Referee Study on Germination of Canarygrass (*Phalaris canariensis*)

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Introduction:

Seed Science and Technology Section (SSTS) of Canadian Food Inspection Agency (CFIA) organized a referee study of seed germination on canarygrass (*Phalaris canariensis* L.) in April-May, 2023. A referee study is to evaluate testing methods, i.e., ISTA, AOSA, and Canadian Methods and Procedures for Testing Seed (Canadian M&P). A referee study is also used for studying the uniformity among participating laboratories and providing information for method improvement or harmonization.

It is noticed that the three testing rules, ISTA, AOSA and M&P, have different germination testing temperatures and counting days required for the germination of canarygrass (Table 1).

Table 1. Comparison of the germination method for canarygrass in three testing rules

Rule	Substrata	Temperature (°C)	First count (days)	Final count (days)	Fresh or Dormant seeds
M&P	BP; RT	15-25	7	10	Prechill, 0.1% KNO_3
ISTA	TP	20-30; 15-25	7	21	KNO_3; Prechill
AOSA	B; T	20-30	3	7	

The Objectives of this Canarygrass Referee Study are:

1. To promote uniformity among seed testing rules regarding the germination testing temperatures and counting days in canarygrass.

2. To provide data as supporting evidence for proposing seed testing rule amendments, such as the harmonization of seed testing procedures and rules of AOSA, ISTA and Canadian M&P.

Materials and Method

Testing Samples

A pre-test was conducted to select seed lots based on germination range. In total, germination of nine seed lots of canarygrass were tested and compared under 20-30 ºC (Figure 1). Three seed lots (lot 2, 4 and 8) with germination range between 74-88% were selected for this referee study.

Figure 1. Pre-test germination results of 9 seed lots of canarygrass in 20-30 ºC.

Each selected seed lot was mixed and divided into the required subsamples following the Canadian M&P procedures. The homogeneity test samples were randomly assigned, and the tolerance was calculated following ISTA PT program in sample preparation. When the seed lots passed the homogeneity test, subsamples were assigned randomly to 28 participating laboratories in Canada and the USA. Each subsample contained approximately 2000 units of pure seeds, corresponding to approximately 13.5 g (AOSA Rules Volume 1, Table 2A, 150 seeds per gram). This quantity ensured there were enough pure seeds in the subsample for all the required germination tests and re-test if necessary.

Testing Methods

For this referee study, the germination method for canarygrass was conducted as indicated in Table 2. Seed germination was counted and recorded in 3, 7, 10, 14 and 21 days after planting in the provided datasheet. Seedling evaluation followed either the Canadian M&P or AOSA rule. No dormancy treatment was applied.

Table 2. Germination method for the seeds of *Phalaris canariensis*

Treatments	Number of seeds x reps	Substrata	Specific requirement
Alternating 15 - 25°C	100 x 4	BP/B*	light
Alternating 20 - 30°C	100 x 4	BP/B*	light

* Between paper (BP) substrata is following the Canadian M&P Rule; Between blotters (B) substrata is following the AOSA Rule.

Data analysis

Significant difference between the two germination temperatures and among five counting dates in normal seedlings, abnormal seedlings and ungerminated seeds was evaluated using Generalized Linear Model (GLM) in SAS software at 95% confidence level, with seed lot and temperature as fixed effects and laboratory and replicate as random effects. Analysis of Variance (ANOVA) was conducted to detect the variation sources. Comparison of means between germination temperatures and among counting days were generated.

Data of normal and abnormal seedlings and ungerminated seeds were analyzed using software *ISTAgermMV* in R package following statistical tools "Inter laboratory tests using ISO 5725-2" developed by ISTA statistical committee. Repeatability and reproducibility were calculated with *ISTAgermMV* program, where repeatability quantifies the average variability of results within a laboratory, and reproducibility quantifies the average variability among laboratories.

Testing result variation of final germination in each participating laboratory was also analyzed using z-scores, which compare the distance of the participants' results from the overall sample mean of all participants under each temperature for each seed lot.

