International Seed Testing Association

Secretariat, Zürichstrasse 50, CH-8303 Bassersdorf, Switzerland
Phone: +41 44 838 60 00 Fax: +41 44 838 60 01
Email: ista.office@ista.ch - http://www.seedtest.org

Document 06-2009-OM

Method Validation Reports for Rules Proposals 2010

Contents

ISTA validation study on germination testing of *Brachiaria brizantha* (A.Rich.) Stapf [Rules Proposal 2010 B.1.]

Ignacio Aranciaga, National Seed Institute, República Argentina

Summary

A validation study on germination testing of *Brachiaria brizantha* was carried out. Six laboratories were involved and each tested 400 seeds of three seed lots. The analyses of the results demonstrate that the following method is of sufficient repeatability and reproducibility to be included in the ISTA Rules:

Table 5A Part 1 Agricultural and vegetable seeds

Species	Prescriptions for:				Additional directions including recommendations for breaking dormancy
	Substrate	Temperature (°C)	First count (d)	Final count (d)	
1	2	3	4	5	6
Brachiaria brizantha	TP	20-35	7	21	KNO_3; predry and KNO_3

1. Plant material

Three seed samples of *Brachiaria brizantha* of commercially traded quality were obtained from the Argentinian seed trade for this study.

The samples obtained were mechanically divided into subsamples by use of a soil divider. An in-house study using confirmed the homogeneity of the seed samples. About 25 g of each sample were sent to each of the participating laboratories on 28 November 2008 with instructions to have the tests completed by 3 February 2009. The seeds were packed as blind samples (lot nos. 1–3); the numbering of the three samples was different for each laboratory.

2. Participating laboratories

Six laboratories from four countries participated in this validation study.

INTA LA CONSULTA (Argentina)	Marilú Makuch, Jorge Valdéz	ISTA Member Lab
Matzuda Semillas (Brazil)	Pedro Henrique	ISTA Accredited Lab
National Seed Institute of Uruguay	Teresita Farras, Deneb Manfrini	ISTA Accredited Lab
Queensland Seed Technology Lab (Australia)	Mrs. Karen A. Hill	ISTA Accredited Lab
SGS Mid-West Seed Services, Inc. (USA)	Kari Fiedler	ISTA Accredited Lab
INASE - National Seed Institute (Argentina)	Ignacio Aranciaga	ISTA Accredited Lab

In this report the laboratories are anonymously numbered as Labs 1–6; the sequence of these numbers is not identical to the alphabetical list given here.

3. Procedure for germination tests

The testing method is described in table 1.

The three different seed lots were tested on top-of-paper (TP) medium using 15-35 °C and 20-35 °C temperature regimes. For each test, a total of 400 seeds were tested in replicates of 100 seeds. Light was supplied for 8 h during the high temperature phase, and germination counts were made at 7 days (first count) and 21 days (final count). Seedlings were assessed according to Seedling Type D – Seedling Group A-1-2-3-1. Any non-germinated seeds were checked as being fresh or dead using tetrazolium in accordance with the method given in the ISTA Rules, Chapter 6.

Since sample homogenization is extremely important, participants were instructed to follow PSD 36 when preparing pure seed from the samples for germination tests.

Table 1. Germination testing methods used in this study to determine a germination procedure for *Brachiaria brazantha* that could be included in the ISTA Rules

Experiment no.	Dormancy breaking pretreatment	Temperature regime	Light	Intermediate count (days)	Final count (days)
1	KNO_3 (0.2%) on germination media	20-35 ºC	8 h (during the high temp.)	7 days	21 days
2	Dry in oven with forced air for 5 days (35-40ºC). KNO_3 (0.2%) on germination media	20-35 ºC	8 h (during the high temp.)	7 days	21 days
3	H_2SO_4 (96%, 36N) for 15 min, after acid draining, seed soaked in water for 1 h, followed by 1 min of washing in tap water and surface drying the seed over blotter paper. KNO_3 (0.2%) on germination media	20-35 ºC	8 h (during the high temp.)	7 days	21 days
4	KNO_3 (0.2%) on germination media	15-35 ºC	8 h (during the high temp.)	7 days	21 days
5	Dry in oven with forced air for 5 days (35-40 ºC) KNO_3 (0.2%) on germination media	15-35 ºC	8 h (during the high temp.)	7 days	21 days
6	H_2SO_4 (96%, 36N) for 15 min, after acid draining, seed soaked in water for 1 h, followed by one minute of washing in tap water and surface drying the seed over blotter paper. KNO_3 (0.2%) on germination media	15-35 ºC	8 h (during the high temp.)	7 days	21 days

Note: The experiments 3 and 6 were not obligatory, since some participants did not have the necessary facilities and/or had no experience of using concentrated sulphuric acid as a dormancy breaking method.

4. Results

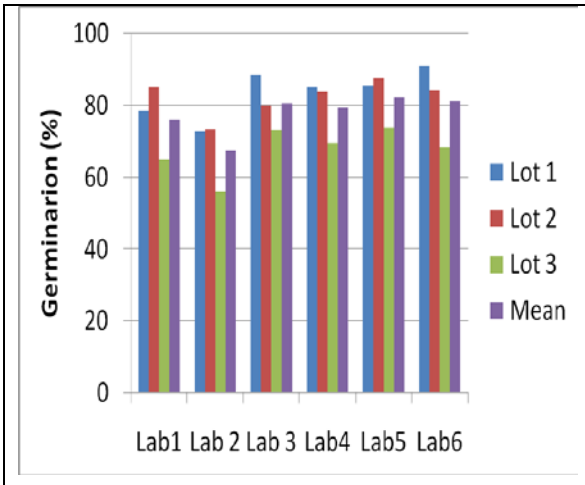
The results of the germination tests were reported between March and July 2007. For all experiments the level of fresh seed was less than 1%.

4.1 KNO_3 Treatment

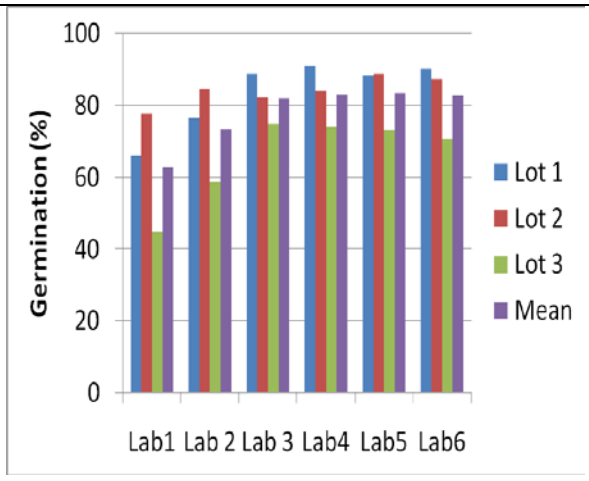
The results for the KNO_3 treatment at both temperatures (experiments 1 and 4) are given in table 2 and shown in figure 1.

Table 2. Germination (total percentage of normal seedlings) as reported for the three *Brachiaria brizantha* seed samples by the 6 participating laboratories (results of the four replicates each containing 100 seeds) KNO_3

	20-35 °C			15-35 °C		
	Lot			Lot		
	1	2	3	1	2	3
Lab 1	80	82	75	66	79	42
	77	83	65	62	79	48
	83	90	63	67	75	36
	74	85	57	69	73	53
Lab 2	73	67	63	82	84	60
	73	74	59	72	76	53
	74	79	50	81	76	52
	71	74	52	71	77	70
Lab 3	88	78	70	86	86	71
	88	76	73	92	87	80
	87	81	72	87	85	75
	91	85	78	90	86	73
Lab 4	89	84	74	93	78	71
	87	84	70	86	80	73
	85	82	71	93	73	77
	79	85	63	92	80	75
Lab 5	90	88	82	88	79	73
	78	86	68	91	84	67
	89	87	71	87	79	78
	85	89	74	87	82	75
Lab 6	92	86	75	95	73	71
	92	84	67	89	81	68
	91	82	63	87	81	68
	89	85	68	90	85	76
Mean	84	82	68	84	80	66



a) 20-35 °C



b) 15-35 °C

Figure 1. Germination results obtained at participating laboratories using 0.2% KNO_3 as a dormancy-breaking treatment at (a) 20-35 °C and (b) 15-35 °C temperature regimes.

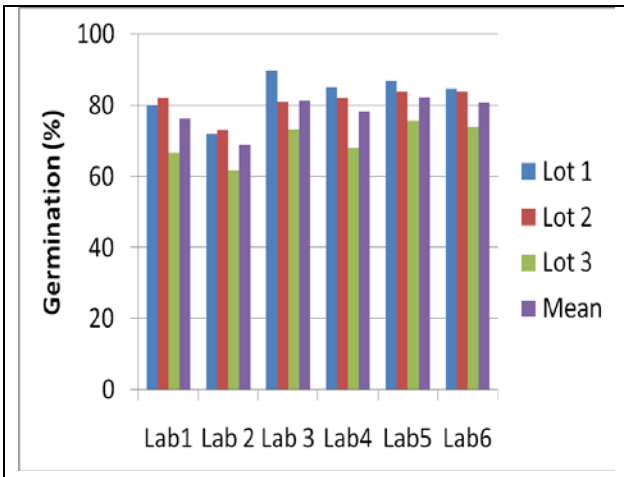
For the 20-35 °C temperature regime, the mean viability was 84 ± 3 % for Lot 1, 82 ± 2 % for Lot 2 and 68 ± 3 % for Lot 3. For the 15-35 °C temperature regime, the mean viability was 84 ± 4 % for Lot 1, 82 ± 2 % for Lot 2 and 66 ± 5 % for Lot 3.

4.2 Heat + KNO_3 treatment

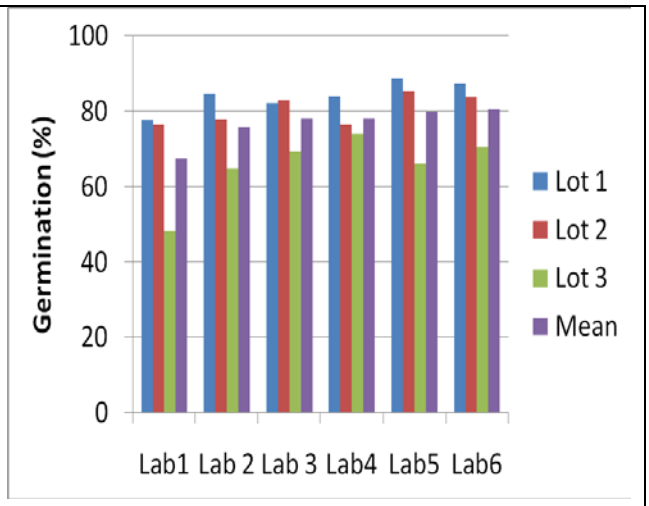
The results for the heat + KNO_3 treatment at both temperatures (experiments 2 and 5) are given in table 3 and shown in figure 2.

Table 3. Germination (total normal seedlings %) as reported for the three *Brachiaria brizantha* seed samples by the 6 participating laboratories (results of the four replicates each containing 100 seeds) Heat + KNO_3

	20-35 °C			15-35 °C		
	Lot			Lot		
	1	2	3	1	2	3
Lab 1	85	79	75	76	79	52
	72	91	63	70	83	43
	83	80	71	86	72	42
	81	78	58	79	72	56
Lab 2	74	74	61	82	76	69
	71	77	64	87	77	57
	72	73	62	86	77	70
	71	68	60	84	82	63
Lab 3	90	79	79	82	84	65
	92	84	76	79	85	73
	90	81	69	84	80	72
	87	80	69	84	83	67
Lab 4	85	78	63	87	74	78
	86	79	66	81	84	73
	89	83	71	86	75	77
	80	88	72	82	73	68
Lab 5	87	84	77	84	89	68
	84	88	71	86	80	68
	91	84	72	94	84	65
	86	79	83	91	88	63
Lab 6	88	83	76	93	85	71
	88	81	66	84	85	73
	83	81	80	84	85	71
	80	90	74	88	80	67
mean	83	81	70	84	81	65



a) 20-35 °C



b) 15-35 °C

Figure 2. Germination results obtained at participating laboratories using heat and 0.2% KNO_3 as a dormancy breaking treatment at (a) 20-35 °C and (b) 15-35 °C temperature regimes

For the 20-35°C temperature regime, the mean viability was 83 ± 3 % for Lot 1, 81 ± 2 % for Lot 2 and 70 ± 3 % for Lot 3. For the 15-35 °C temperature regime, the mean viability was 84 ± 4 % for Lot 1, 81 ± 2 % for Lot 2 and 65 ± 4 % for Lot 3.

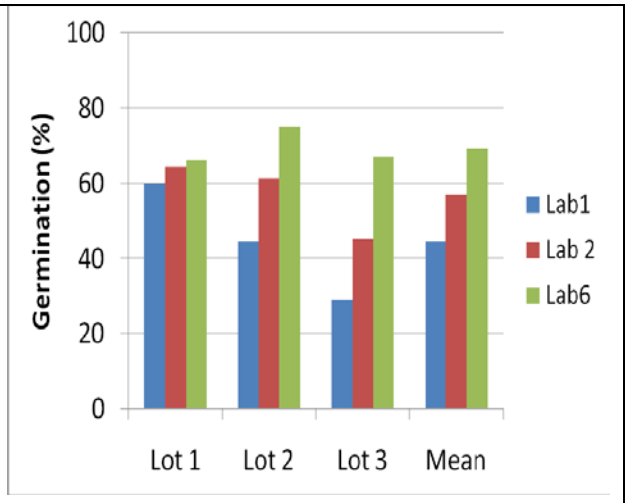
4.1 H_2SO_4 + KNO_3 treatment

Only three laboratories participated in experiments 3 and 6, in which H_2SO_4 + KNO_3 was used, and their results are given in table 4 and shown in figure 3.

Table 4. Germination (total percentage of normal seedlings) as reported for the three *Brachiaria brizantha* seed samples by the 3 participating laboratories (results of the four replicates each containing 100 seeds) H_2SO_4 + KNO_3

	20-35 °C			15-35 °C		
	Lot			Lot		
	1	2	3	1	2	3
Lab 1	59	47	23	60	45	28
	63	39	28	60	45	29
	66	32	36	58	47	25
	64	36	30	62	41	34
Lab 2	79	76	59	66	69	36
	69	63	59	59	56	46
	69	64	68	68	60	46
	68	68	73	65	60	53
Lab 6	61	79	72	68	66	66
	69	74	75	63	85	72
	68	86	68	67	74	61
	70	77	67	67	75	69
mean	67	62	55	64	60	47

a) 20-35 °C



b) 15-35 °C

Figure 3. Germination results obtained at participating laboratories using H_2SO_4 and 0.2% KNO_3 as a dormancy breaking treatment at (a) 20-35 °C and (b) 15-35 °C temperature regimes

For the 20-35 °C temperature regime, the mean viability was 67 ± 3 % for Lot 1, 62 ± 12 % for Lot 2 and 55 ± 13 % for Lot 3. For the 15-35 °C temperature regime, the mean viability was 64 ± 2 % for Lot 1, 60 ± 9 % for Lot 2 and 47 ± 11 % for Lot 3.

5. Statistical Analysis

5.1 Data exploration with side-by-side boxplots

As the first step in the analysis, the data was explored using side-by-side boxplots (figures 4–10).

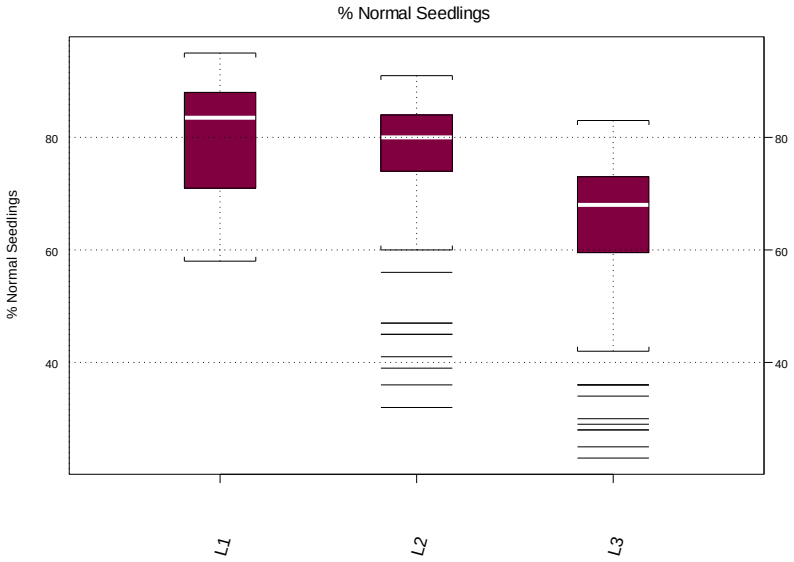


Figure 4. Boxplot (grouping factor: seed lots) showing germination results obtained at participating laboratories on the 3 seed lots used in this validation study.

Grouping factor: Temperature

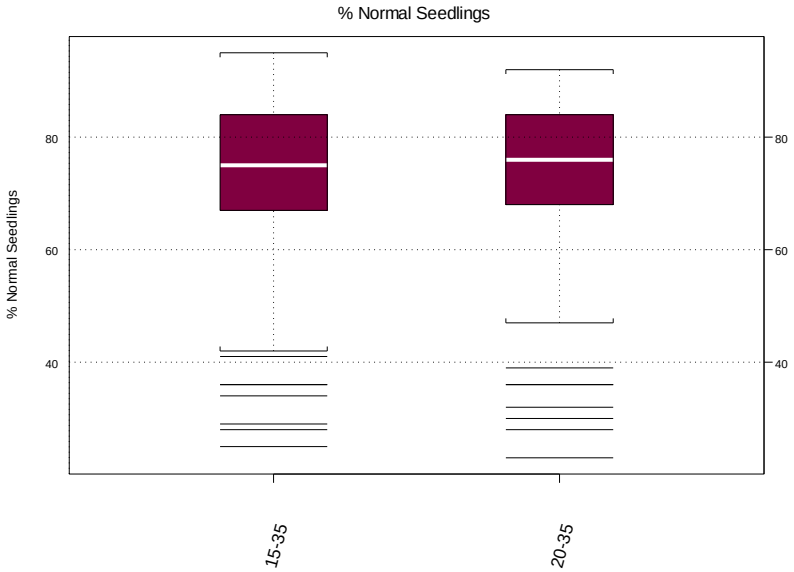


Figure 5. Boxplot (grouping factor: temperature regime) showing germination results obtained at participating laboratories using 15-35 °C and 20-35 °C temperature regimes.

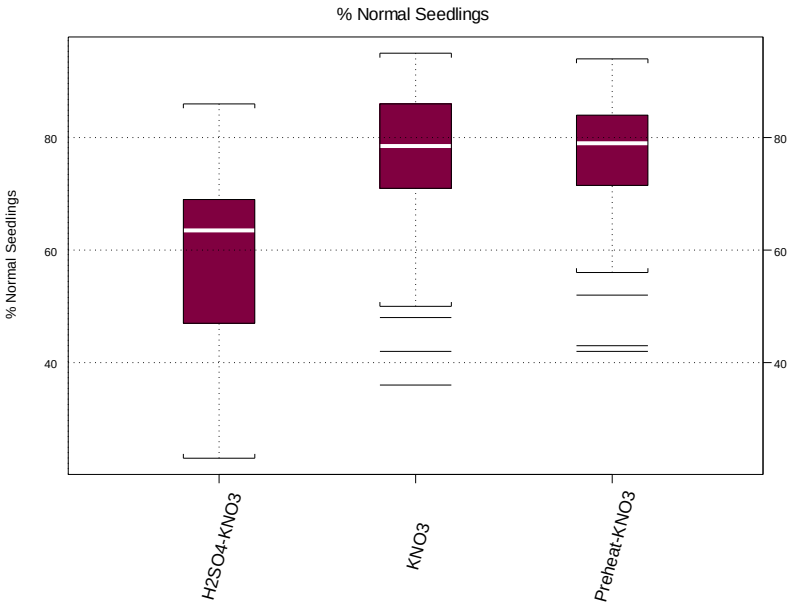


Figure 6. Boxplot (grouping factor: dormancy-breaking treatment) showing germination results obtained at participating laboratories using H_2SO_4 + KNO_3, KNO_3, and heat + KNO_3 dormancy-breaking treatments.

