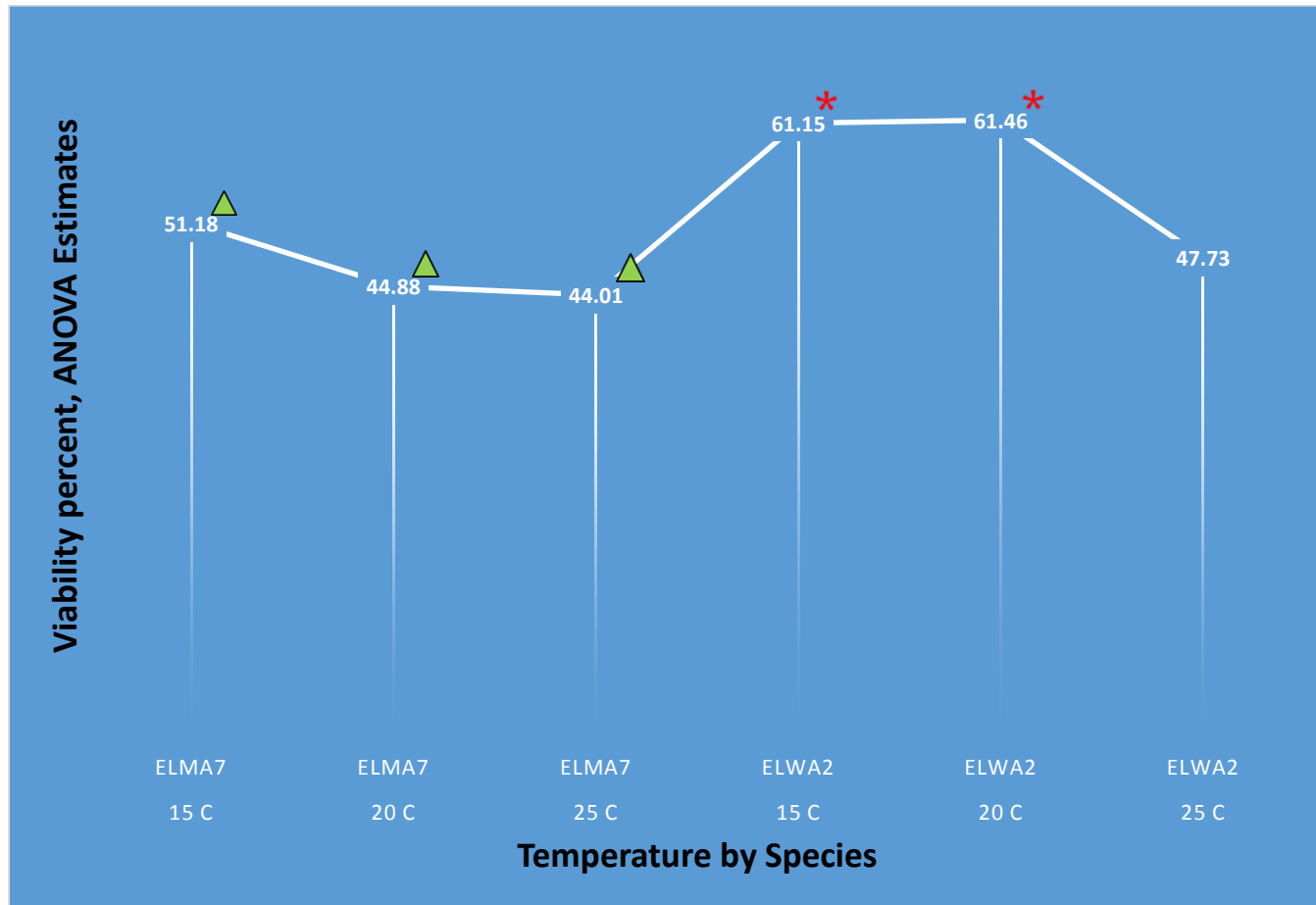
Figure 1. Number of normal seedlings over time at temperatures 15°C, 20°C and 25°C



ANOVA ( $P < 0.05$ ) was done with SAS 9.2 (2013). There was no statistical variation for the number of abnormal seedlings ( $P = 0.7839$ ) or dead seeds ( $P = 0.6793$ ) observed in this experiment for all species. ELMA7 had no significant variation between all treatments. There was no significant difference in number of normal seedlings produced in ELWA2 at 15°C and 20°C.