Test Results

Analysis of Variance and the Effect of Germination Temperature

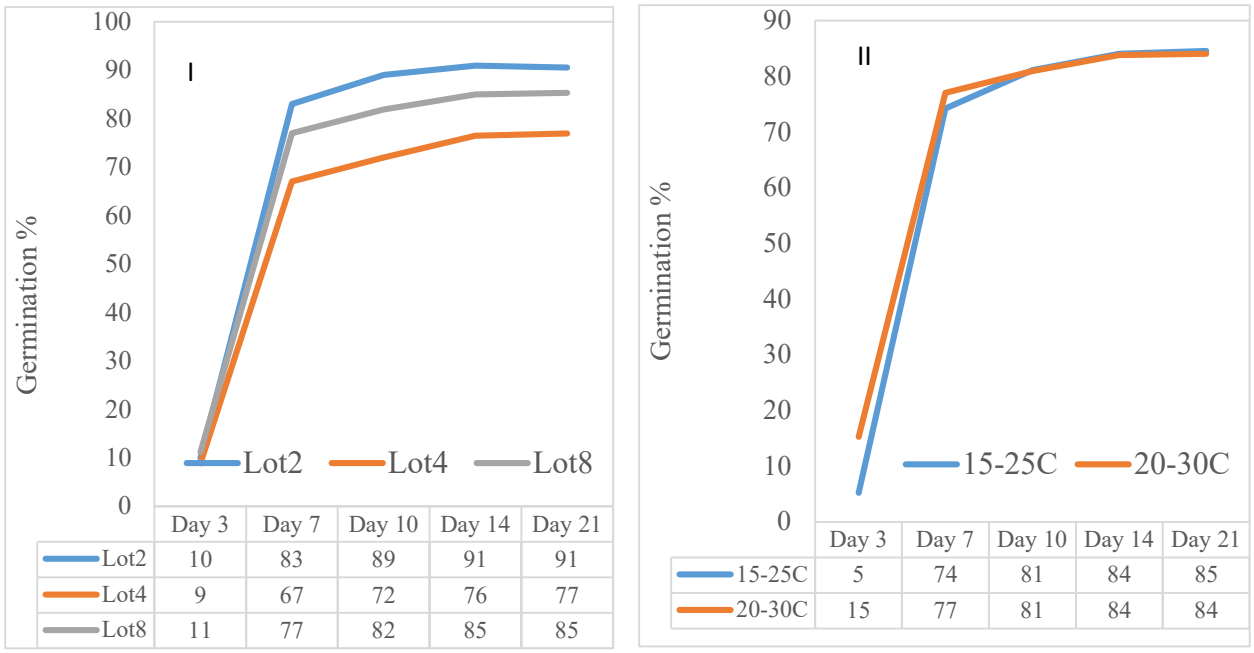
The analysis of variance for temperature and seed lot effects under different counting days (Table 3) indicated that temperature did not have significant effect on normal seedlings from day 7 and onwards. Abnormal seedlings were also not significantly affected by the germination temperatures. Seed lots had significant effect on normal and abnormal seedlings in all different counting days, which demonstrates designed variation of seed lots.

Table 3: Analysis of variance of temperature and seed lot effects in different final counting days for canarygrass.

		Normal Seedling				Abnormal Seedling
		Day 7	Day 10	Day 14	Day 21	Day 21
Source	DF	**Pr > F**	**Pr > F**	**Pr > F**	**Pr > F**	**Pr > F**
Temp	1	0.2346	0.8160	0.6003	0.2843	0.3780
Seed lot	2	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Temp*seed lot	2	0.2956	0.0101	0.0288	0.0724	0.0005

The Effect of Counting Days

The germination trends of canarygrass were similar among the three tested seed lots (Figure 2I) and between the two tested germination temperatures (Figure 2II). Overall, germination percentage increased rapidly until day 7, and then slowed down after day 10 and stopped increasing after day 14. Averaged germination percentage did not differ significantly from day 10 (Figure 3).



	Day 3	Day 7	Day 10	Day 14	Day 21
Lot2	10	83	89	91	91
Lot4	9	67	72	76	77
Lot8	11	77	82	85	85

	Day 3	Day 7	Day 10	Day 14	Day 21
15-25C	5	74	81	84	85
20-30C	15	77	81	84	84

Figure 2. Average germination curve in different counting days in three canarygrass seed lots (I) and under 15-25 and 20-30ºC (II).