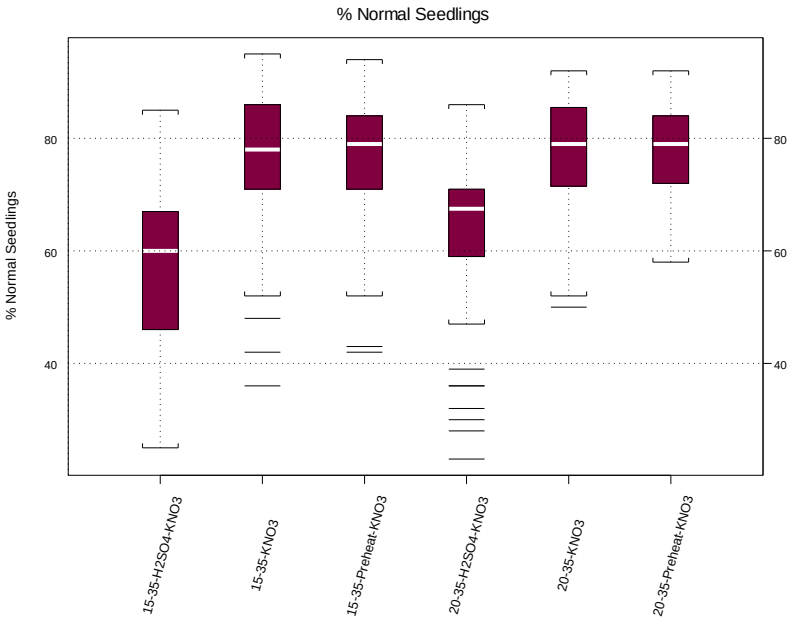


Figure 7. Boxplot (grouping factor: temperature regime and dormancy-breaking treatment) showing germination results obtained at participating laboratories using 15-35 °C and 20-35 °C temperature regimes and H_2SO_4 + KNO_3, KNO_3, and heat + KNO_3 dormancy-breaking treatments.

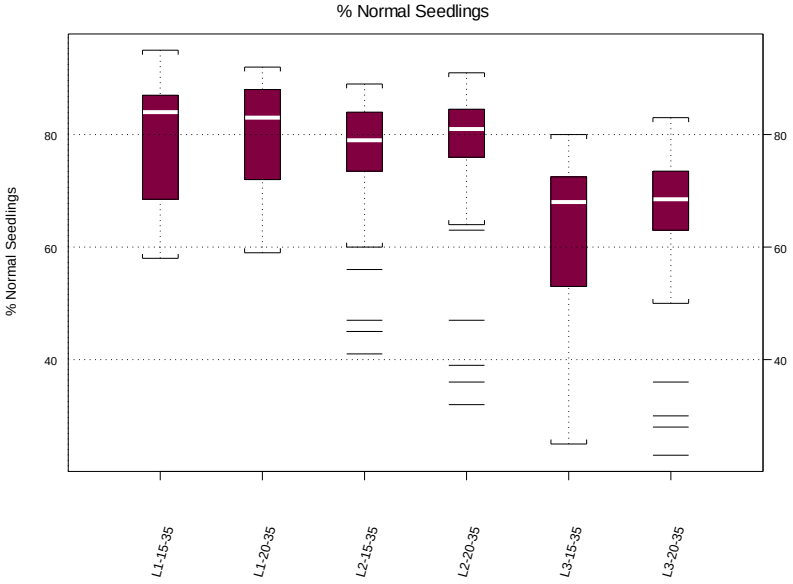


Figure 8. Boxplot (grouping factor: seed lot and temperature regime) showing germination results obtained at participating laboratories on seed lots 1, 2 and 3 using 15-35 °C and 20-35 °C temperature regimes.

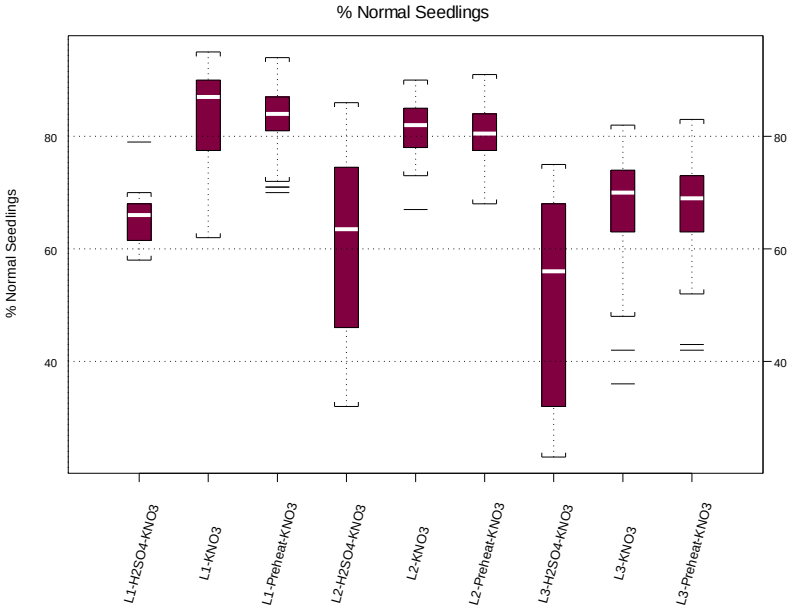


Figure 9. Boxplot (grouping factor: seed lot and dormancy-breaking treatment) showing germination results obtained at participating laboratories on seed lots 1, 2 and 3 using H_2SO_4 + KNO_3, KNO_3, and heat + KNO_3 dormancy-breaking treatments.

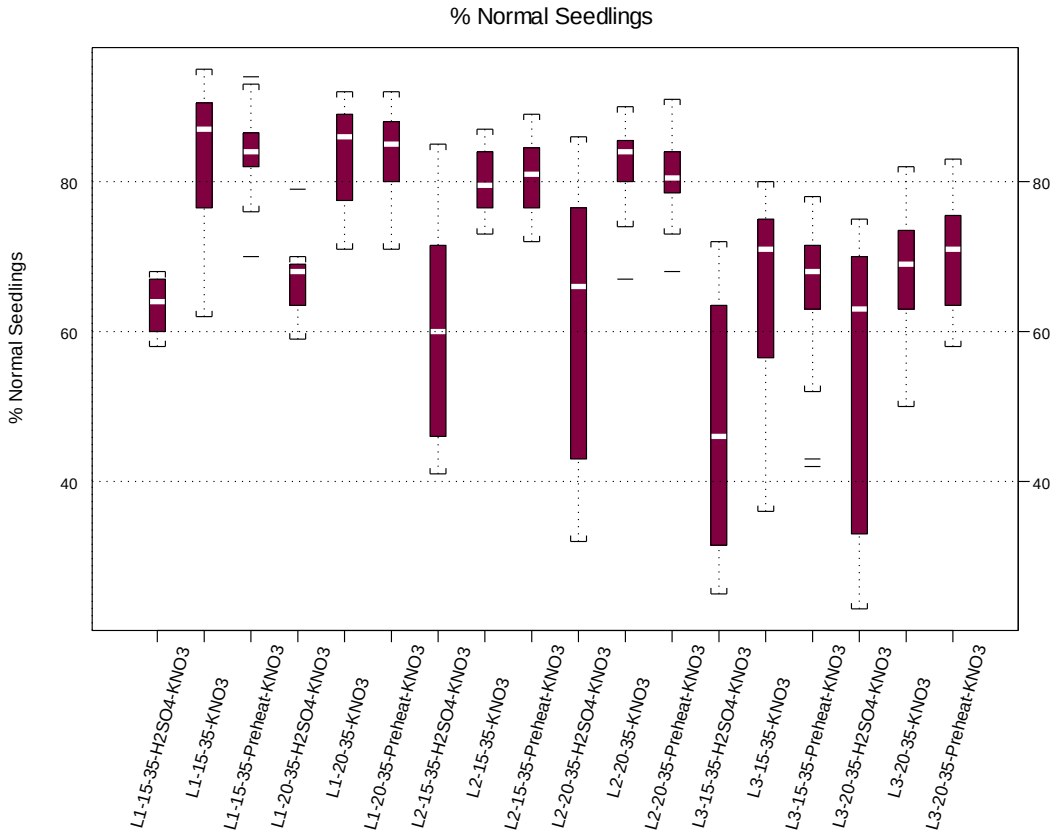


Figure 10. Boxplot (grouping factor: seed lot, temperature regime and dormancy-breaking treatment) showing germination results obtained at participating laboratories on seed lots 1, 2 and 3 using H_2SO_4 + KNO_3, KNO_3, and heat + KNO_3 dormancy-breaking treatments and 15-35 °C and 20-35 °C temperature regimes.

As expected from the germination results (binomial data), both heteroscedasticity and non-symmetry are exhibited in these side-by-side boxplots.

The H_2SO_4 + KNO_3 dormancy-breaking treatment exhibits lower values and higher variability of the results. In addition, only 3 laboratories out of 6 used this dormancy-breaking treatment, and the results of this dormancy breaking treatment were excluded from further analysis.

5.2 Modeling 1: assessing significance of the effects

Data considered: pretreatment H_2SO_4-KNO_3 removed from the original dataset.

Model: generalized linear mixed-effect model

$Normal\_\ seedlings\_counts_{ijklm} \sim \text{Binomial}(100,\ \pi_{ijklm})$

$$\text{logit}\left(\pi_{ijklm}\right) = \log\left(\frac{\pi_{ijklm}}{1-\pi_{ijklm}}\right)$$

$$= \mu + \alpha_i + \beta_j + \delta_k + \left(\alpha\beta\right)_{ij} + \left(\alpha\delta\right)_{ik} + \left(\beta\delta\right)_{jk} + \left(\alpha\beta\delta\right)_{ijk} + c_l + \left(\alpha c\right)_{il} + \left(\beta c\right)_{jl} + \left(\delta c\right)_{kl}$$

where:

- $i = 1, 2, 3$ $j = 1, 2$ $k = 1, 2$ $l = 1, 2, 3, 4, 5, 6$ $m = 1, 2, 3, 4$
- μ is the general effect.

- α_i is the fixed effect of lot *i*.
- β_j is the fixed effect of temperature *j*.
- δ_k is the fixed effect of pretreatment *k*.
- $(\alpha\beta)_{ij}$ is the fixed interaction effect between lot *i* and temperature *j*.
- $(\alpha\delta)_{ik}$ is the fixed interaction effect between lot *i* and pretreatment *k*.
- $(\beta\delta)_{jk}$ is the fixed interaction effect between temperature *j* and pretreatment *k*.
- $(\alpha\beta\delta)_{ijk}$ is the fixed interaction effect between lot *i*, temperature *j* and pretreatment *k*.
- c_l is the random effect of lab *l*. The c_l are iid N(0, σ^2_{Lab}).
- $(\alpha c)_{il}$ is the random interaction effect between lot *i* and lab *l*.

The $(\alpha c)_{il}$ are iid N(0, $\sigma^2_{Lot\times Lab}$).

- $(\beta c)_{jl}$ is the random interaction effect between temperature *j* and lab *l*.

The $(\beta c)_{jl}$ are iid N(0, $\sigma^2_{Temp\times Lab}$).

- $(\delta c)_{kl}$ is the random interaction effect between pre-treatment *k* and lab *l*.

The $(\delta c)_{kl}$ are iid N(0, $\sigma^2_{Pre\_treat\times Lab}$).

This model was fitted using the SAS GLIMMIX procedure.

Results

Table 5 gives the results of the analysis.

Table 5. Results of generalized linear mixed-effect model analysis

Variance components:

Lab	0.05235
Lot x Lab	0.02074
Temp x Lab	0.03771
Pretreat x Lab	0.00546

Factor	**Numerator degrees of freedom**	**Denominator degrees of freedom**	**F Value**	**Probability > F**
Lot	2	10	59.62	**<.0001**
Temperature	1	5	0.28	**0.6165**
Pre-treatment	1	5	0.00	**0.9693**
Lot x temperature	2	251	3.45	**0.0333**
Lot x pretreatment	2	251	0.54	**0.5819**
Temperature x pretreatment	1	251	0.18	**0.6755**
Lot x Temperature x pretreatment	2	251	2.19	**0.1139**

The lot main effect is highly significant, which is not surprising. The other significant effect is the lot x temperature interaction. However, looking at the interaction plot for this interaction term (figure 11), there is no cross-over between the effects:

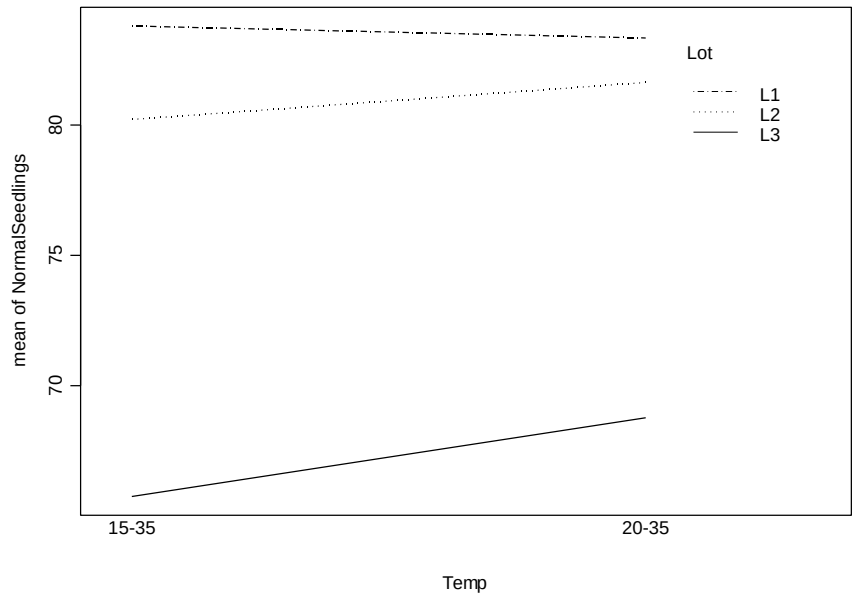


Figure 11. Interaction plot showing relationship between temperature regime and seed lot.

5.3 Modeling 2: assessing repeatability/reproducibility

Pretreatment H_2SO_4-KNO_3 removed from the original dataset.

Model: for a given temperature x pretreatment level:

Normal_ seedlings_counts$_{ij}$ ~ Binomial(400, π_{ij})

$$\text{logit}\left(\pi_{ij}\right) = \log\left(\frac{\pi_{ij}}{1-\pi_{ij}}\right) = \mu + \alpha_i + b_j$$

where:

- i = 1, 2, 3 j = 1, 2, 3, 4, 5, 6
- μ is the general effect.
- α_i is the fixed effect of lot i.
- b_j is the random effect of lab j. The b_j are iid N(0, σ^2_{Lab}).

Repeatability and reproducibility computations:

The repeatability and the reproducibility variances are computed for three nominal probabilities π_i ("gold" standards), 0.7, 0.8 and 0.9 using the following formulas:

Repeatability variance: $S_r^2 = \dfrac{\hat{\phi}\,\pi_i\left(1-\pi_i\right)}{400}$

Reproducibility variance: $S_R^2 = S_r^2 + \pi_i^2\left(1-\pi_i\right)^2\hat{\sigma}^2_{Lab}$

where $\hat{\phi}$ is the scale parameter estimate.

Results

Table 6 gives the results of the analysis.

Table 6. Results of repeatability and reproducibility computations of validation study involving the germination of *Brachiaria brizantha* using 15-35 °C and 20-35 °C temperature regimes and KNO_3 and heat + KNO_3 dormancy-breaking treatments

	Gold standard	Repeatability std-dev	Reproducibility std-dev	Binomial std-dev
15-35 °C KNO_3	70%	0.046621	0.101896	0.022913
	80%	0.040694	0.080135	0.020000
	90%	0.030520	0.049390	0.015000
20-35 °C KNO_3	70%	0.033597	0.071622	0.022913
	80%	0.029326	0.056414	0.020000
	90%	0.021994	0.034909	0.015000
15-35 °C Preheat-KNO_3	70%	0.035718	0.064287	0.022913
	80%	0.031177	0.051289	0.020000
	90%	0.023383	0.032734	0.015000
20-35 °C Preheat-KNO_3	70%	0.024464	0.061794	0.022913
	80%	0.021354	0.048220	0.020000
	90%	0.016016	0.029119	0.015000

Repeatability standard deviations appear to be greater for temperature 15-35 °C and reproducibility standard deviations greater for (temperature 15-35 °C x pretreatment KNO_3).

5.4 Experimental error of replicate results

The experimental error of replicate results from individual participating laboratories is quantified by the ratio f between the observed standard deviation (SD observed) and the expected standard deviation (SD expected) based on the binomial distribution:

$f = SD_{(obs.)} / SD_{(exp.)}$

$$SD_{(\exp.)} = \sqrt{(p \times q)/n} \qquad \textbf{Equation 1}$$

p : % Germination as mean;

q : 100 – p;

n = number of seeds.

The germination tolerances and ranges for tests in different laboratories allow for between-laboratory variation as well as for the random variation given in Equation 1. This is reflected in Equation 2:

$$f = SD_{(obs.)} / SD_{(exp.)} = 2.38 - 008321p \qquad \textbf{Equation 2}$$

Tolerated ranges for comparing germinations from different laboratories are computed using Equation 3:

S = Equation 1 x Equation 2 x F

$$S = (\sqrt{(p \times q)/n})(2.38 - 008321p)F \qquad \textbf{Equation 3}$$

Where S is the tolerated range and F is the factor for estimating the range from the expected standard deviation; from Pearson and Hartley (1954) table 22 (as referenced by Miles 1963). In this case with 6 participating laboratories (for KNO_3 and heat + KNO_3 germinations) F = 5.62, and with 3 participating laboratories (for H_2SO_4 germinations) F = 5.06.

Results

Experimental error among the replicates:

In table 7. the factors f for experimental error among the 4 replicates within a germination test in each of the 6 laboratories are given. The average factor f for 6 labs and 4 lots is below 1.00 for the following combinations of dormancy-breaking treatments and temperature regimes:

- temperature regime 15-35 °C in combination with dormancy-breaking treatments KNO_3, heat + KNO_3 and H_2SO_4 + KNO_3;
- temperature regime 20-35 °C in combination with dormancy-breaking treatment KNO_3.

For temperature regime 20-35 °C and dormancy-breaking treatment heat + KNO_3, the f value was very close to 1 (1.02), but for dormancy-breaking treatment H_2SO_4 + KNO_3, the f value was high at 1.20, indicating significant variation between replicates.