[Key: ELMA7 = Tufted Wheatgrass; ELWA2 = Snake River Wheatgrass]

Figure 2. Statistical analysis of viability at three constant temperatures 15°C, 20°C and 25°C



▲ There was no significant difference in all three temperature treatments for ELMA7 (P = 0.6651).

\* There was no significant difference between temperatures 15°C and 20°C for ELWA2 (P= 0.0059).

[Key: ELMA7 = Tufted Wheatgrass; ELWA2 = Snake River Wheatgrass]

Table 2. Analysis of Variation (ANOVA) model for ELMA7 and ELWA2

The SAS System

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The Mixed Procedure

Model Information

```
Data Set                WORK.GERM
Dependent Variable      ABNORM
Covariance Structure    Variance Components
Estimation Method       REML
Residual Variance Method Profile
Fixed Effects SE Method Model-Based
Degrees of Freedom Method Satterthwaite
```

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TEMP	2	20.3	3.24	0.0602
Species	3	9.04	0.36	0.7839
Species*TEMP	6	258	1.62	0.1423

The SAS System

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The Mixed Procedure

Model Information

```
Data Set                WORK.GERM
Dependent Variable      DEAD
Covariance Structure    Variance Components
Estimation Method       REML
Residual Variance Method Profile
Fixed Effects SE Method Model-Based
Degrees of Freedom Method Satterthwaite
```

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TEMP	2	15.9	5.62	0.0143
Species	3	9	0.52	0.6793
Species*TEMP	6	258	4.55	0.0002

The SAS System

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The Mixed Procedure

Model Information

```
Data Set                WORK.GERM
```

Dependent Variable **D35NORM**  
 Covariance Structure Variance Components  
 Estimation Method REML  
 Residual Variance Method Profile  
 Fixed Effects SE Method Model-Based  
 Degrees of Freedom Method Satterthwaite

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TEMP	2	23.5	2.54	<b>0.1005</b>
Species	3	9.05	1.02	0.4281
Species*TEMP	6	258	2.42	0.0270

The SAS System

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The Mixed Procedure

Model Information

Data Set WORK.GERM  
 Dependent Variable **VIABLE**  
 Covariance Structure Variance Components  
 Estimation Method REML  
 Residual Variance Method Profile  
 Fixed Effects SE Method Model-Based  
 Degrees of Freedom Method Satterthwaite

----- Effect=Species\*TEMP Method=LSD(P<.05) Set=3 -----

Obs	Species	TEMP	Estimate	Standard Error	Letter Group
8	ELELb	20	89.2141	26.1852	ABC
9	ELELb	15	85.4681	26.3087	ABC
10	ELELc	15	71.2372	15.5440	ABD
11	ELELc	20	69.8441	15.4348	ABD
12	ELELb	25	64.1883	26.1852	DE
13	ELWA	20	61.4559	13.5020	<b>ACD</b>
14	ELWA	15	61.1514	13.6125	<b>ACD</b>
15	ELMA	15	51.1784	12.3090	<b>ABCD</b>
16	ELWA	25	47.7343	13.5020	BE
17	ELMA	20	44.8840	12.1962	<b>ABCD</b>
18	ELMA	25	44.0098	12.1962	<b>ABCD</b>
19	ELELc	25	41.0679	15.4348	CE

## Referee Panel:

A referee panel was established with 4 to 7 laboratories. The referee for ELMA7 evaluation had four participating laboratories. The ELWA2 referee evaluation had seven participating laboratories. The referee experiment employed a split-plot, nested design with the laboratory as the main plot and replicated samples as a subplot on the number of seedlings counted as normal germination by AOSA published standards. The main parameter tested was normal seedlings at 7, 14 and 21 d between all laboratories. The seed sources for the referee included fresh seed samples when available which were less than one year old, Table 3. 2015 Referee seed samples of ELMA7 and ELWA2. The fresh seed lot ELWA2 was regenerated from seed stock during the 2013-2014 growing season at the USDA-ARS, Central Ferry Research Farm, located on the Snake River in Washington. The regeneration harvest yielded no pure live seed of ELMA7. The harvest was primarily of inflorescence with immature seed. No fresh seed of ELMA7 was available for the referee study.