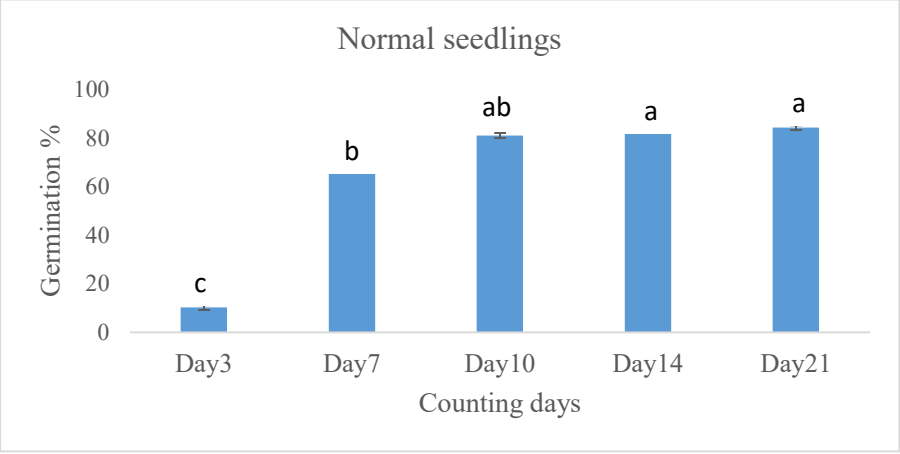


Figure 3. Average germination percentage over three seed lots and two germination temperatures at 5 different counting days.

Repeatability and Reproducibility

Repeatability of canarygrass indicated more variation when seeds were germinated in 20-30°C than in 15-25°C when the seedlings were counted at day 7 and day 10, however, the variations within labs were similar between the two temperatures when counted at day 14 and 21 (Figure 4). The dispersal factors were both around 1.0 (data not shown) for seedlings in both temperatures counted at day 14 and 21.

The reproducibility indicating the variation among laboratories was much higher in 15-25°C when counted early at day 7 and day 10, but the variations got smaller between the two temperatures when the seedlings were counted at day 14 and 21, even though the variation was still higher in 15-25°C (Figure 4).

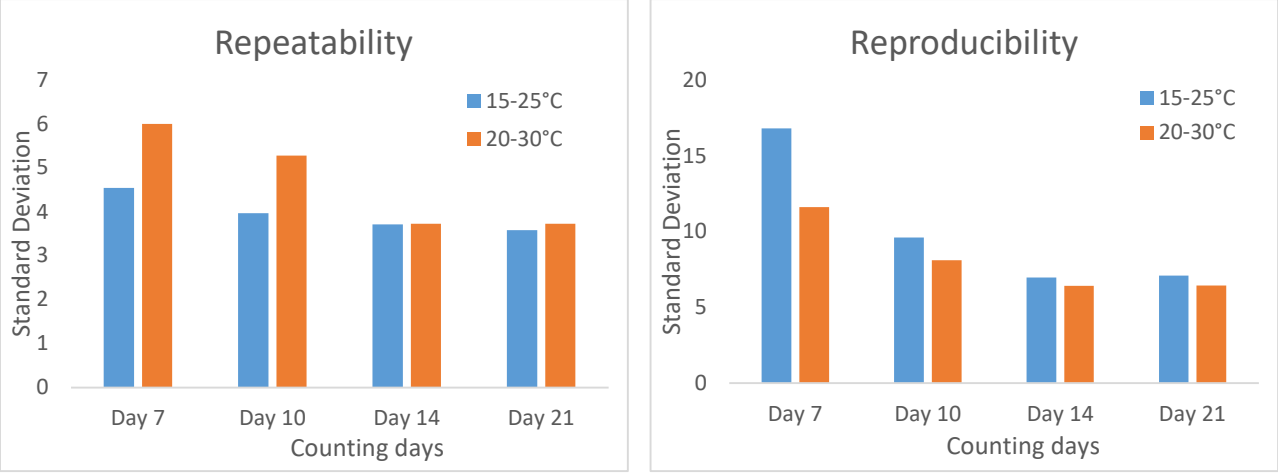


Figure 4. Repeatability and reproducibility of canarygrass seeds germinated in 15-25°C and 20-30°C and counted at day 7, 10, 14 and 21.