Table 7: Experimental errors within the tests. For each lot/laboratory combination the mean, the observed standard deviation between the 4 replicates, the expected standard deviation (based on the binomial distribution) and the f values are shown

(a) 20-35 °C KNO_3

	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	mean
Lot 1							
Mean	78.5	72.75	88.5	85.0	85.5	91	
SD obs.	3.87	1.26	1.73	4.32	5.45	1.41	
SD exp.	4.11	4.45	3.19	3.57	3.52	2.86	
f	**0.94**	**0.28**	**0.54**	**1.21**	**1.55**	**0.49**	**0.84**
Lot 2							
Mean	85.0	73.5	80.0	83.75	87.5	84.25	
SD obs.	3.56	4.93	3.92	1.26	1.29	1.71	
SD exp.	3.57	4.41	4.00	3.69	3.31	3.64	
f	**1.00**	**1.12**	**0.98**	**0.34**	**0.39**	**0.47**	**0.72**
Lot 3							
Mean	65.0	56.0	73.25	69.5	73.75	68.25	
SD obs.	7.45	6.06	3.40	4.66	6.02	4.99	
SD exp.	4.77	4.96	4.42	4.60	4.40	4.66	
f	**1.57**	**1.22**	**0.77**	**1.01**	**1.37**	**1.07**	**1.17**
total average							**0.91**

(b) 20-35 °C heat + KNO_3

	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	mean
Lot 1							
Mean	80.25	72.0	89.75	85.0	87.0	84.75	
SD obs.	5.74	1.41	2.06	3.74	2.94	3.95	
SD exp.	3.98	4.49	3.03	3.57	3.36	3.60	
f	**1.44**	**0.32**	**0.68**	**1.05**	**0.88**	**1.10**	**0.91**
Lot 2							
Mean	82.0	73.0	81.0	82.0	83.75	83.75	
SD obs.	6.06	3.74	2.16	4.55	3.69	4.27	
SD exp.	3.84	4.44	3.92	3.84	3.69	3.69	
f	**1.58**	**0.84**	**0.55**	**1.83**	**1.00**	**1.16**	**1.05**
Lot 3							
Mean	66.75	61.75	73.25	68.0	75.75	74.0	
SD obs.	7.68	1.71	5.06	4.24	5.50	5.89	
SD exp.	4.71	4.86	4.43	4.67	4.29	4.39	
f	**1.63**	**0.35**	**1.14**	**0.91**	**1.28**	**1.34**	**1.11**
total average							**1.02**

(c) 20-35 °C H_2SO_4 + KNO_3

	Lab 1	Lab 2	Lab 6	mean
Lot 1				
Mean	63.0	71.25	67.0	
SD obs.	2.94	5.19	4.08	
SD exp.	4.83	4.53	4.70	
f	**0.61**	**1.15**	**0.87**	**0.87**
Lot 2				
Mean	38.5	67.75	79.0	
SD obs.	6.35	5.91	5.10	
SD exp.	4.87	4.67	4.07	
f	**1.31**	**1.26**	**1.25**	**1.27**
Lot 3				
Mean	29.25	64.75	70.5	
SD obs.	5.34	6.95	3.70	
SD exp.	4.55	4.78	4.56	
f	**1.18**	**1.45**	**0.81**	**1.45**
total average				**1.20**

(d) 15-35 °C KNO_3

	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	mean
Lot 1							
Mean	66.0	76.5	88.75	91.0	88.25	90.25	
SD obs.	2.94	5.80	2.75	3.37	1.89	3.40	
SD exp.	4.74	4.24	3.16	2.86	3.22	2.97	
f	**0.62**	**1.37**	**0.87**	**1.18**	**0.59**	**1.15**	**0.96**
Lot 2							
Mean	76.5	78.25	86.0	77.75	81.0	80.0	
SD obs.	3.00	3.86	0.82	3.30	2.45	5.03	
SD exp.	4.24	4.13	3.47	4.16	3.92	4.00	
f	**0.71**	**0.94**	**0.24**	**0.79**	**0.62**	**1.26**	**0.76**
Lot 3							
Mean	44.75	58.75	74.75	74.0	73.25	70.75	
SD obs.	7.37	8.30	3.86	2.58	4.65	3.77	
SD exp.	4.97	4.92	4.34	4.39	4.43	4.55	
f	**1.48**	**1.69**	**0.89**	**0.59**	**1.05**	**0.83**	**1.09**
total average							**0.94**

(e) 15-35 °C heat + KNO_3

	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	mean
Lot 1							
Mean	77.75	84.75	82.25	84.0	88.75	87.25	
SD obs.	6.65	2.22	2.36	2.94	4.57	4.27	
SD exp.	4.16	3.60	3.82	3.67	3.16	3.34	
f	**1.60**	**0.62**	**0.62**	**0.80**	**1.45**	**1.28**	**1.06**
Lot 2							
Mean	76.5	78.0	83.0	76.5	85.25	83.75	
SD obs.	5.45	2.71	2.16	5.07	4.11	2.50	
SD exp.	4.24	4.14	3.76	4.24	3.55	3.69	
f	**1.28**	**0.65**	**0.58**	**1,20**	**1.16**	**0.68**	**0.92**
Lot 3							
Mean	48.25	64.75	69.25	74.0	66.0	70.5	
SD obs.	6.84	6.02	3.86	4.55	2.45	2.52	
SD exp.	4.99	4.78	4.62	4.39	4.74	4.56	
f	**1.37**	**1.26**	**0.84**	**1.04**	**0.52**	**0.55**	**0.93**
total average							**0.97**

(f) 15-35 °C H_2SO_4 + KNO_3

H_2SO_4	Lab 1	Lab 2	Lab 6	mean
Lot 1				
Mean	60.0	64.5	66.25	
SD obs.	1.63	3.87	2.22	
SD exp.	4.90	4.79	4.73	
f	**0.33**	**0.81**	**0.47**	**0.54**
Lot 2				
Mean	44.5	61.25	75.0	
SD obs.	2.52	5.50	7.79	
SD exp.	4.97	4.87	4.33	
f	**0.51**	**1.12**	**1.80**	**1.15**
Lot 3				
Mean	29.0	45.25	67.0	
SD obs.	3.74	6.99	4.69	
SD exp.	4.54	4.98	4.70	
f	**0.83**	**1.41**	**1.00**	**1.08**
total average			**0.92**	

The maximum tolerated ranges for the mean viabilities were calculated by the formula S = f × SD × F, given by Miles (1963) (see Table 8). For seed lots 2 and 3 the range obtained experimentally was greater than the tolerated range for H_2SO_4 + KNO_3 at both 15-35 °C and 20-35 °C. For temperature regime 15-35 °C, the experimental range for dormancy-breaking treatments KNO_3 and heat + KNO_3 was greater than the tolerated range for seed lot 3 (KNO_3) and seed lots 1 and 3 (heat + KNO_3). For temperature regime 20-35 °C, the results for KNO_3 and heat + KNO_3 were all within the tolerated range.

Table 8. Maximum tolerated ranges S(%) according to Miles (1963)

KNO_3												
	20-35 °C						**15-35 °C**					
lot	**Mean**	**f**	**SD exp.**	**F**	**S (%)**	**Range**	**Mean**	**f**	**SD exp.**	**F**	**S (%)**	**Range**
Lot 1	83.50	1.85	1.68	5.62	18	18	84.13	1.83	1.68	5.62	17	11
Lot 2	82.33	1.91	1.70	5.62	18	14	80.50	1.98	1.71	5.62	19	9
Lot 3	67.63	2.34	1.82	5.62	24	18	65.46	2.38	1.84	5.62	25	26
Heat +KNO_3												
	20°C - 35°C						**15°C - 35°C**					
lot	**Mean**	**f**	**SD exp.**	**F**	**S (%)**	**Range**	**Mean**	**f**	**SD exp.**	**F**	**S (%)**	**Range**
Lot 1	83.13	1.87	1.68	5.62	**18**	18	83.46	1.86	1.69	5.62	18	25
Lot 2	80.92	1.97	1.70	5.62	**19**	11	79.92	2.00	1.71	5.62	19	10
Lot 3	69.92	2.29	1.82	5.62	**23**	14	66.04	2.37	1.83	5.62	24	30
H_2SO_4												
	20°C - 35°C						**15°C - 35°C**					
lot	**Mean**	**f**	**SD exp.**	**F**	**S (%)**	**Range**	**Mean**	**f**	**SD exp.**	**F**	**S (%)**	**Range**
Lot 1	67.08	2.35	1.82	5.05	22	8	63.58	2.37	1.83	5.06	22	6
Lot 2	61.75	2.43	1.87	5.05	23	41	60.25	2.45	1.88	5.06	23	31
Lot 3	54.83	2.49	1.92	5.05	24	41	47.08	2.50	1.95	5.06	24	38

... **Experimental result out of tolerance**

6. Conclusions

The f factors in table 7 indicate an acceptable experimental error among the 4 replicates within the tests for all dormancy-breaking pretreatment and temperature regime combinations, apart from H_2SO_4 + KNO_3 and 20-30 °C.

Dormancy-breaking treatment H_2SO_4 + KNO_3 gives significantly lower germinations for all seed lots in both temperature regimes.

H_2SO_4 + KNO_3 is not recommended as a dormancy breaking treatment for *Brachiaria brizantha*.

When the H_2SO_4 + KNO_3 results are excluded:

- There is no difference in the means over the 6 laboratories between the different temperature regimes (15-35 °C and 20-35 °C) and the different dormancy-breaking treatments (KNO_3 and heat plus KNO_3) and their combinations.
- There are significant differences between the results obtained from the 3 seed lots.
- There is a significant seed lot x temperature regime interaction but no cross-over between effects.
- Repeatability standard deviations are quantitatively higher for temperature regime 15-35 °C and reproducibility standard deviation higher for the combination of temperature regime 15-35 °C and dormancy-breaking treatment KNO_3.
- The range of results obtained using temperature regime 20-35 °C and dormancy-breaking treatments KNO_3 and heat + KNO_3 are all within the theoretical tolerated range.
- The range of results obtained using temperature regime 15-35 °C and dormancy-breaking treatments KNO_3 and heat + KNO_3 are outside the theoretical tolerated range for seed lot 3 (KNO_3) and seed lots 1 and 3 (heat + KNO_3).

The use of the temperature regime 15-35 °C is not recommended for the germination of *Brachiaria brazantha*.

The temperature regime 20-35 °C in combination with dormancy-breaking treatment KNO_3 or heat + KNO_3 should be introduced in the ISTA Rules for the germination of *Brachiaria brazantha*.

7. References

Miles S R (1963). Handbook of Tolerances and Measures of Precision for Seed Testing. Proc. Int. Seed Test. Ass. 28 (3)

Acknowledgements

Sincere thanks are due to Jean-Louis Laffont, who carried out the statistical analysis using the generalized linear mixed-effect model and calculated repeatability and reproducibility variances. Thanks are also due to Michael Kruse, who advised in the calculation of f factors and tolerated ranges, and to Ronald Don, who edited the validation report.

Validation of a cargo sampler and sampling stick without compartments for seed sampling in small seeded species

Validation Report

Approved and submitted by the
ISTA Bulking and Sampling Committee (BSC)
19. March 2009

Test organizer:	**Michael Kruse, Leena Pietila**
First reviewer:	**Ronald Don**
Second reviewer:	**John Hampton**
Statistical reviewer:	**Jean-Louis Laffont**
Sponsors:	**DLF-Trifolium A/S and Hunsballe Frø A/S**
Author Validation report :	**Michael Kruse**

Summary

The object is to validate the sampling stick without compartments for vertical use and the cargo sampler. The reference method was the ISTA approved sampling stick with compartments. Submitted samples were taken by two participants from in total 12 seed lots of chaffy and non-chaffy small seeded species with the three triers in five replications. Trueness and repeatability in purity, other seed count and germination were evaluated. Calculations of acceptable variation were based on Miles (1963). The results showed that for all three triers variability in purity testing was lower than the variation calculated by Miles (1963). However, in other seed count and in germination testing variability was significantly greater. Even the ISTA approved sampling stick did not fulfil the requirements. The explanation may be that in the formula for purity testing several sources of variation are included unlike in the formulas for other seed count and germination that include random sampling variation only. Consequently, additional factors were added for the calculation of acceptable standard deviations for germination and other seed count. Recalculated reference values showed that the ISTA approved trier and the new triers did not exceed acceptable variation. Based on the recalculated results, both new triers are recommended to be included into the ISTA Rules for small seeded species.

Introduction

The ISTA Rules, Chapter 2 allow to use the Nobbe trier and sampling stick for sampling manually seeds in bags or other containers. If the sampling stick shall be used vertically, it has to have compartments. In many countries and companies also other types of triers are widely used in cases where an ISTA International Seed Lot Certificate is not requested, like e.g. the cargo sampler. Cargo samplers can be used when the container is deeper than 2 - 3 meters where the Nobbe trier or the sampling stick can not be used anymore. Also, sampling sticks without compartments are used vertically, since they are considered to be more practical for emptying the seed.

Objective

The objective of this validation project is to validate new types of triers for sampling seed as a requirement for the submission of ISTA Rules change proposals. In this study, the sampling stick without compartments (the spiral spear) was used vertically

for sampling small seeds and the cargo sampler was tested also for sampling small seeds.

Materials and Methods

Each new trier was tested by two participants (DLF-Trifolium A/S and Hunsballe Frø A/S). Each participant tested the triers in three seed lots of small (smaller than seed size of *Triticum aestivum*) seeded chaffy species and three seed lots of small seeded non-chaffy species. Additionally, all seed lots were sampled with an ISTA approved sampling stick. Each sampling was repeated five times. Thus, in total 12 seed lots (2 participants x 2 species groups x 3 seed lots) were sampled, 180 sampling operations (3 triers x 2 participants x 2 species groups x 3 seed lots x 5 replicates) were performed and 180 submitted samples were tested. Table 1 provides further information about the seed lots sampled in this study.

Table 1: Seed lot identification and description

Code	Company	Species	Lot Size (kg)	Containers
C1	1	*Dactylis glomerata*	9 012	14 boxes
C2	1	*Festuca rubra*	10 000	17 boxes
C3	1	*Lolium perenne*	10496	11 boxes
C4	2	*Lolium perenne*	900	1 box
C5	2	*Dactylis glomerata*	690	1 box
C6	2	*Phleum pratense*	1143	1 box
N1	1	*Brassica napus*	10 000	8 boxes
N2	1	*Trifolium pratense*	1000	1 box
N3	1	*Trifolium repens*	1689	2 boxes
N4	2	*Trifolium repens*	981	1 box
N5	2	*Trifolium repens*	1100	1 box
N6	2	*Trifolium repens*	1025	1 box

Sampling was carried out by persons authorised to take samples for ISTA International Seed Lot Certificates according to the ISTA Accreditation Standard. The

persons were engaged in sampling routinely and were familiar with the new sampling methods as well as the ISTA sampling method.

Sampling with the sampling sticks (with and without compartments) was done according to the ISTA Rules. The cargo samplers were those described in the ISTA Handbook for Seed Sampling (5.2.2.5.1. and Figure 5.7.D in the Handbook) and they were used as described in the same Handbook and in the submitted Rules change proposal. In all cases sampling intensity and sample reduction were done according to the ISTA Rules.

On each submitted sample a purity analysis, an other seed count and a germination test were performed according to the ISTA Rules. All the tests on the fifteen submitted samples from one seed lot were done by the same analyst in the same laboratory within a short time period to avoid laboratory bias as best as possible.

The results within the two groups were evaluated independently from each other.

Sampling methods

Cargo sampler *(bulk sampler).* The cargo sampler consists of a special type of chamber that is fixed to a shaft. The lower part of the chamber is cone-shaped with a pointed end. To reach a greater depth, the shaft may be lengthened by screwing on successive extensions. For all species, the minimum inside diameter can be about 35 mm and the depth 75 mm. When using the cargo sampler, it is inserted in the closed position into the container and pushed vertically into the seed so that the point reaches the required position. When the cargo sampler is in the right position it is pulled back about 10 cm or it is turned (depending on the closing system) and agitated slightly to allow it to fill completely. Then the cargo sampler is closed if possible and withdrawn from the container. The primary sample is then emptied into a sample container. The cargo sampler used in this study could not be closed.

Sampling stick without compartments (*model used here: spiral spear).* The model used in this study is the “spiral spear”. This sampling stick consists of an inner tube which fits loosely inside an outer tube, but tightly enough so that seed or impurities do not slip between them. The outer tube has a solid pointed end. Both tubes have slots cut into their walls. In the inner tube they are in a spiral arrangement so that the cavity of the inner tube can be opened from the bottom by slowly twisting the inner tube inside the outer tube. Further twisting opens further slots towards the top while slots at the bottom are closed already. The spiral spear was used vertically.

ISTA reference method: A sampling stick with compartments according to the ISTA Rules was used by both participants as an ISTA approved method.

A B C

Figure 1: Photos of the triers used in this validation study: A: spiral spear, B: cargo sampler, C: sampling stick with compartments (ISTA method)

Statistical analysis

The statistical analyses were done as described in the approved method validation test plan as reviewed by the statistical reviewer. Based on the results obtained with the ISTA reference method, the statistical analysis was adapted as described below. Two statistical characteristics were used in this evaluation: Trueness and repeatability.

Trueness describes whether the mean quality obtained by using the new trier is not significantly different from the mean quality obtained by using the ISTA Method. The analysis was done according to the principle used in the ISTA tolerance tables.

Repeatability describes the variation of test results when sampling and testing is done under “the same” conditions, i.e. by the same method and the same person with no time delay.

The two characteristics were analysed as follows:

Trueness

The mean purity, other seed count and germination were calculated from the 5 replicate submitted samples for the new two triers ($\overline{x}_{t1}$ and $\overline{x}_{t2}$) and the ISTA

sampling method ($\overline{x}_{ISTA}$). Then, the difference D between the trier and the ISTA method was calculated (e.g. $D_1 = \overline{x}_{t1} - \overline{x}_{ISTA}$). In addition, the overall mean $\overline{x}_{total}$ was calculated (e.g. ($\overline{x}_{t1} + \overline{x}_{ISTA}$)/2) and the maximum difference on the basis of sampling errors according to Miles (!963) was calculated as:

$$D_{max} = 1.96 \times \sqrt{2 \times \left(\left(\frac{B}{10} \times \frac{50-10}{50} \right) + \frac{C}{10} + \frac{W}{5} + \frac{A}{1} + \frac{I}{1} \right)}$$ in the case of purity

with: B = $\frac{\overline{x}_{total} \times (100 - \overline{x}_{total})}{3500}$ in case of non chaffy seed

B = $\frac{\overline{x}_{total} \times (100 - \overline{x}_{total})}{500}$ in case of chaffy seed

C = $\frac{\overline{x}_{total} \times (100 - \overline{x}_{total})}{14000}$ in case of non chaffy seed

C = $\frac{\overline{x}_{total} \times (100 - \overline{x}_{total})}{2000}$ in case of chaffy seed

W = $\frac{\overline{x}_{total} \times (100 - \overline{x}_{total})}{2750}$ in the case of non chaffy seed

W = $\frac{\overline{x}_{total} \times (100 - \overline{x}_{total})}{2300}$ in the case of chaffy seed

A = $\frac{\overline{x}_{total} \times (100 - \overline{x}_{total})}{7500}$ in the case of non chaffy and chaffy seed

I = $\frac{\overline{x}_{total} \times (100 - \overline{x}_{total})}{7500}$ in the case of non chaffy and chaffy seed

$$D_{max} = 1.96 \times \sqrt{2 \times \overline{x}_{total}}$$ in the case of other seed count

$$D_{max} = 1.96 \times \sqrt{2 * \frac{\overline{x}_{total} \times (100 - \overline{x}_{total})}{5 \times 400}}$$ in the case of germination

These calculations of the sampling errors are based on Miles (1963).
Under the six seed lots per species group there may be no more than 1 seed lot, in which the difference D per quality characteristic is greater than D_{max} (1 in 6 is 16 % and 16 % is greater than 5 % (which is the type 1 error when using 1.96), so the test is quite conservative.).