The referee test procedures started with saturation of standard blotter paper with distilled water. A single saturated blotter paper was placed in a germination box. The size of the germination box varied among laboratories, but had no impact on the outcome. For each provided sample, a minimum of four replicates were placed in an individual germination box and labeled for each species. The chamber temperature was established at a constant 20°C. Chamber temperatures were monitored to assure they were within 2°C of targeted parameter. One laboratory reported a spike in temperature and made note of the variation on the data recording sheet. This single occurrence did not alter the output. All germination boxes were monitored for the moisture content of the blotter paper. The counts for germinated seedlings were done on day 7, 14 and 21. If seeds were determined as ungerminated by 21 d, it was held over for an additional 2 d of testing per AOSA rules (2014a). A total test period of 23 d was possible. Following a 21-day-test period, remaining ungerminated seed were TZ tested and calculated TZ viable results were added to the total viability. The samples and sources of the seed samples are in Table 3. 2015 Referee seed samples of ELMA7 and ELWA2.



Table 3. 2015 Referee seed samples of ELMA7 and ELWA2

<b>TAXON SYMBOL</b>	<b>ID #</b>	<b>CULTIVAR OR NAME</b>	<b>SOURCE</b>	<b>YEAR</b>
ELMA7	1	sgo-05-05pmc138	Alaska Plant Material Center, Palmer, Alaska	2005
ELMA7	2	sfd-07-07PMC115	Alaska Plant Material Center, Palmer, Alaska	2007
ELMA7	3	sfd-09-11pmc12	Alaska Plant Material Center, Palmer, Alaska	2011
ELMA7	4	sfd-11-11pmc148	Alaska Plant Material Center, Palmer, Alaska	2011
ELWA2	1	'SECAR'	United States Department of Agriculture – Forest and Range Research Laboratory, Logan Utah	1995
ELWA2	2	'SECAR'	United States Department of Agriculture – Forest and Range Research Laboratory, Logan Utah	2003
ELWA2	3	'DISCOVERY'	United States Department of Agriculture – Forest and Range Research Laboratory, Logan Utah	2011
ELWA2	4	PI 440921, 'SECAR'	USDA-ARS, Central Ferry Research Farm, Washington	2013
ELWA2	5	PI 506266	USDA-ARS, Central Ferry Research Farm, Washington	2013
ELWA2	6	'SECAR'	USDA-ARS, Central Ferry Research Farm, Washington	2014

A comparison of the results among laboratories, between replicates and test days were statistically analyzed using SPSS v10.0 software (2015). Two assumptions were made in the analysis; all laboratories evaluated the abnormal seedlings and dead seedlings correctly according to the AOSA Rules standards. A paired sample test (t-test) was performed for comparison of the replicated results of seedling counts for 14 versus 21 d. The paired t-test for ELMA7 had a lower mean at 14 d (7.3594) than for 21 d (14.0625). A comparison of each replicate for all laboratories and samples had a significant difference between 14 and 21 d ( $P = 0.000$ ) with a high standard deviation (10.56601). The 95% Confidence Interval of the Difference for each replicate for all laboratories and samples was high at 5.27863%. Both of these values indicate the results were inconsistent between laboratories and samples. A comparison of viability percentages, from the pooled four replicates was similar with results of an 11.1321% difference between the upper and lower Confidence Interval with a standard deviation (10.44558) greater than the mean germination (7.3594) at 7 d. The comparison of viability from the pooling of four replicates between 14 and 21 d had a significant difference ( $P = 0.021$ ). The Univariate Analysis of Variance indicates results between laboratories at 21 d had no significant difference ( $P = 0.097$ ). The replicates had no differences on 7 d ( $P = 0.468$ ), 14 d ( $P = 0.462$ ) and 21 d ( $P = 0.529$ ). The largest interaction between laboratories and samples was at 21 d ( $P = 0.011$ ). The test sample size of four is very small. Therefore, a more reliable statistic is the Confidence Interval of Difference which indicates the germination increased over time and a significant difference in viability occurred by 21 d evaluation. Thus, the final evaluation for optimal viability was most appropriate at 21 d. The statistical analysis for ELMA7 was summarized in Table 4. Statistical analysis of 2015 referee project of ELMA7. A germination test period of > 14 d is not unusual for other *Elymus* species published within AOSA Rules for Testing Seeds. For instance, the final count for *Elymus smithii* (*Pascopyrum smithii*) is listed as 28 d. (2014b).