Performance of Participating Laboratories

The performance evaluation is based on the sum of absolute values of z-score and other parameters for the three seed lots (Appendix 1). This is the feedback to participating laboratories comparing to peer labs for testing uniformity. See Appendix 2 for more details.

The performance rating of the laboratories based on sum of z-scores at each germination condition was displayed in Figure 5. Overall, the performance in the A and B rating categories

was between 89 and 96 % over four germination conditions. The lower performance results (C rating) were treatment-specific, e.g. germination under 15-25°C had higher C ratings (11%).

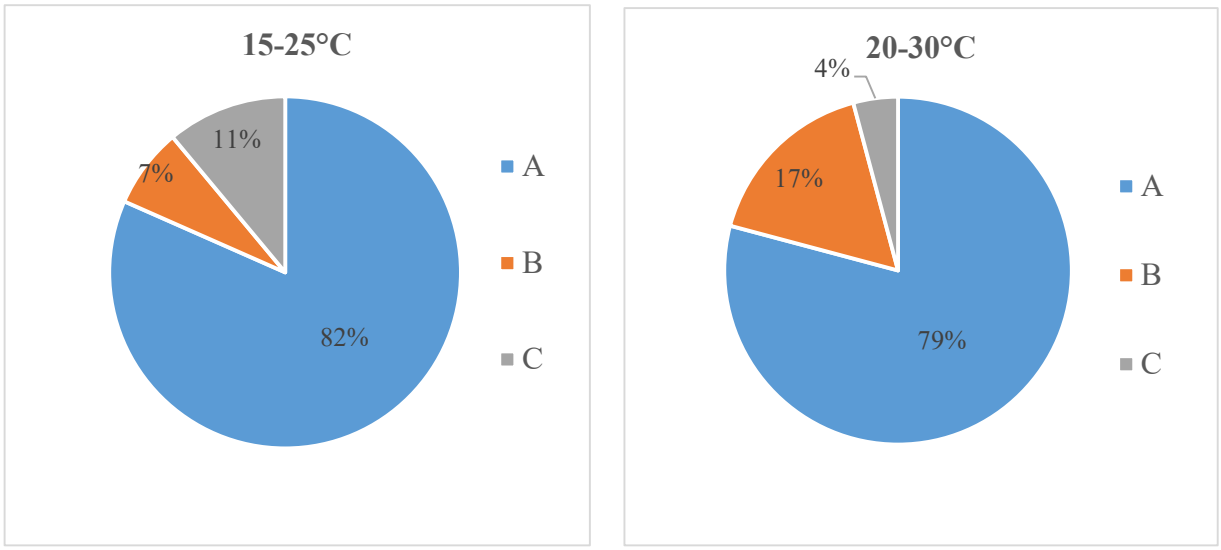


Figure 5. Percentage distribution of overall performance rating over 28 laboratories at two temperature germination conditions. The performance rating is based on sum of z-scores.

Summary and Conclusion

Current M&P has 15-25°C as required germination regime for canarygrass, AOSA has 20-30°C while ISTA has both temperatures listed in the rule. This study indicates the germination of canarygrass was not significantly different between the two germination temperatures from day 7 (Table 3). For the counting days (Table 1), the referee result indicated germination percentage had no significant difference from day 10 (Figures 2 and 3). The comparison of repeatability and reproducibility between the two germination temperatures also showed that the variation within labs and among labs were similar when the seedlings were counted at day 14 and day 21, but not earlier (Figure 5). These results provided supporting evidence for day 7 as the first counting day and day 14 as the final counting day, and both temperature regimes (15-25°C and 20-30°C) for canarygrass. This result and support evidence can be used in testing Rule harmonization and amendment.

Appendix 1. Category standards used for performance evaluation.