Repeatability

For this criteria, the means and standard deviations between the 5 replicate submitted samples were calculated for each trier ($\overline{x}_{t1}$, $\overline{x}_{t2}$, $\overline{x}_{ISTA}$ and SD_{t1}, SD_{t2}, SD_{ISTA}). Then, the standard deviations were calculated by the following formulas developed by Miles (1963):

$$SD_{random} = \sqrt{\left(\left(\frac{B}{10} \times \frac{50-10}{50}\right) + \frac{C}{10} + \frac{W}{1} + \frac{A}{1} + \frac{I}{1}\right)}$$ in the case of purity

with: B = $\frac{\overline{x}_{t1} \times (100 - \overline{x}_{t1})}{3500}$ in case of non chaffy seed

B = $\frac{\overline{x}_{t1} \times (100 - \overline{x}_{t1})}{500}$ in case of chaffy seed

C = $\frac{\overline{x}_{t1} \times (100 - \overline{x}_{t1})}{14000}$ in case of non chaffy seed

C = $\frac{\overline{x}_{t1} \times (100 - \overline{x}_{t1})}{2000}$ in case of chaffy seed

W = $\frac{\overline{x}_{t1} \times (100 - \overline{x}_{t1})}{2750}$ in the case of non chaffy seed

W = $\frac{\overline{x}_{t1} \times (100 - \overline{x}_{t1})}{2300}$ in the case of chaffy seed

A = $\frac{\overline{x}_{t1} \times (100 - \overline{x}_{t1})}{7500}$ in the case of non chaffy and chaffy seed

I = $\frac{\overline{x}_{t1} \times (100 - \overline{x}_{t1})}{7500}$ in the case of non chaffy and chaffy seed

$SD_{random} = \sqrt{\overline{x}_{t1}}$ in the case of other seed count and

$SD_{random} = \sqrt{\frac{\overline{x}_{t1} \times (100 - \overline{x}_{t1})}{400}}$ in the case of germination.

Calculations of the standard deviations are based on Miles (1963).

The observed standard deviations may exceed $SD_{max} = SD_{random} \times \sqrt{F_{0.05,4,\infty}} = SD_{random} \times 1.54$ (the 4 degrees of freedom are from 5 replicates -1 and infinite degrees of freedom are considered assuming SD_{random} is a true population variance) in no more than one out of the six seed lots per species

group per test (1 in 6 is 16 % and 16 % is greater than 5 % (which is the type 1 error when using 1.96), so the test is quite conservative).

Results and Discussion

Tables 2 and 3 show the means of the five replicates per seed lot and trier. The seed lots are of commercial quality and most of them of even good quality. Tables 4 and 5 show the standard deviations among the 5 replicates per seed lot and trier.

Table 6 shows the repeatability of sampling when using the ISTA approved method with a sampling stick. In purity testing, the variation among the 5 test results per seed lot was never significantly higher than the sampling variation as calculated according to Miles (1963). However, in the other seed count test, in four out the 12 seed lots, the variation was significantly higher than the random sampling variation as calculated by the Poisson-distribution according to Miles (1963). In germination testing, even in six out of the 12 seed lots the standard deviation was significantly higher in at least one category (normal, abnormal, non germinated) than the random sampling variation as calculated by the binomial distribution according to Miles (1963).

These results were not expected and show that either the sampling stick is not fit for purpose, or the way how the expected standard variations are calculated is not appropriate for sampling seed lots, or even both. The fact, that the variation is not conspicuous in purity testing but in other seed count and germination requires further attention. Miles (1963) developed a formula for calculating the expected variation of purity test results by taking into account all relevant sources of errors, starting from the variations among and within bags up to the seed analyst (see formula above). So his formula is not expecting that a purity test is a random sampling experiment but the expected variation includes experimentally determined additional sources of errors. Quite surprisingly, he did not follow this strategy in the case of other seed count and germination. His argumentation was, that for these traits variations e.g. caused by segregation within bags or silos are negligible. Therefore, he introduced factors for additional variation only for germination tests done in different laboratories and no such a factor in other seed count test at all.

Table 2: Means of the 5 replicates per trier and seed lot obtained from small chaffy seed

	Pure seeds (%)	Other species (%)	Inert matter (%)	# other seeds	Normal seedlings (%)	Abnormal seedlings (%)	Non germ. seeds (%)
Seed lot C1							
Spiral spear	95,60	0,08	4,32	33,0	92,4	2,4	5,2
Cargo sampler	95,12	0,16	4,72	25,0	93,6	1,2	5,2
ISTA stick	95,32	0,22	4,46	40,0	94,4	1,4	4,2
Seed lot C2							
Spiral spear	99,88	0	0,12	58,4	90,0	1,2	8,8
Cargo sampler	99,80	0	0,20	67,8	88,4	1,0	10,6
ISTA stick	99,70	0,06	0,24	64,0	90,0	1,2	8,8
Seed lot C3							
Spiral spear	95,10	0	4,9	10,6	82,0	1,4	16,8
Cargo sampler	94,70	0	5,3	16,8	82,2	1,0	16,8
ISTA stick	95,18	0,02	4,8	7,6	82,6	1,0	16,4
Seed lot C4							
Spiral spear	99,66	0,12	0,22	85,0	96,6	0,2	3,2
Cargo sampler	99,68	0,02	0,30	79,0	97,4	0,4	2,2
ISTA stick	99,68	0,06	0,26	82,6	97,4	0,4	2,2
Seed lot C5							
Spiral spear	97,36	0,50	2,14	178,0	94,4	1,4	4,2
Cargo sampler	97,08	0,58	2,34	173,4	92,6	1,6	5,8
ISTA stick	97,22	0,64	2,14	183,8	92,0	2,2	5,8
Seed lot C6							
Spiral spear	99,04	0,44	0,52	128,8	84,2	2,2	13,6
Cargo sampler	99,28	0,34	0,38	134,0	88,0	1,2	10,8
ISTA stick	99,24	0,42	0,34	132,4	83,4	1,4	15,2

Table 3: Means of the 5 replicates per trier and seed lot obtained from small non-chaffy seed

	Pure seeds (%)	Other species (%)	Inert matter (%)	# other seeds	Normal seedlings (%)	Abnormal seedlings (%)	Non germ. seeds (%)
Seed Lot N1							
Spiral spear	99,94	0	0,06	2,4	86,2	5,0	8,8
Cargo sampler	99,90	0	0,10	3,0	78,4	8,2	13,4
ISTA stick	99,92	0	0,08	2,8	82,0	5,8	12,2
Seed lot N2							
Spiral spear	98,3	1,58	0,12	1057,0	86,6	7,4	6,0
Cargo sampler	98,3	1,62	0,08	971,0	88,6	4,8	6,6
ISTA stick	98,2	1,66	0,14	992,2	89,4	5,6	5,0
Seed lot N3							
Spiral spear	99,40	0,44	0,16	83,6	82,8	5,8	11,4
Cargo sampler	99,26	0,40	0,34	81,6	83,0	6,8	10,2
ISTA stick	99,66	0,12	0,22	54,0	86,0	4,8	9,2
Seed lot N4							
Spiral spear	98,70	0,20	1,10	127,0	86,2	2,8	11,0
Cargo sampler	99,14	0,18	0,68	81,4	88,2	3,0	8,8
ISTA stick	98,74	0,22	1,04	125,2	83,6	3,2	13,2
Seed lot N5							
Spiral spear	99,02	0	0,98	28,6	50,2	8,0	41,8
Cargo sampler	99,10	0	0,90	16,2	51,6	8,6	39,8
ISTA stick	98,90	0,02	1,08	28,0	51,4	7,8	40,8
Seed lot N6							
Spiral spear	97,46	0,10	2,44	80,8	84,2	3,2	12,6
Cargo sampler	97,64	0,12	2,24	63,6	83,4	3,8	12,8
ISTA stick	97,64	0,10	2,26	75,4	83,2	4,0	12,8

Table 4: Standard deviation of the 5 replicates per trier and seed lot obtained from small chaffy seed.

	Pure seeds (%)	Other species (%)	Inert matter (%)	# other seeds	Normal seedlings (%)	Abnormal seedlings (%)	Non germ. seeds (%)
Seed lot C1							
Spiral spear	0,122	0,130	0,045	7,969	1,140	1,140	0,447
Cargo sampler	0,497	0,114	0,444	6,205	1,140	0,447	1,304
ISTA stick	0,277	0,164	0,207	6,819	1,517	0,548	1,304
Seed lot C2							
Spiral spear	0,110	0,000	0,110	25,560	2,236	0,447	2,168
Cargo sampler	0,071	0,000	0,071	11,862	0,894	0,000	0,894
ISTA stick	0,100	0,089	0,114	13,928	2,236	0,447	2,168
Seed lot C3							
Spiral spear	0,141	0,000	0,141	3,782	2,121	0,548	2,049
Cargo sampler	0,346	0,000	0,346	2,864	2,490	0,707	2,864
ISTA stick	0,130	0,045	0,141	3,435	2,510	1,000	2,074
Seed lot C4							
Spiral spear	0,114	0,110	0,045	13,491	1,140	0,447	0,837
Cargo sampler	0,164	0,045	0,141	9,434	0,894	0,548	0,447
ISTA stick	0,164	0,089	0,089	6,768	0,894	0,548	0,447
Seed lot C5							
Spiral spear	0,182	0,158	0,114	13,946	0,548	0,548	0,837
Cargo sampler	0,444	0,259	0,195	9,423	2,702	0,894	1,924
ISTA stick	0,327	0,230	0,207	18,089	1,732	0,447	1,304
Seed lot C6							
Spiral spear	0,182	0,251	0,130	11,841	1,095	0,837	1,140
Cargo sampler	0,130	0,055	0,110	3,808	2,000	0,447	1,924
ISTA stick	0,207	0,110	0,114	11,760	1,140	0,548	1,304

Table 5: Standard deviation of the 5 replicates per trier and seed lot obtained from small non-chaffy seed.

	Pure seeds (%)	Other species (%)	Inert matter (%)	# other seeds	Normal seedlings (%)	Abnormal seedlings (%)	Non germ. seeds (%)
Seed Lot N1							
Spiral spear	0,055	0,000	0,055	1,140	1,924	1,732	1,304
Cargo sampler	0,000	0,000	0,000	1,414	1,673	2,168	1,817
ISTA stick	0,045	0,000	0,045	1,483	2,236	1,304	1,304
Seed lot N2							
Spiral spear	0,255	0,239	0,084	84,448	1,517	1,673	0,707
Cargo sampler	0,367	0,377	0,045	132,639	2,074	1,304	1,673
ISTA stick	0,316	0,297	0,055	69,780	1,949	0,894	1,871
Seed lot N3							
Spiral spear	0,316	0,344	0,055	22,843	2,168	1,095	1,817
Cargo sampler	0,182	0,122	0,114	16,562	2,449	1,304	1,483
ISTA stick	0,152	0,084	0,110	17,564	1,732	0,837	1,095
Seed lot N4							
Spiral spear	0,122	0,071	0,141	11,467	1,924	0,837	1,581
Cargo sampler	0,241	0,045	0,217	10,691	2,280	1,225	2,049
ISTA stick	0,230	0,084	0,152	14,307	2,302	1,483	2,490
Seed lot N5							
Spiral spear	0,192	0,000	0,192	10,359	2,387	1,414	2,775
Cargo sampler	0,141	0,000	0,141	3,033	4,722	3,435	4,147
ISTA stick	0,292	0,045	0,277	8,000	5,225	1,095	4,764
Seed lot N6							
Spiral spear	0,241	0,000	0,241	8,927	2,490	0,837	2,510
Cargo sampler	0,152	0,045	0,152	7,893	2,408	1,304	1,304
ISTA stick	0,207	0,000	0,207	19,087	2,775	0,707	2,588

There are already several indications that this way of quantifying and modelling sources of errors in seed sampling does not reflect the reality and the present data obtained with the ISTA sampling stick also confirm this. Variations in other seed count and germination testing are higher than to expected in a random sampling experiment.

The problem for the present study is now that the reference method does not fulfil the expectation regarding repeatability. The main reason for this is that the expectation is not adequate. Consequently, also the new methods do not need to fulfil the expectation either. To be fair to the new methods, the inaccuracy in the statistical system needs to be taken into account. This was done in the following way:

In germination testing, the expected variation among the 5 replicates was re-calculated by including the factor for variation among laboratories. This factor was quantified by Miles (1963) and ranges between 1.56 and 1.96 as depending on the mean percentage of the considered category (normal, abnormal or dead) seed. In fact, this factor is taken into account in table 5.2 of the ISTA Rules also when tests are made in the same laboratory, so the use may be well justified. To keep it simple, constant values were used: for normal seedlings the factor was set to 1,64 corresponding to 92% normal seedlings, for abnormal seeds the factor was set to 1,57 corresponding to 1% abnormal seedlings, and for non-germinated seeds the factor was set to 1,62 corresponding to 7 % non germinated seeds.

In other seed count, there is no factor used by Miles (1963) at all. Therefore, as a makeshift, a factor of 1.5 was used. The recalculated expected standard deviations and conclusions for repeatability for the ISTA sampling stick are shown in table 7.

The repeatability and the trueness for the two new triers were calculated by using these additional factors and the results are shown in tables 8 to 11.

Table 6: Results for testing the **repeatability** of **ISTA sampling method** based on **expected variations according to Miles (1963)**.

	Pure seed			Other species			Inert matter			Seed count			Normal seedlings			Abnormal seedlings			Non germ. seeds		
Lot	SD max	SD	SD>SD max	SD max	SD	SD>SD max	SD max	SD	SD>SD max	SD max	SD	SD>SD max	SD max	SD	SD>SD max	SD max	SD	SD>SD max	SD max	SD	SD>SD max
C1	0,99	0,28	No	0,22	0,16	No	0,95	0,21	No	9,73	6,82	No	1,77	1,52	No	0,91	0,55	No	1,54	1,30	No
C2	0,26	0,10	No	0,11	0,09	No	0,23	0,11	No	12,32	13,93	Yes	2,31	2,24	No	0,83	0,45	No	2,19	2,17	No
C3	1,00	0,13	No	0,06	0,04	No	1,00	0,14	No	4,25	3,44	No	2,93	2,51	No	0,77	1,00	Yes	2,85	2,07	No
C4	0,26	0,16	No	0,11	0,09	No	0,23	0,09	No	14,00	6,77	No	1,23	0,89	No	0,49	0,55	Yes	1,12	0,45	No
C5	0,77	0,33	No	0,37	0,23	No	0,68	0,21	No	20,88	18,09	No	2,09	1,73	No	1,12	0,45	No	1,80	1,30	No
C6	0,40	0,21	No	0,31	0,11	No	0,28	0,11	No	17,73	11,76	No	2,86	1,14	No	0,91	0,55	No	2,77	1,30	No
N1	0,11	0,04	No	0,00	0,00	No	0,11	0,04	No	2,57	1,48	No	2,96	2,24	No	1,80	1,30	No	2,53	1,30	No
N2	0,52	0,32	No	0,51	0,30	No	0,15	0,05	No	48,51	69,78	Yes	2,37	1,95	No	1,77	0,89	No	1,68	1,87	Yes
N3	0,23	0,15	No	0,14	0,08	No	0,18	0,11	No	11,32	17,56	Yes	2,66	1,73	No	1,65	0,84	No	2,23	1,10	No
N4	0,45	0,23	No	0,18	0,08	No	0,40	0,15	No	17,23	14,31	No	2,85	2,30	No	1,36	1,48	Yes	2,60	2,49	No
N5	0,42	0,29	No	0,06	0,04	No	0,42	0,28	No	8,15	8,00	No	3,85	5,22	Yes	2,06	1,10	No	3,79	4,76	Yes
N6	0,60	0,21	No	0,12	0,00	No	0,59	0,21	No	13,37	19,09	Yes	2,88	2,77	No	1,51	0,71	No	2,57	2,59	Yes

Table 7: Results for testing the **repeatability** of **ISTA sampling method** based on **recalculated expected variations (see Text)**.

	Pure seed			Other species			Inert matter			Seed count			Normal seedlings			Abnormal seedlings			Non germ. seeds		
Lot	SD max	SD	SD>SD max	SD max	SD	SD>SD max	SD max	SD	SD>SD max	SD max	SD	SD>SD max	SD max	SD	SD>SD max	SD max	SD	SD>SD max	SD max	SD	SD>SD max
C1	0,99	0,28	No	0,22	0,16	No	0,95	0,21	No	14,60	6,82	No	2,90	1,52	No	1,43	0,55	No	2,49	1,30	No
C2	0,26	0,10	No	0,11	0,09	No	0,23	0,11	No	18,48	13,93	No	3,79	2,24	No	1,30	0,45	No	3,55	2,17	No
C3	1,00	0,13	No	0,06	0,04	No	1,00	0,14	No	6,38	3,44	No	4,81	2,51	No	1,21	1,00	No	4,62	2,07	No
C4	0,26	0,16	No	0,11	0,09	No	0,23	0,09	No	21,00	6,77	No	2,02	0,89	No	0,77	0,55	No	1,81	0,45	No
C5	0,77	0,33	No	0,37	0,23	No	0,68	0,21	No	31,32	18,09	No	3,43	1,73	No	1,76	0,45	No	2,92	1,30	No
C6	0,40	0,21	No	0,31	0,11	No	0,28	0,11	No	26,60	11,76	No	4,69	1,14	No	1,43	0,55	No	4,49	1,30	No
N1	0,11	0,04	No	0,00	0,00	No	0,11	0,04	No	3,86	1,48	No	4,85	2,24	No	2,83	1,30	No	4,10	1,30	No
N2	0,52	0,32	No	0,51	0,30	No	0,15	0,05	No	72,77	69,78	No	3,89	1,95	No	2,78	0,89	No	2,72	1,87	No
N3	0,23	0,15	No	0,14	0,08	No	0,18	0,11	No	16,98	17,56	Yes	4,36	1,73	No	2,59	0,84	No	3,61	1,10	No
N4	0,45	0,23	No	0,18	0,08	No	0,40	0,15	No	25,85	14,31	No	4,67	2,30	No	2,14	1,48	No	4,21	2,49	No
N5	0,42	0,29	No	0,06	0,04	No	0,42	0,28	No	12,23	8,00	No	6,31	5,22	No	3,23	1,10	No	6,14	4,76	No
N6	0,60	0,21	No	0,12	0,00	No	0,59	0,21	No	20,06	19,09	No	4,72	2,77	No	2,37	0,71	No	4,16	2,59	No

Table 8: Results for testing the **trueness** of sampling by the **cargo sampler**.