ELWA2 paired test of each replicate for all laboratories and samples had a small standard deviation (1.45) and a very small 95% Confidence Interval of the Difference (0.44). Similarly, the paired test of viability percent from pooled four replicates had a small standard deviation (1.11) and very small 95% Confidence Interval of the Difference (0.69). The data had a consistent and real difference ( $P = 0.000$ ) although very small. The laboratories differed among each other between evaluation days ( $P = 0.000$ ). However, these differences did not appear in the replicates for 7 d ( $P = 0.195$ ), 14 d ( $P = 0.703$ ) or 21 d ( $P = 0.081$ ), which indicates the laboratory results were similar when pooled for the replicates at each count day. An interaction of laboratories and samples without the effect of replicates was observed at all evaluation days ( $P = 0.000$ ). The statistical analysis for ELWA2 was summarized in Table 5. Statistical analysis of 2015 referee project of ELWA2.

**Conclusion:** The preliminary study had strong evidence supporting 20°C as an appropriate germination temperature for ELMA7 and ELWA2. The referee project results using the Confidence Interval of the Difference indicated a small difference of viability between 14 and 21 d for ELWA2. For a small sample size, the Confidence Interval of Difference was an accurate statistical measurement. The mean viability of

the replicates individually and pooled for ELWA2 were similar which indicated there was little difference between a 14 or 21 d counts. Therefore, the 14 d was proposed for the final count for ELWA2. A high standard deviation and larger Confidence Interval of Difference suggested that the final count for ELMA7 should be made at 21 d.

The proposed new rules are reasonable and similar in content to other *Elymus* species currently published within AOSA Rules for Testing Seeds, Table 6A (2014a). The addition of *Elymus macrourus* (ELMA7, Tufted Wheatgrass) and *Elymus wawawaiensis* (ELWA2, Snake River Wheatgrass) germination standards to the AOSA Rules for Testing Seeds will establish uniformity among native grass seed laboratories. These elementary standards start to fill in gaps where germination standards were lacking.

Assistance with the statistical analysis of the preliminary germination study was provided by Dr. Richard Johnson, USDA-ARS, Western Regional Plant Introduction Station, Pullman, Washington. Statistical analysis and assistance of the referee project was provided by Dr. Riad Baalbaki, Plant Pest Diagnostics Branch, California Department of Food & Agriculture, Sacramento, California.

Table 4. Statistical analysis of 2015 referee project of ELMA7

**Paired Samples Statistics of Each Replicate from All Labs and Samples**

	Mean	N	Std. Deviation	Std. Error Mean
Pair 1 14 days	7.3594	64	14.28215	1.78527
21 days	14.0625	64	23.45470	2.93184

**Paired Samples Test of Each Replicate from All Labs and Samples**

		Paired Differences							
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		t	df	Sig. (2-tailed)
					Lower	Upper			
Pair 1	14 days-21 days	-6.70313	10.56601	1.32075	-9.34244	-4.06381	-5.075	63	.000

**Paired Samples Statistics of Germination % from Total of Four Replicates**

	Mean	N	Std. Deviation	Std. Error Mean
Pair 1 % germination 14 days	7.3594	16	14.13977	3.53494
% germination 21 days	14.0625	16	23.89587	5.97397