Σ absolute z- Score	Performance rating	Feedback Action Suggestions
0.00 – 3.49	**A**	**Acceptable, no action needed.**
3.50- 5.29	**B**	**Review of methods is recommended.**
5.30 – 6.99	**C**	**Lab should investigate possible problem**
> 6.99	**D**	**Lab should investigate possible problem. It is Below Minimum Performance of expected as Canadian or ISTA accredited laboratory**

a. Z-score. The Z-score compares the distance of the participant's result from the overall sample mean to the average difference from the mean of all participants. A Z-score of zero indicates the participant's result equaled the overall mean. A high number indicates the participant's result was far away from the mean.

b. Bias. The bias is the average Z-score for a lab and is an indicator of a systematic error which is causing results to be consistently high or low. A value of zero indicates no bias. As the value gets farther from zero the possibility of a bias towards high or low results increases. No significant value has been identified, but a value greater than **1.5** (ignoring the sign) should cause a lab to review its procedures.

c. Precision. Precision is a measure of consistency. A lab which has consistent results will have a low precision value, regardless of how far from the mean these results are. A low precision value indicates consistent performance, while a high value indicates variable performance.

d. Accuracy. Accuracy, as used here, is a combination of bias and precision. Low values indicate the lab is consistently near the overall mean. Increasing values indicate that the lab has a bias in one direction and/or is inconsistent. A value greater than **1.5** is cause for concern; a value greater than **2.0** is an indication that the lab may have a serious problem.

Appendix 2: Evaluation of results for three seed lots from participating laboratories under each of two germination temperatures.

Lab number	Performance parameter				Performance evaluation
	Bias	Precision	Accuracy	Z-score	
			15-25 °C		
1	-0.25	0.25	0.35	0.88	A
2	-0.44	1.43	1.50	3.59	B
3	-1.01	0.45	1.10	3.02	A
4	0.35	0.23	0.42	1.04	A
5	-1.49	0.95	1.77	4.48	B
6	0.39	0.18	0.43	1.18	A
7	0.14	2.29	2.29	5.89	C
8	0.95	0.81	1.25	3.18	A
9	-0.57	0.21	0.61	1.71	A
10	-0.30	0.26	0.40	0.91	A
11	0.17	0.27	0.31	0.91	A
12	0.26	0.60	0.66	1.94	A
13	-2.10	0.76	2.24	6.31	C
14	-0.07	0.34	0.35	0.89	A
15	0.88	0.34	0.95	2.65	A
16	-0.12	0.29	0.32	0.69	A
17	-0.81	0.82	1.15	2.93	A
18	0.33	0.37	0.49	1.23	A
19	-0.08	2.39	2.39	6.16	C
20	0.58	0.19	0.61	1.74	A
21	0.16	0.12	0.20	0.50	A
22	0.23	0.14	0.27	0.69	A
23	-0.50	0.72	0.87	2.12	A
24	0.73	0.08	0.73	2.18	A
25	0.50	0.24	0.56	1.51	A
26	-0.34	0.22	0.41	1.03	A
27	0.39	0.16	0.42	1.16	A
28	0.44	0.18	0.48	1.33	A

Lab number	Performance parameter				Performance evaluation
	Bias	Precision	Accuracy	Z-score	
			20-30 °C		
1	1.03	1.26	1.63	4.53	B
3	-0.85	0.55	1.01	2.56	A
4	-0.28	0.35	0.45	1.16	A
5	-1.39	1.08	1.76	4.20	B
6	0.11	0.57	0.58	1.43	A
7	-0.05	2.31	2.31	6.03	C
8	1.08	0.89	1.40	3.23	A
9	-0.81	0.74	1.09	2.42	A
10	0.78	0.44	0.89	2.33	A
12	-0.60	0.33	0.68	1.79	A
14	0.44	0.38	0.58	1.32	A
15	0.59	0.30	0.66	1.76	A
16	-0.20	0.31	0.36	1.03	A
17	-1.19	0.56	1.32	3.56	B
18	-0.02	0.68	0.68	1.90	A
19	-1.64	1.12	1.99	4.92	B
20	0.37	0.31	0.49	1.24	A
21	-0.13	0.15	0.20	0.43	A
22	-0.14	0.39	0.41	1.09	A
23	-0.20	0.55	0.59	1.63	A
24	0.27	0.28	0.39	0.93	A
25	0.23	0.60	0.64	1.63	A
26	-0.46	0.69	0.83	2.38	A
27	0.11	0.56	0.57	1.47	A
28	0.71	0.18	0.73	2.13	A