Lot	Pure seed			Other species			Inert matter			Seed count			Normal seedlings			Abnormal seedlings			Non germ. seeds		
	D_{max}	D	$\|D\|>D_{max}$	D_{max}	D	$\|D\|>D_{max}$	D_{max}	D	$\|D\|>D_{max}$	D_{max}	D	$\|D\|>D_{max}$	D_{max}	D	$\|D\|>D_{max}$	D_{max}	D	$\|D\|>D_{max}$	D_{max}	D	$\|D\|>D_{max}$
C1	1,40	-0,20	No	0,29	-0,06	No	1,38	0,26	No	23,70	-15,00	No	2,41	-0,80	No	1,10	-0,20	No	2,12	1,00	No
C2	1,44	-0,48	No	0,07	-0,02	No	1,44	0,50	No	14,52	9,20	No	3,87	-0,40	No	0,97	0,00	No	3,74	0,40	No
C3	0,33	0,10	No	0,11	-0,06	No	0,31	-0,04	No	33,75	3,80	No	3,15	-1,60	No	1,02	-0,20	No	2,96	1,80	No
C4	0,37	0,00	No	0,13	-0,04	No	0,35	0,04	No	37,38	-3,60	No	1,62	0,00	No	0,61	0,00	No	1,47	0,00	No
C5	1,09	-0,14	No	0,51	-0,06	No	0,97	0,20	No	55,56	-10,40	No	2,71	0,60	No	1,33	-0,60	No	2,35	0,00	No
C6	0,56	0,04	No	0,40	-0,08	No	0,39	0,04	No	47,99	1,60	No	3,56	4,60	Yes	1,10	-0,20	No	3,37	-4,40	Yes
N1	0,16	-0,02	No	0,00	0,00	No	0,16	0,02	No	7,08	0,20	No	4,05	-3,60	No	2,48	2,40	No	3,35	1,20	No
N2	0,70	0,10	No	0,68	-0,04	No	0,18	-0,06	No	130,26	-21,20	No	3,18	-0,80	No	2,17	-0,80	No	2,35	1,60	No
N3	0,39	-0,40	No	0,27	0,28	Yes	0,28	0,12	No	34,23	27,60	No	3,67	-3,00	No	2,28	2,00	No	2,96	1,00	No
N4	0,81	0,00	No	0,18	0,02	No	0,79	-0,02	No	34,67	-11,80	No	3,79	0,20	No	1,88	-0,20	No	3,35	0,00	No
N5	0,53	0,20	No	0,05	-0,02	No	0,53	-0,18	No	19,55	-11,80	No	5,08	0,20	No	2,67	0,80	No	4,92	-1,00	No
N6	0,55	0,40	No	0,24	-0,04	No	0,49	-0,36	No	42,26	-43,80	No	3,54	4,60	Yes	1,68	-0,20	No	3,14	-4,40	Yes

Table 9: Results for testing the **repeatability** of sampling by the **cargo sampler**.

Lot	Pure seed			Other species			Inert matter			Seed count			Normal seedlings			Abnormal seedlings			Non germ. seeds		
	SD *max*	*SD*	SD>SD *max*	SD *max*	*SD*	SD>SD *max*	SD *max*	*SD*	SD>SD *max*	SD *max*	*SD*	SD>SD *max*	SD *max*	*SD*	SD>SD *max*	SD *max*	*SD*	SD>SD *max*	SD *max*	*SD*	SD>SD *max*
C1	1,00	0,50	No	0,18	0,11	No	0,99	0,44	No	11,55	6,20	No	3,08	1,14	No	1,30	0,45	No	2,77	1,30	No
C2	0,20	0,07	No	0,00	0,00	No	0,20	0,07	No	19,01	11,86	No	4,03	0,89	No	1,21	0,00	No	3,84	0,89	No
C3	1,05	0,35	No	0,00	0,00	No	1,05	0,35	No	9,47	2,86	No	4,82	2,49	No	1,21	0,71	No	4,67	2,86	No
C4	0,26	0,16	No	0,06	0,04	No	0,26	0,14	No	20,54	9,43	No	2,02	0,89	No	0,77	0,55	No	1,81	0,45	No
C5	0,79	0,44	No	0,35	0,26	No	0,71	0,19	No	30,42	9,42	No	3,31	2,70	No	1,52	0,89	No	2,92	1,92	No
C6	0,40	0,13	No	0,28	0,05	No	0,29	0,11	No	26,75	3,81	No	4,08	2,00	No	1,30	0,45	No	3,87	1,92	No
N1	0,12	0,00	No	0,00	0,00	No	0,12	0,00	No	3,99	1,41	No	5,20	1,67	No	3,31	2,17	No	4,24	1,82	No
N2	0,51	0,37	No	0,49	0,38	No	0,11	0,04	No	71,99	132,64	Yes	4,02	2,07	No	2,59	1,30	No	3,09	1,67	No
N3	0,34	0,18	No	0,25	0,12	No	0,23	0,11	No	20,87	16,56	No	4,76	2,45	No	3,05	1,30	No	3,77	1,48	No
N4	0,37	0,24	No	0,17	0,04	No	0,32	0,22	No	20,84	10,69	No	4,07	2,28	No	2,06	1,22	No	3,55	2,05	No
N5	0,37	0,14	No	0,00	0,00	No	0,37	0,14	No	9,29	3,03	No	6,31	4,72	No	3,39	3,44	No	6,11	4,15	No
N6	0,60	0,15	No	0,14	0,04	No	0,59	0,15	No	18,41	7,89	No	4,69	2,41	No	2,32	1,30	No	4,16	1,30	No

Table 10: Results for testing the **trueness** of sampling by the **spiral spear**.

Lot	Pure seed			Other species			Inert matter			Seed count			Normal seedlings			Abnormal seedlings			Non germ. seeds		
	D_{max}	D	$\|D\|>D_{max}$	D_{max}	D	$\|D\|>D_{max}$	D_{max}	D	$\|D\|>D_{max}$	D_{max}	D	$\|D\|>D_{max}$	D_{max}	D	$\|D\|>D_{max}$	D_{max}	D	$\|D\|>D_{max}$	D_{max}	D	$\|D\|>D_{max}$
C1	1,37	0,28	No	0,25	-0,14	No	1,35	-0,14	No	25,13	-7,00	No	2,53	-2,00	No	1,33	1,00	No	2,12	1,00	No
C2	1,42	-0,08	No	0,07	-0,02	No	1,41	0,10	No	12,54	3,00	No	3,89	-0,60	No	1,05	0,40	No	3,74	0,40	No
C3	0,30	0,18	No	0,11	-0,06	No	0,28	-0,12	No	32,52	-5,60	No	3,05	0,00	No	1,05	0,00	No	2,85	0,00	No
C4	0,38	-0,02	No	0,20	0,06	No	0,32	-0,04	No	38,06	2,40	No	1,74	-0,80	No	0,53	-0,20	No	1,62	1,00	No
C5	1,07	0,14	No	0,50	-0,14	No	0,95	0,00	No	55,92	-5,80	No	2,56	2,40	No	1,29	-0,80	No	2,19	-1,60	No
C6	0,61	-0,20	No	0,43	0,02	No	0,43	0,18	No	47,52	-3,60	No	3,74	0,80	No	1,29	0,80	No	3,53	-1,60	No
N1	0,14	0,02	No	0,00	0,00	No	0,14	-0,02	No	6,71	-0,40	No	3,72	4,20	No	2,20	-0,80	No	3,08	-3,40	Yes
N2	0,70	0,10	No	0,67	-0,08	No	0,19	-0,02	No	133,10	64,80	No	3,30	-2,80	No	2,40	1,80	No	2,28	1,00	No
N3	0,36	-0,26	No	0,28	0,32	Yes	0,23	-0,06	No	34,49	29,60	No	3,69	-3,20	No	2,18	1,00	No	3,05	2,20	No
N4	0,82	-0,18	No	0,17	0,00	No	0,81	0,18	No	36,75	5,40	No	3,76	1,00	No	1,81	-0,80	No	3,34	-0,20	No
N5	0,54	0,12	No	0,05	-0,02	No	0,54	-0,10	No	22,13	0,60	No	5,08	-1,20	No	2,62	0,20	No	4,94	1,00	No
N6	0,60	-0,04	No	0,24	-0,02	No	0,55	0,06	No	46,70	1,80	No	3,64	2,60	No	1,66	-0,40	No	3,27	-2,20	No

Table 11: Results for testing the **repeatability** of sampling by the **spiral spear**.

Lot	Pure seed			Other species			Inert matter			Seed count			Normal seedlings			Abnormal seedlings			Non germ. seeds		
	SD max	SD	SD>SD max	SD max	SD	SD>SD max	SD max	SD	SD>SD max	SD max	SD	SD>SD max	SD max	SD	SD>SD max	SD max	SD	SD>SD max	SD max	SD	SD>SD max
C1	0,95	0,12	No	0,14	0,13	No	0,94	0,04	No	13,26	7,97	No	3,33	1,14	No	1,87	1,14	No	2,77	0,45	No
C2	0,15	0,11	No	0,00	0,00	No	0,15	0,11	No	17,66	25,56	Yes	3,79	2,24	No	1,30	0,45	No	3,55	2,17	No
C3	1,00	0,14	No	0,00	0,00	No	1,00	0,14	No	7,53	3,78	No	4,85	2,12	No	1,43	0,55	No	4,67	2,05	No
C4	0,28	0,11	No	0,15	0,11	No	0,22	0,04	No	21,30	13,49	No	2,30	1,14	No	0,53	0,45	No	2,20	0,84	No
C5	0,74	0,18	No	0,32	0,16	No	0,68	0,11	No	30,81	13,95	No	2,90	0,55	No	1,43	0,55	No	2,49	0,84	No
C6	0,45	0,18	No	0,31	0,25	No	0,34	0,13	No	26,22	11,84	No	4,59	1,10	No	1,76	0,84	No	4,26	1,14	No
N1	0,09	0,05	No	0,00	0,00	No	0,09	0,05	No	3,59	1,14	No	4,35	1,92	No	2,64	1,73	No	3,55	1,30	No
N2	0,51	0,25	No	0,49	0,24	No	0,14	0,08	No	75,11	84,45	Yes	4,30	1,52	No	3,17	1,67	No	2,96	0,71	No
N3	0,31	0,32	Yes	0,26	0,34	Yes	0,15	0,05	No	21,12	22,84	Yes	4,77	2,17	No	2,83	1,10	No	3,97	1,82	No
N4	0,45	0,12	No	0,17	0,07	No	0,42	0,14	No	26,04	11,47	No	4,35	1,92	No	1,98	0,84	No	3,89	1,58	No
N5	0,39	0,19	No	0,00	0,00	No	0,39	0,19	No	12,36	10,36	No	6,31	2,39	No	3,28	1,41	No	6,16	2,77	No
N6	0,62	0,24	No	0,12	0,00	No	0,62	0,24	No	20,76	8,93	No	4,59	2,49	No	2,14	0,84	No	4,15	2,51	No

Based on these recalculated reference values for accountable variation, the new triers were within acceptable deviations from the ISTA reference method. Only the spiral spear showed a lower repeatability in the characteristic “other seed count” in non chaffy seed (in two seed lots standard deviations were too high). However, in all these cases, the observed standard deviations were only slightly higher than the acceptable standard deviation. The results of the 5 replicates of the relevant tests are shown in table 12. These data are not indicating a severe problem with this trier.

Table 12: Results of the five replicates in tests where the standard deviation exceeds in two seed lots per test the maximum standard deviation.

sampler	test	lot	rep 1	rep 2	rep 3	rep 4	rep 5	SD	SD_{max}
spiral	other seed count	N2	939	1066	1174	1033	1073	84,45	75,11
		N3	81	47	106	85	99	22,84	21,12

Conclusions

The spiral spear and the cargo sampler did not cause any problem during sampling seed lots by the two participants. In fact, the advantages of the two triers were confirmed. They are easier to be emptied and easier to push into the seed, respectively, than the ISTA approved sampling stick with compartments. Therefore, also during the validation study the motivation grew to submit the proposal for inclusion of the two types of triers into the ISTA Rules since these tools achieve an improvement in the practical work.

Unfortunately, the results obtained by using the ISTA approved sampling stick with compartments were showing that in general sampling and testing seed under “repeatability-conditions” is not as precise as the ISTA Rules expect. The seed lots came from commercial production and standard processing, persons doing sampling were trained and ISTA accredited, tests were done according to the ISTA Rules in ISTA accredited laboratories. So we need to assume that the variation found is representative for what is achievable in an ISTA system in practice.

When this is accepted for this validation study, the statistical evaluation needs to be adapted. The differences between purity and other seed count + germination regarding repeatability of the ISTA approved sampling stick are corresponding with the amount of additional variation taken into account in the formulas by Miles (1963) for setting up tolerance tables. Therefore, it is justified if not even indicated to adjust the maximum tolerated variation.

Based on the re-calculated acceptable variations, all two new triers were found to achieve results in sufficient agreement with results obtained with the ISTA approved sampling stick in small chaffy and non chaffy seed. Therefore, the triers are proposed for being included into the ISTA Rules for all small seeded species (smaller than seed size of *Triticum aestivum*).

Finally, the contrasting results obtained with the ISTA approved method in purity testing on the one side and germination testing and other seed count on the other side clearly indicate that the statistical concept of quantifying and modelling variations caused by sampling needs to revised thoroughly.

Acknowledgements

The input of the companies DLF-Trifolium A/S and Hunsballe Frø A/S is greatly acknowledged. Special thanks are due to Jean-Louis Laffont for his help in data analysis. Ronald Don, John Hampton and Jean-Louis Laffont gave valuable comments on design of the study and reporting the results.

References

Miles, S. R. (1963). Handbook of tolerances and of measures of precision for seed testing. *Proceedings of the International Seed Testing Association*, **28** (3), 525-686.

Evaluation of Creped Cellulose Paper Covered with Sand as an ISTA Medium for *Glycine max, Helianthus annuus, Phaseolus vulgaris* and *Zea mays* [Rules Proposal 2010 C.5.1.]

Fiedler, K. A., T. J. Gutormson, K. A. Brix-Davis, A. L. Patin

SGS Mid-West Seed Services, Inc.

Brookings, South Dakota USA

Summary

TCS (On top of creped cellulose paper without a blotter and covered with ½ to ¾ inch layer of sand) is currently utilized in AOSA Rules as a growing medium for six species: Glycine max, Gossypium species, Helianthus annuus, Phaseolus vulgaris, Pisum sativum, and Zea mays. TPS (top of cellulose paper with sand) was adopted for Pisum sativum in June 2008 by ISTA

A peer validation study showed no significant differences between currently used ISTA media and TPS (top of crepe paper with sand) media for Glycine max and Zea mays. However, for Phaseolus vulgaris, the TPS medium produced significantly different but higher results than the BP (between paper) method. For Helianthus annuus, the S (Sand) media results were not significantly different than TPS results; however, TPS results were significantly higher than BP and O (organic growing media) media results. These results support the inclusion of TPS as a new media testing option for ISTA laboratories.

Introduction

A significant germination media disparity exists between the International Seed Testing Association (ISTA) *International Rules for Seed Testing* and Association of Official Seed Analysts (AOSA) *Rules for Testing Seeds*. The difference is the AOSA option of conducting germination tests on creped cellulose paper (TC) and on top of creped cellulose paper without a blotter and covered with ½ to ¾ inch layer of sand (TCS) on the following species: *Glycine max, Gossypium species, Helianthus annuus, Phaseolus vulgaris,* and *Zea mays.* A number of laboratories utilizing AOSA methods have adopted the TCS medium because it allows precise calibration of moisture levels through the use of calibrated water spraying tables (Figure 1) and dry sand.

Figure 1. Germination tray covered with creped cellulose paper moving through a calibrated water spraying table.

Typically, TCS tests are conducted by moistening a sheet of creped paper, planting seed on the moistened medium and covering the seed and paper with 2 cm of dry sand. Initial discussions (September 2003) with the ISTA Germination committee chairperson, Ronald Don, suggested the TC medium was already covered as top of paper (TP) as an ISTA medium: however the TCS medium (combination of paper and sand (S) as a

medium) would require comparative testing before consideration for inclusion in the *International Rules for Seed Testing*.

In response, a multi-laboratory comparative test was completed on the use of TCS as a germination media for *Pisum sativum*. After the completion of this study the ISTA germination committee approved a proposal for the inclusion of TPS, the equivalent to the AOSA TCS method, as a germination medium for *Pisum sativum*. This rule proposal was adopted in June 2008 at the ISTA Ordinary meeting. However, the AOSA Rules contain five additional species: *Glycine max, Gossypium species, Helianthus annuus, Phaseolus vulgaris,* and *Zea mays* that allow use of the TCS medium. Allowing the use of the TPS medium within the ISTA Rules for these additional species is the next logical topic of interest.

Materials and methods

A peer validation study was designed to compare germination results of *Glycine max, Phaseolus vulgaris* and *Zea mays* on three media types (*Table 1).* The *Helianthus annuus* germination results utilized four media types *(Table 1). Gossypium species* was considered for the study, but was removed since the TCS method on *Gossypium species* is not widely utilized. The study included four seed lots per species. The germination levels for the four seed lots of *Glycine max, Phaseolus vulgaris, Zea mays, Helianthus annuus* was 88.9%, 89.6%, 93.3%, 88.8% respectively. Germination regimes followed ISTA germination Table 5A Part 1. Four hundred seeds were used for each substrate with four, 100 seed observations. Two ISTA accredited laboratories participated. Participants included: Kari Fiedler, SGS Mid-West Seed Services, Inc., Brookings, SD, USA (AOSA/ISTA); and Victor Vankus, National Tree Seed Laboratory, Dry Branch, GA USA (AOSA/ISTA).

Table 1. Species, media, and temperatures used in the validation study.

Species	Substrate	Temperature (C)	Final Count in Days
Glycine max	BP, S, TPS	25	8
Helianthus annuus	BP, S, O, TPS	25	10
Phaseolus vulgaris	BP, S, TPS	25	9
Zea mays	BP, S, TPS	25	7

BP=between paper, S=Sand, TPS= top of crepe paper with sand, O=organic growing media

The effects of the different factors (laboratory, seed lot, test and their interactions) were assessed utilizing the Analysis of Variance technique with square root transformation. Obtained differences were tested by LSD. Computations were performed using MSUSTAT (1991).

Results

Media type mean germination percentages for *Glycine max and Zea mays* are shown in Table 2. ANOVA results conducted on square root transformed mean germination percentages for *Glycine max* and *Zea mays* were not statistically significant for media types (Table 3 and 4). LSD's were not performed due to the p value being greater than 0.05 for the media type. ANOVA results (Table 5) demonstrated *Phaseolus vulgaris* method mean germination percentages were statistically significant. *Phaseolus vulgaris* r^2LSD showed (Table 6) methods S and TPS mean germination percentages were 92.1% and 91.2%, respectively and significantly higher than BP 85.7%. The ANOVA (Table 7) demonstrated *Helianthus annuus* method mean germination percentages were statistically significant. Square root transformed LSD results demonstrated mean *Helianthus annuus* germination percentage for TPS was 94.4%, significantly higher than BP medium and O medium, 91.0% and 92.9% respectively but not significantly different than the S media, 94.3% (Table 8).

Table 2. Mean germination percentages averaged across two laboratories, four seed lots, and three media types for *Glycine max* and *Zea mays*.

	Mean Germination Percentage	
	Glycine max	Zea mays
Media	---------- % ----------	
BP	88.6	94.8
S	88.2	94.1
TPS	88.1	93.9

Table 3. Analysis of variance for the square root of the normal germination responses for *Glycine max*.

Source	df	S.S.	M.S.	F-Value	P-Value
Blocks	3	0.0200	0.0067		
Lab	1	0.1067	0.1067	2.59	0.1123
Sample	3	0.2285	0.0762	1.85	0.1466
Lab*Sample	3	1.5613	0.5204	12.62	0.0000
Method	2	0.0131	0.0065	0.16	0.8537
Lab*Method	2	0.3868	0.1934	4.69	0.0123
Sample*Method	6	0.8074	0.1346	3.26	0.0070
Lab*Sam*Method	6	0.6093	0.1016	2.46	0.0323
Residual	69	2.8446	0.0412		

Table 4. Analysis of variance for the square root of the normal germination responses for *Zea mays*

Source	df	S.S.	M.S.	F-Value	P-Value
Blocks	3	0.0492	0.0164		
Lab	1	0.2272	0.2272	14.11	0.0000
Sample	3	1.3801	0.4600	28.58	0.0000
Lab*Sample	3	0.5860	0.1953	12.13	0.0000
Method	2	0.0394	0.0197	1.22	0.3005
Lab*Method	2	0.1224	0.0612	3.80	0.0272
Sample*Method	6	0.1031	0.0172	1.07	0.3904
Lab*Sam*Method	6	0.1332	0.0222	1.38	0.2355
Residual	69	1.1107	0.0161		

Table 5. Analysis of variance for the square root of the normal germination responses for *Phaseolus vulgaris*

Source	df	S.S.	M.S.	F-Value	P-Value
Blocks	3	0.4109	0.1370		
Lab	1	4.2001	4.2001	67.93	0.0000
Sample	3	1.9050	0.6350	10.27	0.0000
Lab*Sample	3	1.1150	0.3717	6.01	0.0110
Method	2	2.3564	1.1782	19.06	0.0000
Lab*Method	2	1.1916	0.5958	9.64	0.0002
Sample*Method	6	0.4984	0.0831	1.34	0.2499
Lab*Sam*Method	6	0.1868	0.0311	0.50	0.8036
Residual	69	4.2661	0.0618		

Table 6. *Phaseolus vulgaris* mean germination percentages and r^2 transformed germination percentage averaged across two laboratories and four seed lots for three media methods.