**Paired Samples Test of Germination % from Total of Four Replicates**

		Paired Differences							
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		t	df	Sig. (2-tailed)
					Lower	Upper			
Pair 1	% germination 14 days - % germination 21 days	-6.70313	10.44558	2.61140	-12.26918	-1.13707	-2.567	15	.021

## Univariate Analysis of Variance

### Between-Subjects Factors

		N
lab	1.00	16
	2.00	16
	4.00	16
	7.00	16
sample	1.00	16
	2.00	16
	3.00	16
	4.00	16
rep	1.00	16
	2.00	16
	3.00	16
	4.00	16

### Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
lab	germ7d	27.375	3	9.125	125.143	.000
	germ14d	309.922	3	103.307	5.603	.003
	germ21d	70.422	3	23.474	2.274	.097
rep(lab)	germ7d	.875	12	.073	1.000	.468
	germ14d	222.938	12	18.578	1.008	.462
	germ21d	115.188	12	9.599	.930	.529
sample	germ7d	36.750	3	12.250	168.000	.000
	germ14d	8979.297	3	2993.099	162.322	.000
	germ21d	6204.922	3	2068.307	200.394	.000
lab * sample	germ7d	82.125	9	9.125	125.143	.000
	germ14d	930.641	9	103.405	5.608	.000
	germ21d	271.266	9	30.141	2.920	.011
Error	germ7d	2.625	36	.073		
	germ14d	663.813	36	18.439		
	germ21d	371.563	36	10.321		
Corrected Total	germ7d	149.750	63			
	germ14d	11106.609	63			
	germ21d	7033.359	63			

Table 5. Statistical analysis of 2015 referee project of ELWA2

**Paired Samples Statistics of Each Replicate from All Labs and Samples**

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	14 days	63.8929	168	37.25716	2.87445
	21 days	65.1607	168	37.10825	2.86296

**Paired Samples Test of Each Replicate from All Labs and Samples**

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	14 days-21 days	-1.26786	1.45377	.11216	-1.48929	-1.04642	11.304	167	.000

**Paired Samples Statistics of Germination % from Total of Four Replicates**

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	% germination 14 days	63.8929	42	37.48199	5.78360
	% germination 21 days	65.1607	42	37.33557	5.76100

**Paired Samples Test of Germination % from Total of Four Replicates**

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	% germination 14 days - % germination 21 days	-1.26786	1.11173	.17154	-1.61430	-.92142	-7.391	41	.000

## Univariate Analysis of Variance

### Between-Subjects Factors

		N
lab	1.00	24
	2.00	24
	3.00	24
	4.00	24
	5.00	24
	6.00	24
sample	1.00	28
	2.00	28
	3.00	28
	4.00	28
	5.00	28
	6.00	28
rep	1.00	42
	2.00	42
	3.00	42
	4.00	42

### Tests of Between-Subjects Effects

Source	Dependent Variable	Type III SS	df	Mean Square	F	Sig.
lab	d7norm	2554.226	6	425.704	28.741	.000
	d14norm	2653.202	6	442.200	40.578	.000
	d21norm	95.488	6	15.915	14.536	.000
rep(lab)	d7norm	402.250	21	19.155	1.293	.197
	d14norm	185.250	21	8.821	.809	.703
	d21norm	35.292	21	1.681	1.535	.081
sample	d7norm	216559.905	5	43311.981	2924.133	.000
	d14norm	2808.262	5	561.652	51.539	.000
	d21norm	15.196	5	3.039	2.776	.021
lab * sample	d7norm	3164.845	30	105.495	7.122	.000
	d14norm	2464.155	30	82.138	7.537	.000
	d21norm	92.012	30	3.067	2.801	.000
Error	d7norm	1555.250	105	14.812		
	d14norm	1144.250	105	10.898		
	d21norm	114.958	105	1.095		
Corrected Total	d7norm	224236.476	167			
	d14norm	9255.119	167			
	d21norm	352.946	167			

## References:

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2014a. [CD] AOSA. Association of Official Seed Analysts, Inc. AOSA Rules for Testing Seeds Volume 1, Table 6A. Principles and Procedures AOSA, 1601 52nd Avenue, Suite #1, Moline, IL 61265, USA.

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