Media	r^2 Transformed Germination %	Mean Germination %
BP	9.241	85.7
S	9.596	92.1
TPS	9.544	91.2
r^2LSD (P=0.05)	0.1240	

Table 7. Analysis of variance for the square root of the normal germination responses for *Helianthus annuus*

Source	df	S.S.	M.S.	F-Value	P-Value
Blocks	3	0.1031	0.0344		
Lab	1	5.3915	5.3915	196.51	0.0000
Sample	3	10.5030	3.5009	127.60	0.0000
Lab*Sample	3	3.9299	1.3100	47.74	0.0000
Method	3	1.4374	0.4791	17.46	0.0000
Lab*Method	3	0.7358	0.2453	8.94	0.0000
Sample*Method	9	0.9621	0.1069	3.90	0.0003
Lab*Sam*Method	9	1.0058	0.1118	4.07	0.0002
Residual	93	2.5516	0.0274		

Table 8. *Helianthus annuus* mean germination percentages and r^2 transformed germination percentages averaged across two laboratories and four seed lots for four media methods.

Media	r^2 Transformed Germination %	Mean Germination %
BP	9.450	91.0
S	9.707	94.3
O	9.624	92.9
TPS	9.712	94.4
r^2 LSD (P =0.05)	0.0822	

Discussion

The *Pisum* validation study, Evaluation of Crepe Cellulose Paper Covered with Sand as an ISTA Medium, was adopted as a medium option in the ISTA rules. The ISTA germination committee determined a peer validation study was necessary to include TPS as a medium option in the ISTA rules for *Glycine max, Helianthus annuus, Phaseolus vulgaris*, and *Zea mays*.

The data generated in this validation study supports the inclusion of TPS as a new medium for ISTA laboratories for these species. The TPS medium utilizes a “Lean Manufacturing” approach to seed testing through sprayer tables, food service trays and carts. The TPS medium has the potential to save time and increase uniformity of results among seed testing laboratories. A key advantage of this medium is the

uniformity in sand based tests allowed by use of dry sand and calibrated moisture application through sprayer tables.

Conclusion and Recommendation

The authors propose ISTA consider adopting TPS as a recommended medium method and recognize TPS as a germination medium option for: *Glycine max, Helianthus annuus, Phaseolus vulgaris,* and *Zea mays.*

Acknowledgments: The authors would like to thank Victor Vankus, National Tree Seed Laboratory for participating in this peer validation study. Thanks also go to Ronald Don for comments on design and editorial review.

References

Association of Official Seed Analysts. 2006. *Rules for Testing Seeds*. Association of Official Seed Analysts. Section 4.10, Table 3. 35p.

Don, Ronald. 2005. Personnel communication from ISTA Germination Committee Chair, September 2003

Fiedler, K A., T. J. Gutormson, K. A. Brix-Davis. 2007. Evaluation of Creped Cellulose Paper Covered with Sand as an ISTA Medium. Presented at ISTA Congress. 2007. Brazil.

Gregoire, Sylvain. 2007. A tool to assess repeatability and reproducibility in inter-laboratory tests according to ISO 5725-2 definitions. *Sylvain GREGOIRE – GEVES – 78285 Guyancourt cedex – France. ISTA, Statistical Tools for Seed Testing Interlaboratory Testing Using ISO 5725 (Online.)* URL: http://www.seedtest.org/en/content---1--1143.html

Gutormson T.J. and Burris, J.S. 1993. Comparison of Creped Cellulose Paper Products as Germination Substrates for Corn (Zea Mays L.) and Soybean [Glycine Max (L.) Merr] Seed. Journal of Seed Technology, Vol. 16, Num. 1.

International Standards Organisation ISO/IEC Guide 43-1 2007 "Proficiency Testing by Interlaboratory Comparison - Part 1: *Development and Operation of Proficiency Testing Schemes*".

Lund, R.E. 1991. A user's guide to MSUSTAT- an interactive statistical analysis package. Microcomputer version 5.02. Montana State University, Bozeman, Montana, USA

The International Seed Testing Association. 2007. *International Rules for Seed Testing.* Table 5A. 23p.

The International Seed Testing Association. 2007. *ISTA Handbook on Seedling Evaluation.* Third Edition.

Patin, A.L. and T.J. Gutormson. 2002. Creped Cellulose Paper Ply Study. The Seed Technologist Newsletter, Vol. 76, Num 1.

Seedling Evaluation Committee. 1992. *Seedling Evaluation Handbook*, Contribution No. 35. Association of Official Seed Analysts

Between-paper method for the germination test of *Brassica* spp. and *Sinapis alba* [Rules Proposal 2010 C.5.2.]

Z. Ripka

Central Agricultural Office, 1024 Budapest, Keleti K. u. 24., Hungary

Email: ripkaz@ommi.hu

Summary

Experiments were carried out to evaluate the germination of *Brassica* spp. and *Sinapis alba* in the between-paper (BP) substrate and compare this to germinations using the top-of-paper (TP) substrate. The experiments were carried out using three different seed samples of each species in three different ISTA accredited laboratories. The results of this peer validation test show that there is no significant difference between the germination test results carried out on TP and BP substrates and that BP can be added to the ISTA Rules as an alternative substrate to TP.

Introduction

In Hungary a large number of *Brassica* spp. and *Sinapis alba* samples are tested in a comparatively short period of time. In 2008 about 320 *Brassica* spp. and 460 *Sinapis alba* samples were tested in the period July till mid September (personal observation). Using the TP substrate prescribed in the ISTA Rules (ISTA 2008) is inefficient when testing large numbers of samples in terms of the space required in the germination facilities. For this reason trials have been carried out with BP substrate, which takes much less space and is a prescribed substrate for *Raphanus sativus* (also Brassicaceae species). The results obtained over several years indicated that there were no significant differences between the test results obtained by the Hungarian laboratory using BP compared to TP.

To test whether BP can be included in the ISTA Rules as a prescribed substrate for *Brassica* and *Sinapis* germinations a peer validation study was carried out. This study involved the comparative testing of three different germination capacity seed samples of *Brassica* spp. and *Sinapis alba* by three different accredited ISTA laboratories. The laboratories tested the samples using both BP and TP method at the two alternative temperatures prescribed in the rules, i.e. 20 and 20-30 °C.

This report gives the results of the comparative test carried out in support of the request to add BP as a prescribed substrate in the ISTA Rules for the germination of *Brassica* spp. and *Sinapis alba*.

Material and methods

Seeds

Details of the three seed samples of both *Brassica* spp. and *Sinapis alba* used in the comparative test are shown in Table 1. These samples were selected on the basis of preliminary testing at the Hungarian laboratory which showed that they had a range of different germination capacities.

Test method

Three ISTA accredited laboratories took part in the comparative test: France (FRDL0200), Italy (ITDL0300) and Hungary (HUDL0100). To test the effect of the substrate on the germination of the seeds independent of the temperature used the germination of each sample was tested using the four methods outlined in Table 2. For each test 4 replicates of 100 seeds were sown on the BP substrate and the participating labs used their own usual procedures for the TP substrate. Preliminary testing by the Hungarian Laboratory indicated that dormancy was not a problem provided a 2 day pre-chilling treatment was applied and laboratories were instructed give a 2 day pre-chill at 7 °C. KNO_3 was not added to the germination media by any of the

laboratories. The germination period (count days) and the evaluation of the seedlings were in accordance to the ISTA Rules.

Data analysis

The raw data for this study was copied to the ISTA Secretariat for archiving. All reported data were evaluated; no data were excluded from the statistical data analysis.

Data exploration using side-by-side boxplots

The number of normal seedlings of *Brassica* spp. and *Sinapis alba* were first explored using boxplots (see Figure 1 for an example and an explanation). Using side-by-side boxplots by terms Lab x Method (Figures 2 and 3), Lot x Method (Figures 4 and 5) and Temperature x Method (Figures 6 and 7) one could visualise that there appeared to be little difference in germination between BP and TP methods or between germinations at 20°C and 20-30°C. Calculation of the mean results for the BP (86.7%) and TP (87.4%) methods and tests conducted at 20°C (87.4%) and 20-30°C (86.7%) confirm this (Table 3). However, there appeared to be interactions between methods and laboratories, particularly for the *Brassica* spp. samples.

An indication of the interactions can be observed when the mean results of the individual laboratories for the four different germination methods (Figures 8 and 9). For the *Brassica* spp. samples, Labs 1 and 2 obtained the highest results with BP at 20°C whereas the highest result for Lab 3 was with TP at 20-30°C and whilst the lowest results for Labs 2 and 3 were obtained using TP at 20°C, the lowest result for Lab 1 was obtained using TP at 20-30°C. For the *Sinapis alba* samples differences between methods and laboratories were not as obvious.

Analysis of variance

In order to investigate the interactions further the data was subject to analysis of variance (Anova). A fixed-effect model was used with the objective to describe the data and not to make inferences based on the possible population of Labs. Detailed Anova tables are given in Tables 4. and Table 5. From these we can see that for the *Brassica* spp. there were significant interactions for Lab x Method, Temperature x Method, Lab x Seed Sample, Lab x Temperature x Method and Laboratory x Seed Sample x Temperature. For *Sinapis alba* the only significant interaction was with Seed Sample x Method. The Anova tables also show that there was no significant difference between the TP and BP methods for either species.

Repeatability and reproducibility

In order to estimate repeatability and reproducibility of the test results for *Brassica* spp. and *Sinapis alba* the following mixed-effect model was fitted to the data:

Fixed effects: Method Lot Temperature

Method x Lot - Method x Temperature - Lot x Temperature

Method x Lot x Temperature

Random effects: Lab

Method x Lab x Lot – Method x Lab x Temperature - Lab x Lot x Temperature

Method x Lab x Lot x Temperature

Residuals

An estimate of the repeatability is then given by the residual variance component estimate and an estimate of the reproducibility by the repeatability estimate plus the sum of the variance component estimates associated to the random terms. Variance components are given in Table 6.

The repeatability estimates are comparable for species, 14.23 for *Brassica* spp. and 12.12 for *Sinapis alba* (Tables 7 and 8). If we compute the binomial variance associated to a germination of 85% and 100 seeds, we find: (85 x 15)/100 = 12.75. We can then say that for the *Brassica* spp. and *Sinapis alba* comparative tests, the repeatability (intra-laboratory variability) is purely associated to the sampling variation.

The reproducibility estimates are very different: 50.5 for *Brassica* spp., 13.84 for *Sinapsis alba*. However, it should be noted that the interaction with Method variance components is relatively small for *Brassica* spp. indicating that the two methods of TP and BP will provide similar results.

Comments of Participants

According to the comments of the other two participating laboratories:

on *Sinapis alba*: "final count could be done after 3 days in BP and after 4 days on TP".

"this experience showed us that the evaluation of seedlings in rolled paper is easier that in other substrates, as the whole seedling is soon available."

The above comments are back up observations of the Hungarian laboratory that *Brassica* spp. and *Sinapis alba* seedlings grow nicely between paper and develop all essential parts for seedling evaluation.

Conclusions and recommendations

The statistical evaluation of the germination test results of this peer validation study show for both *Brassica* spp. and *Sinapis alba*, there is no significant difference between TP and BP germination method. In addition the repeatability of the test method is purely associated with sampling variation and the interaction with method variance components is relatively small even for *Brassica* spp. indicating that the two methods of TP and BP will provide similar results. It is therefore recommended that BP is added to the ISTA Rules as an alternative prescribed germination media for *Brassica* spp. and *Sinapis alba*.

References

ISTA Rules 2008 Chapter 5.

No publications or reports of studies relating to a comparison of the effects of different substrates on the germination of *Brassica* and *Sinapis* seeds were found.

Acknowledgements

I would like to thank to the participating laboratories to carry out the tests and providing the data for this validation report.

I would like to acknowledge the help I got from Jean-Louis Laffont, who made the detailed statistical evaluation of this study with kind professional help.

I also owe many thanks to Ronnie Don, chair of the ISTA Germination Committee, who always gave his kind support and advice during the whole validation procedure.

Tables and figures

Table 1: List of the seed samples used for the peer validation study in which different germination substrates were compared.

Sample	Seed lot	Variety	Purity %	Germination %
	Brassica napus			
1	H-7-101/283*	GK Gabriella	100,0	94
2	H-7-101/284	GK Gabriella	99,8	88
3	H-7-184/73	Heros	91,6	77
	Sinapis alba			
1	H-7-51/227	Bea	100,0	97
2	H-7-51/98	Carwella	99,7	89
3	H-7-5/105	Twist	99,9	82

* seed lot treated with Vitavax 2000 (carboxin + thiram) and Sepiret (colour)

Table 2: The different germination methods used in the peer validation study.

Method	Substrate	Temperature (°C)	Prechill (days/°C)
1	TP	20	2/7
2	TP	20-30	2/7
3	BP	20	2/7
4	BP	20-30	2/7

Table 3: The mean germinations of three *Brassica* spp. and three *Sinapis alba* seed samples germinated in 3 ISTA accredited laboratories using BP and TP and at 20°C and 20-30°C

Species	TP	BP	20°C	20 -30°C
Brassica spp.	83.2	84.2	83.3	84.1
Sinapis alba	91.1	90.6	91.4	89.4
Brassica spp. and Sinapis alba	87.2	87.4	87.4	86.8

Table 4: Anova table of *Brassica* spp. (significant effects at the 5% level are indicated in red):

Source	DF	Type I SS	Mean Square	F Value	Pr > F
METHOD	1	36.00	36.00	2.53	0.1147
LAB	2	2267.17	1133.58	79.65	<.0001
LOT	2	1910.29	955.15	67.11	<.0001
TEMP	1	18.78	18.78	1.32	0.2532
LAB*METHOD	2	219.50	109.75	7.71	0.0007
LOT*METHOD	2	65.63	32.81	2.31	0.1046
TEMP*METHOD	1	56.25	56.25	3.95	0.0493
LAB*LOT	4	704.67	176.17	12.38	<.0001
LAB*TEMP	2	48.39	24.19	1.70	0.1875
LOT*TEMP	2	45.01	22.51	1.58	0.2104
LAB*LOT*METHOD	4	31.25	7.81	0.55	0.7002
LAB*TEMP*METHOD	2	145.50	72.75	5.11	0.0076
LOT*TEMP*METHOD	2	69.79	34.90	2.45	0.0909
LAB*LOT*TEMP	4	256.44	64.11	4.50	0.0021
LAB*LOT*TEMP*METHOD	4	114.08	28.52	2.00	0.0990

Table 5: Anova table of *Sinapis alba* (significant effects at the 5% level are indicated in red):

Source	DF	Type I SS	Mean Square	F Value	Pr > F
METHOD	1	0.03	03	0.00	0.9619
LAB	2	112.06	56.03	4.62	0.0118
LOT	2	2154.89	1077.44	88.93	<.0001
TEMP	1	235.11	235.11	19.41	<.0001
LAB*METHOD	2	43.06	21.53	1.78	0.1741
LOT*METHOD	2	83.39	41.69	3.44	0.0356
TEMP*METHOD	1	1.78	1.78	0.15	0.7024
LAB*LOT	4	66.94	16.74	1.38	0.2452
LAB*TEMP	2	19.39	9.69	0.80	0.4519
LOT*TEMP	2	57.06	28.53	2.35	0.0998
LAB*LOT*METHOD	4	73.53	18.38	1.52	0.2023
LAB*TEMP*METHOD	2	17.06	8.53	0.70	0.4969
LOT*TEMP*METHOD	2	45.72	22.86	1.89	0.1565
LAB*LOT*TEMP	4	65.44	16.36	1.35	0.2561
LAB*LOT*TEMP*METHOD	4	58.69	14.67	1.21	0.3104

Table 6: Variance components of *Brassica* spp. and *Sinapis alba* germination test results

	Brassica spp:	Sinapsis alba:
LAB	18.05	0.71
LAB*METHOD	2.45	0.18
LAB*LOT	7.73	0.00
LAB*TEMP	0.00	0.00
LAB*LOT*METHOD	0.00	0.53
LAB*TEMP*METHOD	2.66	0.00
LAB*LOT*TEMP	4.19	0.08
LAB*LOT*TEMP*METHOD	1.17	0.23
Residual	14.23	12.12

Table 7: Tests of the fixed effects (inferences for a population of laboratories) for *Brassica* spp:

Effect	Num DF	Den DF	F Value	Pr > F
METHOD	1	2	0.33	0.6246
LOT	2	4	5.42	0.0726
TEMP	1	2	0.22	0.6836
LOT*METHOD	2	4	1.73	0.2869
TEMP*METHOD	1	2	1.11	0.4033
LOT*TEMP	2	4	0.43	0.6780
LOT*TEMP*METHOD	2	4	1.84	0.2707

Table 8: Tests of the fixed effects (inferences for a population of laboratories) for *Sinapis alba*

Effect	Num DF	Den DF	F Value	Pr > F
METHOD	1	2	0	0.9746
LOT	2	4	60.13	0.0010
TEMP	1	2	17.19	0.0535
LOT*METHOD	2	4	2.41	0.2054
TEMP*METHOD	1	2	0.14	0.7474
LOT*TEMP	2	4	2.09	0.2396
LOT*TEMP*METHOD	2	4	1.75	0.2840

Explanation of boxplot diagrams

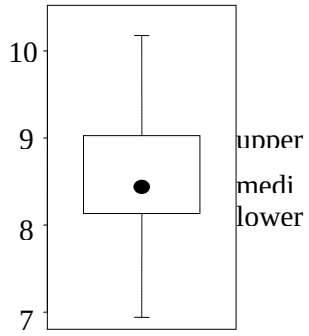


Figure 1: An example of a boxplot.

The boxplot is used to portray the distribution of a set of data. The box is limited by the quartiles (25% of the observations fall below the lower quartile and 25% fall above the upper quartile). The median (50% of the observations fall below the median, 50% fall above) is portrayed by a large point within the rectangle. From each end of the box, a dashed line extends out to the farthest observation that is not beyond the cutoff value (the position of these cutoff values depends on the length of the box). Any observation that lies below the lower cutoff or above the upper cutoff appears as a separate point. The median shows the location of the distribution and the spread of the central 50% of the data is seen as the length of the box. The individual points identify potential outliers.

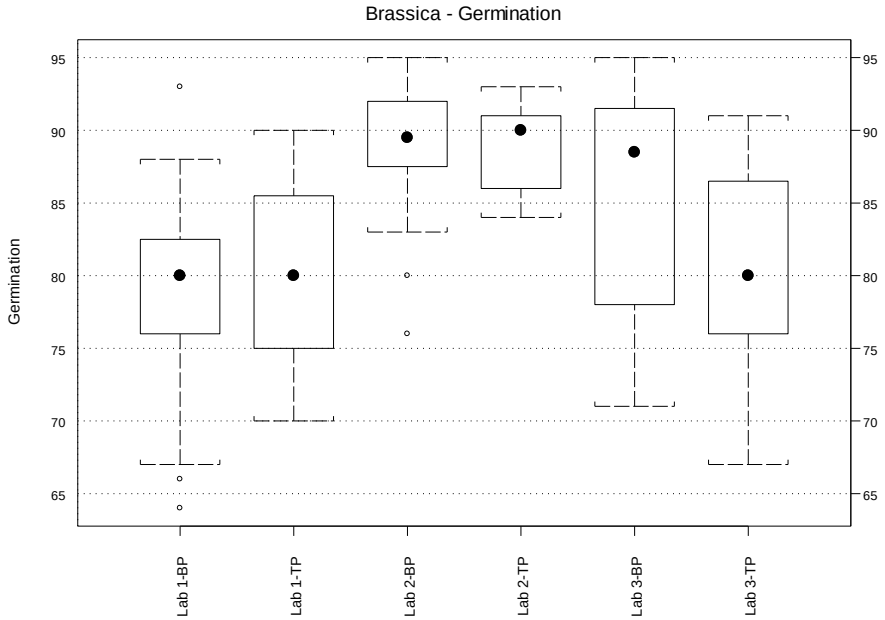


Figure 2: Side-by-side boxplots by Lab x Method for *Brassica* spp.

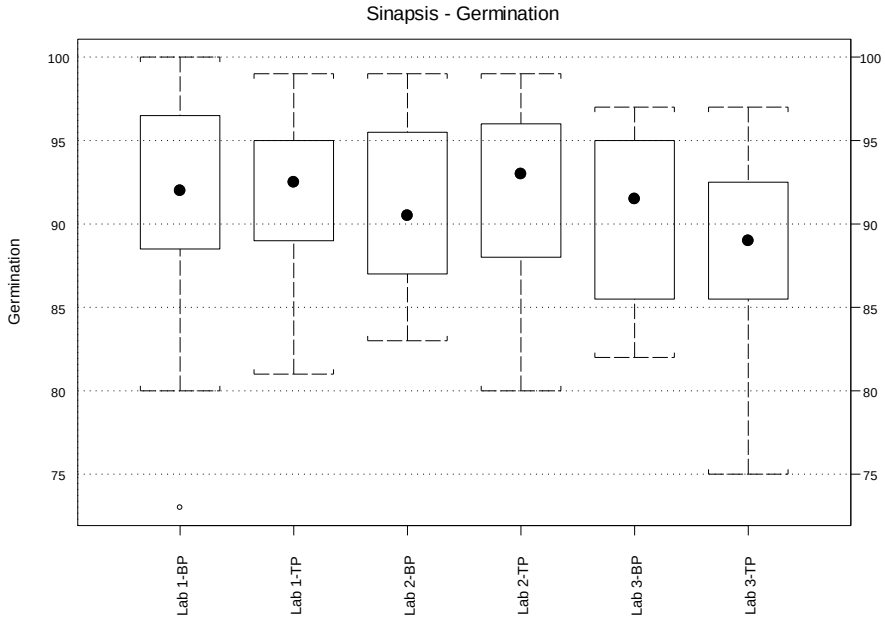


Figure 3: Side-by-side boxplots by Lab x Method for *Sinapis alba*

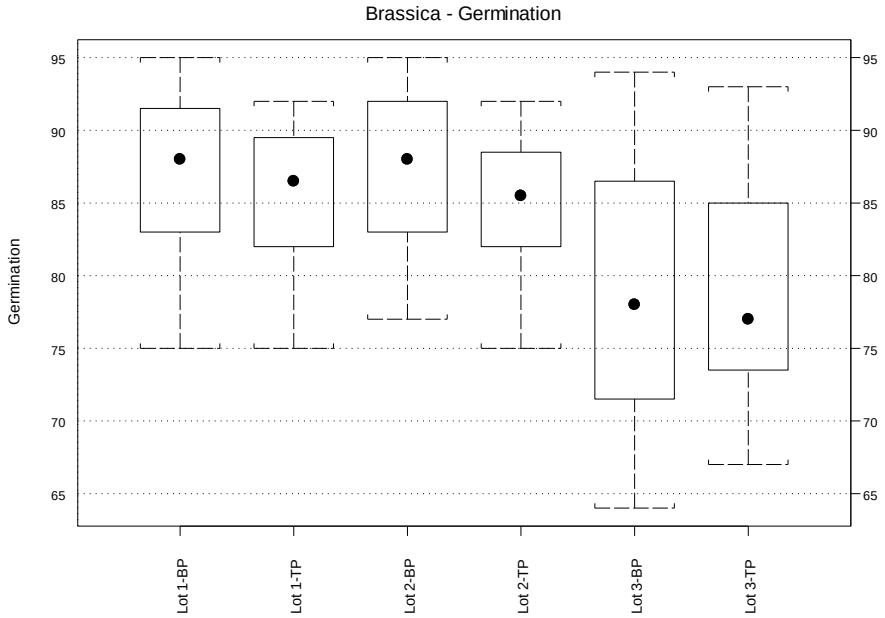


Figure 4: Side-by-side boxplots by Lot x Method for *Brassica* spp.

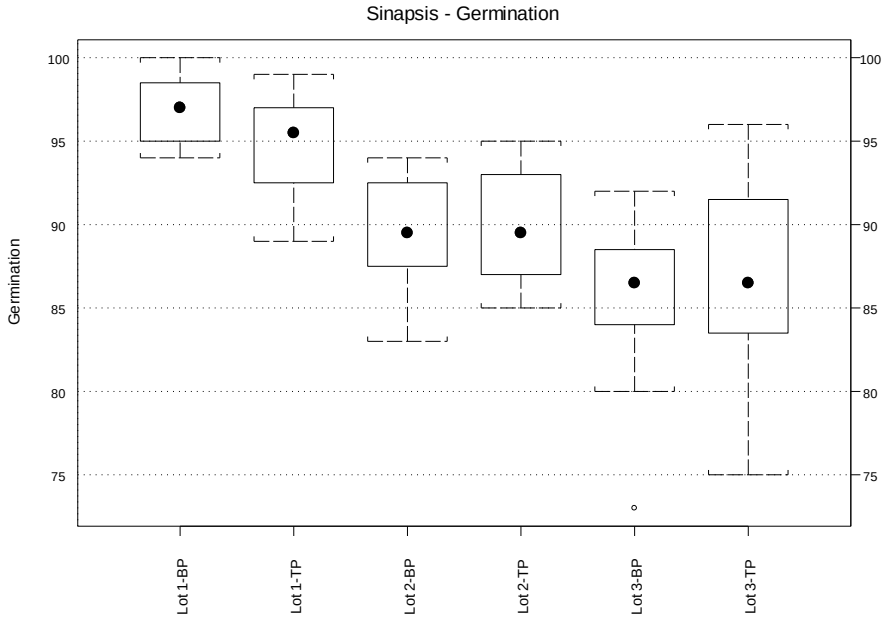


Figure 5: Side-by-side boxplots by Lot x Method for *Sinapis alba*

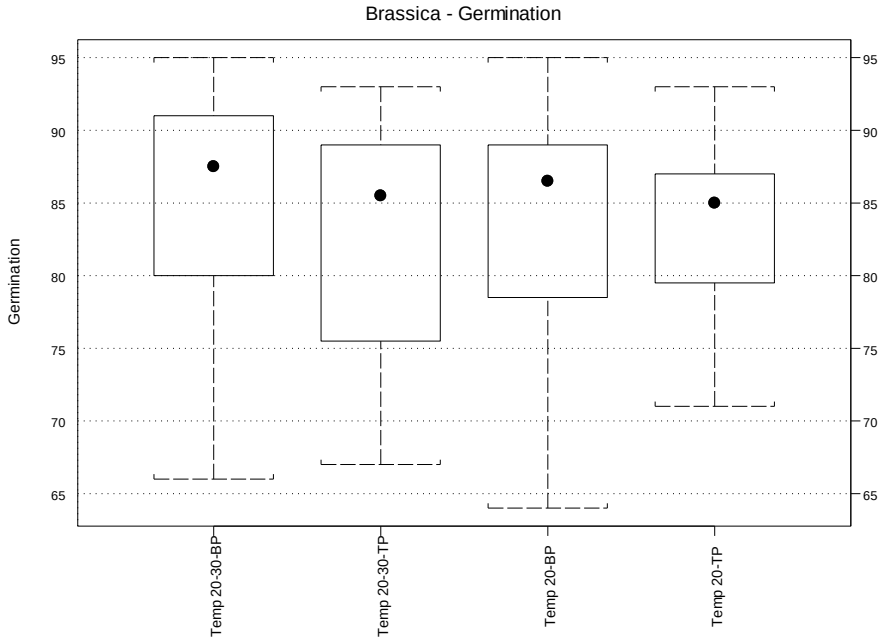


Figure 6: Side-by-side boxplots by Temperature x Method for *Brassica* spp.

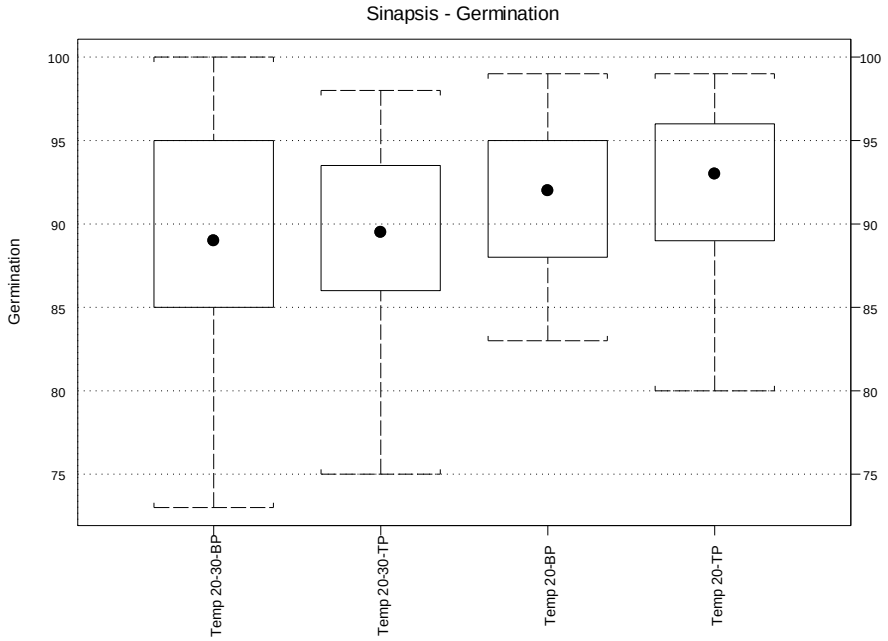


Figure 7: Side-by-side boxplots by Temperature x Method for *Sinapis alba*

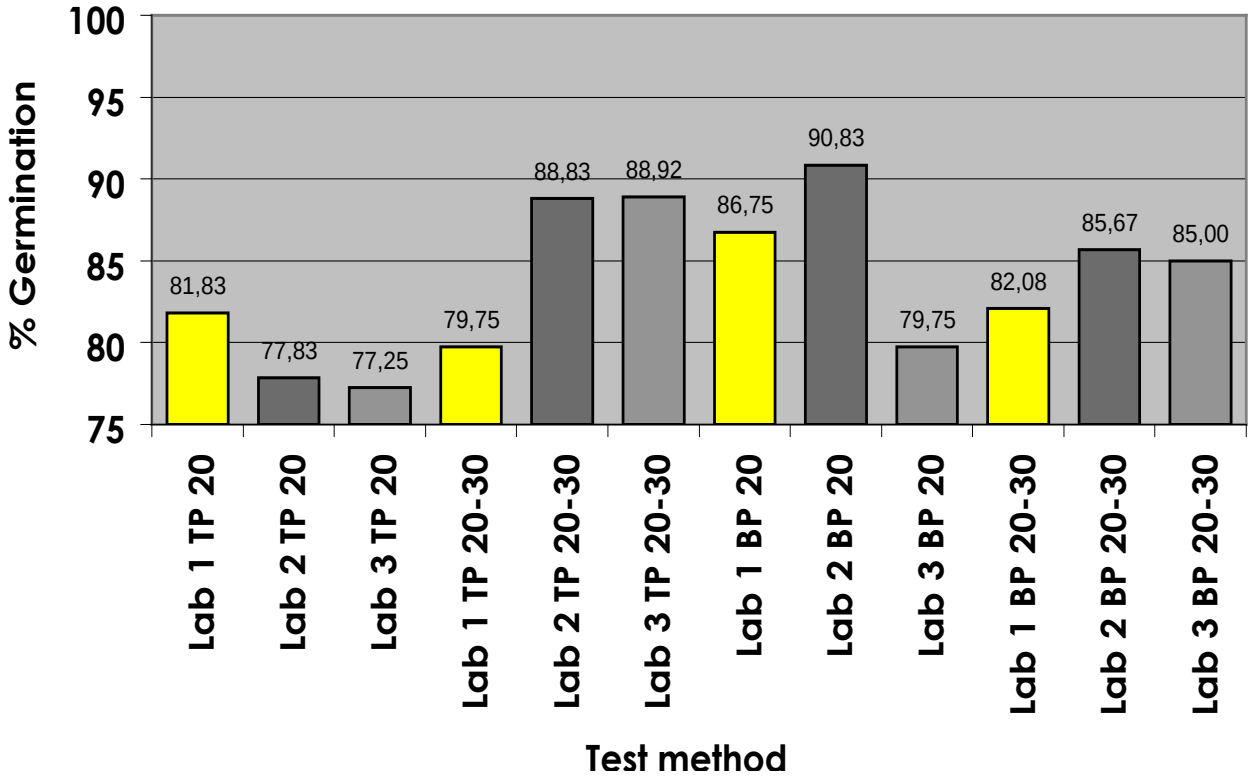


Figure 8: Detailed results of the three labs and the four test methods of the *Brassica* spp. samples.

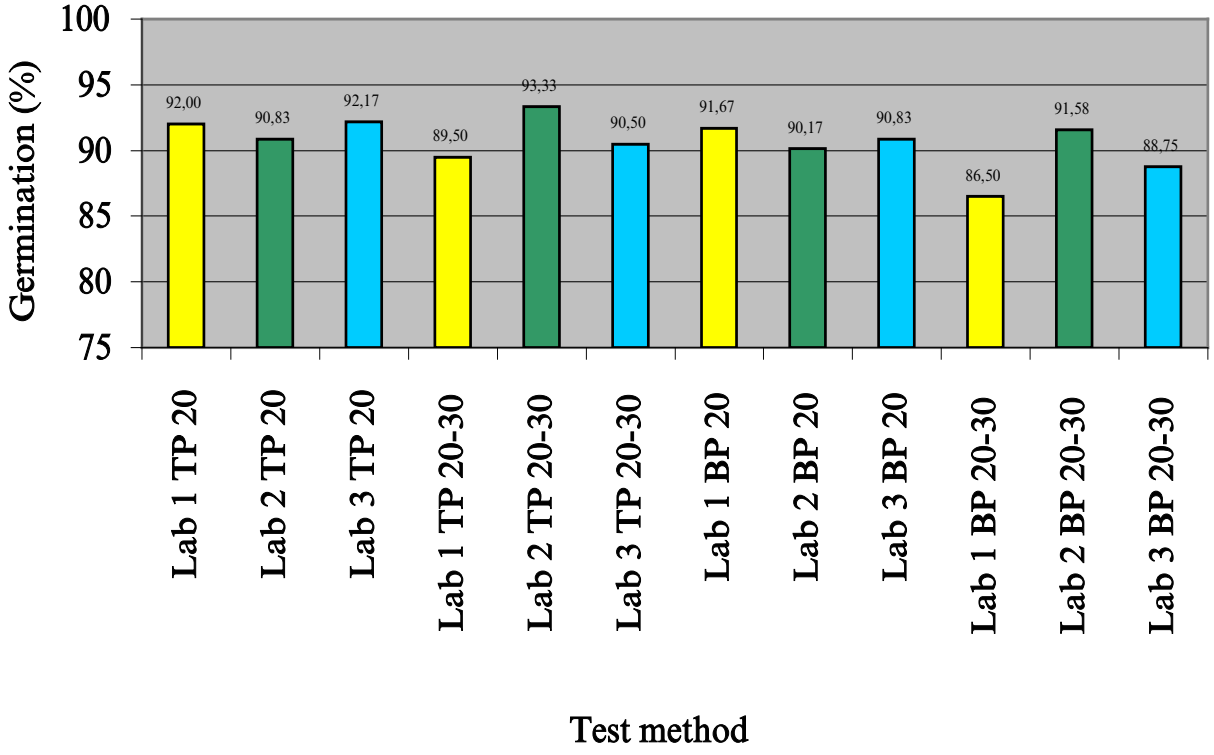


Figure 9: Detailed results of the three labs and the four test methods of the *Sinapis alba* samples.

Use of Organic Growing Media as primary substrate for the germination of *Vicia faba* L. seeds [Rules Proposal 2010 C.5.3.]

Ducournau S., Garreau P., Mallet L., Léchappé J.

GEVES-SNES, Beaucouzé, France

Summary

Experiments were carried out to evaluate the germination of *Vicia faba* L. in organic growing media compared to sand and between paper substrates. The experiments were carried out using three different seed samples in seven different ISTA accredited laboratories. The results of this comparative test show that repeatability and reproducibility is higher with organic growing media than with the other media sand and between paper. Results of normal seedlings also increased with the use of organic growing media compared to the two other substrates. Organic growing media can be proposed as an additional media for the germination of *Vicia faba* L. seeds in ISTA Rules.

Introduction

Following the inclusion of the definition of the organic growing media in the ISTA Rules in 2007, the Germination Committee of ISTA suggested to extend the use of organic growing media as primary substrate when necessary.

The French Seed Testing Station work on *Vicia faba* L. seeds and demonstrated in a national study in 2000 that normal germination was lower with sand substrate than with organic growing media (3% difference in average on 40 samples). This difference was explained by more abnormal seedlings and in particular fractured seedlings that were generated by a more rapid imbibition in sand.

Based on these first results, it has been decided to organise a validation study in order to see if organic growing media can be introduced as another primary substrate for the germination of this species.

Material and methods

A comparative test has been set up in order to compare the results obtained with Organic Growing Media for the germination of some samples of *Vicia faba* L., to the other media already allowed for this species (Between Paper and Sand).

Seed material

Three samples of *Vicia faba* L. seeds were used in this study. Samples with various levels of germination quality (between 80% and 95% germination) have been selected.

Participants

Samples have been sent to 7 accredited laboratories in France (FRDL0200), Netherlands (NLDL0300), Scotland (GBDL0400), Germany (DEDL1800), USA (USML0600), Norway (NODL0100) and Israel (ILDL0100).

Germination methods

Each participant has been asked to test the samples with three different germination methods based on ISTA germination conditions:

BP ; 20°C

Sand ; 20°C

Organic Growing Media ; 20°C

ISTA Rules recommends first count after 4 days and final count after 14 days.

For tests carried out in Sand and Organic Growing Media, first count has been done between 6 and 7 days as seedlings were not enough developed after 4 days.

Statistical analysis

Repeatability and reproducibility have been analysed with the statistical tool developed by S. Grégoire according to ISO 5725-2.

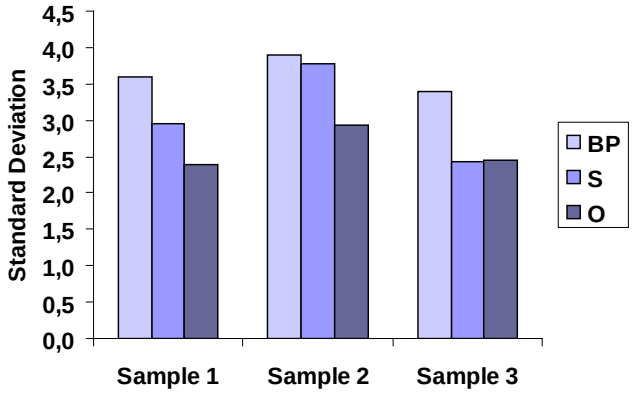
Effect of the different factors (laboratory, sample, growing media) has been analysed by variance analysis with Statgraphics.

Results

Repeatability of the results

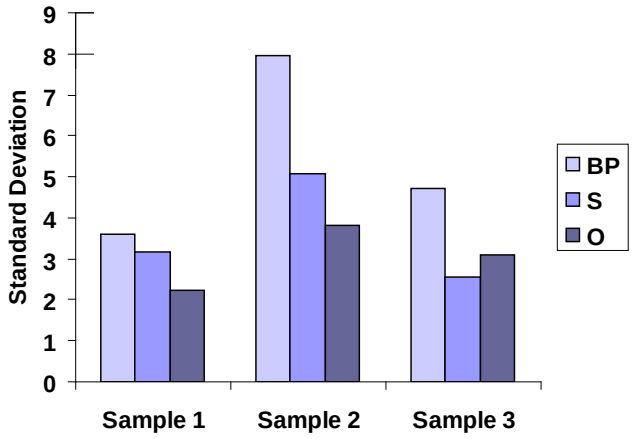
Results of repeatability are calculated from the statistical tool developed by S. Grégoire based on ISO 5725-2. When the standard deviation is low, the repeatability of the method is high.

Results presented in figure 1 and figure 2 show that repeatability of the results of normal seedlings is increased with the use of Organic Growing Media compared to the two other media sand and between paper. Difference in repeatability is lower between Organic Growing Media and Sand than between Organic Growing Media and Between Paper. The tendency is similar for all the 3 samples tested.



Reproducibility of the results

Results of reproducibility for normal seedlings are reported in the same way as for the results of repeatability. When the standard deviation is low, reproducibility of the method is high.


Standard Deviation
9
8
7
6
5
4
3
2
1
0
Sample 1
Sample 2
Sample 3
BP
S
O

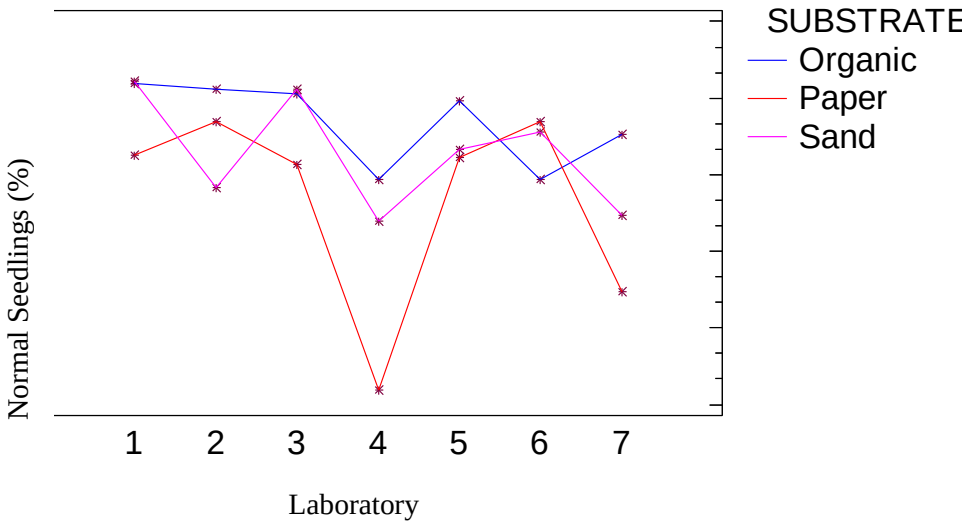


Figure 6: Normal seedling % depending on the laboratory for all the samples tested and for each the growing media.

Figure 6 presents the results obtained from the interaction between laboratories and growing media. It shows that Laboratories 4 and 7 obtain poor results with BP Media.

As a consequence of this, effect of growing media will be analysed with and without the results of the 2 laboratories 4 and 7.

Effect of the samples

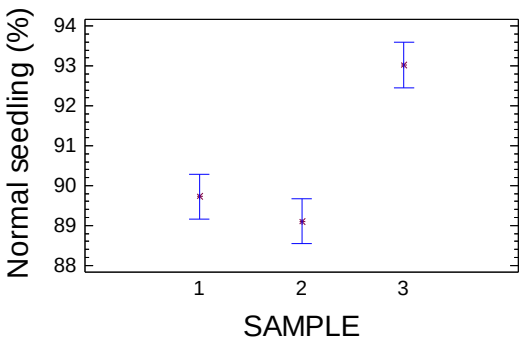


Figure 7: Normal seedling % depending on the samples tested for all the laboratories and all the substrates

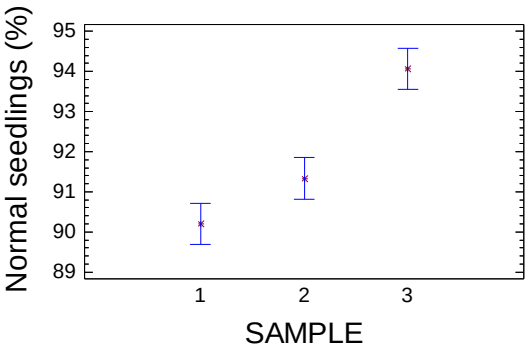


Figure 8: Normal seedling % depending on the samples tested for all the substrates and all the laboratories except laboratories 4 and 7

Figures 7 and 8 present the results obtained on the 3 samples of *Vicia faba* L. When all the laboratories are taken into account, results obtained on samples 1 and 2 are not statistically different. When the results of laboratories 4 and 7 are extracted, the 3 samples ranged from sample 1 to sample 3 according to their increased normal germination %.

Effect of the growing media

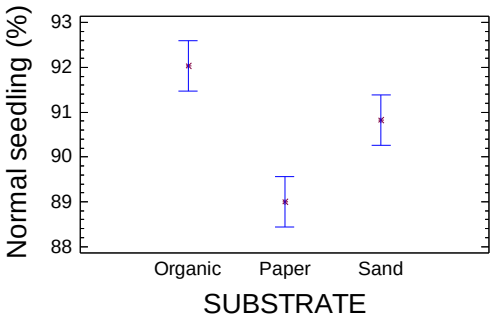


Figure 9: Normal seedling % depending on the growing media used, for all the laboratories and all the samples tested

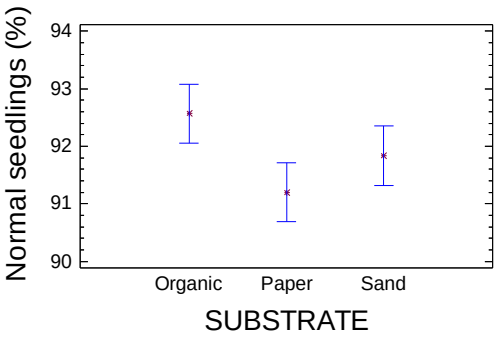


Figure 10: Normal seedling % depending on the growing media used, for all the laboratories except laboratories 4 and 7 and for all the samples tested

The result presented in figures 9 and 10 indicate that normal seedling % is higher with the use of organic growing media than with the use of sand or between paper. The difference between the results when using sand and paper is significant when all the laboratories are taking into account. The difference is not significant when the laboratories 4 and 7 are excluded.

Conclusion

Results obtained in this comparative test indicate that organic growing media can be proposed as an additional primary substrate for the germination of *Vicia faba* L. seeds.

Results of normal seedlings are more repeatable and more reproducible with organic growing media than with the other media sand and between paper. Results of normal seedlings are also increased with the use of organic growing media compared to the two other substrates.

Annexes

Annexe 1: Mean values of repeatability and reproducibility of the results

	Repeatability (sr2)			Reproducibility (sR2)		
	Between Paper	Sand	Organic Growing Media	Between Paper	Sand	Organic Growing Media
Sample 1	3,59	2,96	2,39	3,60	3,18	2,24
Sample 2	3,91	3,78	2,94	7,94	5,09	3,82
Sample 3	3,40	2,44	2,45	4,72	2,54	3,09

Annexe 2: Anova table (significant effects at the 5% level are indicated in blue)

Source	DF	Mean square	Sum of squares	F value	Pr>F
Laboratory	6	183,49	1100,93	18,55	0,0000
Sample	2	372,37	744,74	37,65	0,0000
Growing media	2	196,11	392,21	19,83	0,0000
Laboratory x Sample	12	35,54	426,43	3,59	0,0001
Laboratory x Growing media	12	48,73	584,79	4,93	0,0000
Sample x Growing media	4	18,98	75,90	1,92	0,1090
Laboratory x Sample x Growing media	24	15,12	362,93	1,53	0,0622

Proposal for a new method for the detection of Cucumber Green Mottle Mosaic Virus (CGMMV), Melon Necrotic Spot Virus (MNSV) and Squash Mosaic Virus (SqMV) in Cucurbits using DAS-ELISA [Rules Proposal 2010 C.7.1.]

H.M.S. Koenraadt

Naktuinbouw, P.O. Box 40, 2370 AA Roelofarendsveen, the Netherlands; Email: h.koenraadt@naktuinbouw.nl

P.M. Remeeus

Parkzichtlaan 368, 3544 MN Utrecht, the Netherlands; Email: bert.petra@freeler.nl

Summary

There is currently no internationally accepted protocol for the detection of cucumber green mottle mosaic virus (CGMMV), melon necrotic spot virus (MNSV) and squash mosaic virus (SqMV) in seeds. An international comparative test was organised to evaluate the use of DAS-ELISA for the detection of CGMMV, MNSV and SqMV. In this test flour of virus-infested seeds was used. The 9 laboratories each received 100 samples of healthy or virus-infested cucurbit flour, a description of the protocol, and critical ELISA components such as microtiter plates, antisera and purified virus. Analysis of the data revealed that most laboratories were able to detect CGMMV, MNSV and SqMV in the medium and heavily infested samples with the prescribed DAS-ELISA protocol. Some laboratories were unable to detect CGMMV, MNSV and SqMV in samples with low virus titers. It should be noted that these low virus titre samples were prepared by considerably diluting the infested flour with healthy flour and were therefore perhaps not representative of naturally contaminated samples. It is recommended that testing for the presence of CGMMV, MNSV and SqMV commence by grinding a sub-sample of 100 seeds to a fine flour. The virus should then be extracted from the flour with a buffer solution and each extract tested individually using DAS-ELISA in separate microtiter plates. Testing 2,000 seeds (20 sub-samples of 100 seeds each) per seed lot will give a 95% probability that a 0.15% infestation of CGMMV or MNSV or SqMV is detected.

Introduction

CGMMV

Cucumber green mottle mosaic virus (CGMMV) is a pathogen of several cucurbits species such as cucumber (*Cucumis sativus*), watermelon (*Citrullus lanatus*), bottle gourd (*Lagenaria siceraria*) and bitter gourd (*Momordica charantia*). CGMMV belongs to the genus *Tobamovirus* (Hollings *et al.*, 1975). Tobamoviruses have rod-shaped virions that contain a single-strand RNA genome. CGMMV is a very stable virus and remains infectious for a long time in contaminated soil, recirculation water (Dorst, 1988; Büttner *et al.*, 1995) and debris. Contaminated tools and humans can act as inoculum vectors since CGMMV is readily spread by mechanical transmission. CGMMV-contaminated seeds are a potential primary source of inoculum. Seed-borne CGMMV in cucumber is mostly found as an external contamination of seeds but can also be found in embryos (Hollings *et al.*, 1975). Several authors report seed transmission varies from 3-17% (Hollings *et al.*, 1975; Faris-Mukhayyish and Makkouk, 1983). CGMMV is widely spread in Europe and Asia. Seed treatment can be used to eliminate infectious tobamoviruses such as CGMMV (Hollings *et al.*, 1975; Macias, 2000). Dry heat treatment is widely used to disinfect CGMMV-contaminated seeds.

MNSV

Melon necrotic spot virus (MNSV) is a pathogen of cucurbit species such as melon (*Cucumis melo*), cucumber (*Cucumis sativus*) and watermelon (*Citrullus lanatus*). MNSV is a member of the genus *Carmovirus*. Carmoviruses have isometric virions that contain a single-strand RNA genome. MNSV can be

mechanically transmitted. Seed transmission in melon can be as high as 40% when externally contaminated seeds are sown (Campbell *et al.*, 1996). For seed transmission, the presence of the fungal vector *Olpidium bornovanus* is very important but even without the vector seed transmission can occasionally occur. Vector assisted seed transmission of MNSV could be reduced by acid treatment of seeds but the efficacy varies between seed lots. In one case transmission was reduced from 50% to 0% after acid treatment (Campbell *et al.*, 1996).

SqMV

Squash mosaic virus (SqMV) is a pathogen of several cucurbit species. Melon (*Cucumis melo*) is the principal host but the virus may also infect other cultivated cucurbits. SqMV belongs to the genus *Comovirus*. SqMV has isometric particles with a bipartite, single-strand RNA genome. The spread of SqMV is possible by mechanical transmission. It is transmitted by at least 14 different species of chewing insects, mostly beetles. SqMV is a seed-borne virus and SqMV contaminated seeds could act as a primary source of inoculum. Seed transmission rates usually range from 0.1 to 10% (Alvarez and Campbell, 1978). SqMV can be present in the seed coat, the papery layer and the embryo. Only the embryo infection leads to transmission of SqMV from seed to seedling (Alvarez and Campbell, 1978; Nolan and Campbell, 1984).

Detection of CGMMV, MNSV and SqMV in seeds

As CGMMV, MNSV and SqMV are seed-transmissible viruses their detection in seeds of cucurbit species is an important tool for disease control. Procedures have been described for the detection of CGMMV (Faris-Mukhayyish and Makkouk, 1983; Kawai *et al.*, 1985), MNSV (Matsuo, 1993) and SqMV (Akanda *et al.*, 1991; Faris-Mukhayyish and Makkouk, 1983; Franken *et al.*, 1990; Hamilton and Nichols, 1978; Kumari and Makkouk, 1993; Nolan and Campbell, 1984; Purcifull *et al.*, 1981).

Enzyme-linked immunosorbent assay (ELISA) is widely used for the detection of plant viruses (Clark and Adams, 1977). Although ELISA is relatively robust and has been applied in many laboratories for a long time, only a few internationally accepted ELISA protocols are used in seed testing. Several laboratories test seeds for the presence of CGMMV, MNSV and SqMV by testing flour of 2,000 seeds in 20 sub-samples of 100 seeds. A sub-sample size of 100 seeds is a safe and conservative choice since one SqMV-contaminated seed was consistently detected in 159 healthy seeds (Franken *et al.*, 1990) and in 400 healthy seeds (Nolan and Campbell, 1984). One CGMMV-infested seed was detected in 800 healthy seeds (Kawai *et al.*, 1985). By testing 2,000 seeds per seed lot there is a 95% probability that a 0.15% infestation will be detected. It is known that for many seed-borne pathogens, including SqMV (Nolan and Campbell, 1984), transmission is highly variable since not every infested seed will give an infected seedling. Therefore, the proposed test using 2,000 seeds with a detection threshold for an infestation of 0.15% will lead to a smaller chance of transmission in the field and gives additional security (Franken *et al.*, 1990)

Detection of cucurbit viruses after a seed treatment

Seed treatments to eradicate infectious viruses can be applied to reduce the chance of seed transmission of CGMMV and MNSV. To our knowledge, eradication of SqMV by seed treatment is impossible since SqMV is very stable and often located in the embryo. It is important to note that DAS-ELISA of ground seeds cannot discriminate between infectious and non-infectious virions, and therefore the efficacy of a seed treatment cannot be evaluated using this method. Alternative assays such as a grow-out followed by evaluation of seedlings for infection or DAS-ELISA of seedlings should be used to detect any transmitted virus.

The use of reference materials in comparative testing

Very few comparative tests for the detection of seed-borne viruses were organized in the past. An important bottleneck was the lack of appropriately infected samples and/or stable reference material. When using naturally contaminated seed lots with a low incidence of infested seeds and a varying amount of virus per infested seed, it was very difficult to provide individual laboratories with identical samples. The laboratory at Naktuinbouw, the Netherlands Inspection Service for Horticulture, has attempted to overcome this problem by using finely ground flour of seeds with variable loads of viral contamination.

Flour of healthy and virus-contaminated seed lots have been used as negative and positive controls for more than five years. Researchers at Naktuinbouw found a relatively even distribution of virus in finely ground flour. Also, the amount of ELISA-detectable virus was relatively stable in time irrespective of the storage temperature. These findings suggested that flour could be a reliable reference material and has the advantage that different laboratories can be provided with identical samples. Predictable distributions of the virus in the flour and its stability has been confirmed in several national comparative tests for the detection of cucurbit viruses CGMMV, MNSV and SqMV and pea seed-borne mosaic virus (PSbMV) and pea early-browning virus (PEBV) in the framework of Naktuinbouw's activity in accrediting laboratories in the Netherlands (H. Koenraadt, pers comm).

The use of reference antisera in comparative testing

Antisera against viruses are available from different suppliers and it is known that the quality of antisera may vary as demonstrated for PepMV (Bert Woudt, pers comm). For detecting viruses in high-quality seed lots, the specificity and affinity/avidity of antibodies is critical since relatively low titres of virus have to be detected in contrast to detecting the virus in symptomatic leaves. Antiserum variation, in addition to inter-laboratory variation, and the lack of reference material, often made it very difficult to judge test results of comparative tests hampering the development of internationally accepted standard methods. To overcome the problem of antisera of differing quality affecting the results of the comparative test, antisera for SqMV, MNSV and CGMMV from one batch and the same supplier were sent to participating laboratories.

The use of microtiter plates in comparative testing

In addition to the quality of reference material and antiserum, the quality of microtiter plates (Greiner microlon) can also lead to confounding results making the comparative test difficult to evaluate. Therefore, microtiter plates were also sent to participating laboratories.

Comparative test for SqMV, MNSV and CGMMV

The aim of the comparative test was to evaluate whether laboratories were able to detect SqMV, MNSV and CGMMV in contaminated samples when following a prescribed DAS-ELISA protocol. The final objective of the project was to obtain consensus on a reliable and internationally accepted ELISA protocol for the detection of SqMV, MNSV and CGMMV in seed lots.

Materials and methods

Seeds

Cucurbit seeds were obtained from the Naktuinbouw collection of naturally contaminated seed lots. The seed lots were stored at 4 ºC and low humidity. The collection numbers were: ZZB9 (healthy control), ZZB67 (SqMV-contaminated), ZZB148 (SqMV-contaminated), ZZB204 (SqMV-contaminated), ZZB144 (MNSV-contaminated), ZZB145 (MNSV-contaminated) and ZZB235 (CGMMV-contaminated).

Design of the comparative test

Several seed lots of cucurbits were tested for the presence of CGMMV, MNSV and SqMV using ELISA. Different ratios of the healthy and CGMMV-, MNSV- and SqMV-contaminated seed lots were prepared with the objective to obtain one CGMMV-, MNSV- or SqMV-contaminated seed per sample of 100 seeds. The samples were ground in a grinder (Retsch-Grindomix GM200) at 10,000 rpm for 20 seconds to obtain a fine flour. The flour was transferred to plastic bags. The grinder was cleaned thoroughly between each sample using a fine brush and a vacuum cleaner to prevent cross-contamination. A total of 100 samples were selected by the organiser as recommended by the ISTA Statistics Committee with the objective of including healthy samples and samples to give low to medium A_{405} values in ELISA. To obtain samples with low A_{405} values, several contaminated flour samples were diluted with healthy flour. Only a few strong positive samples were selected since these were considered less discriminative for assessing the ability of a laboratory to use the ELISA protocol.

From each ground sample, 0.45-0.55 gram of flour was transferred to a labelled tube. Ten labelled tubes were prepared per sample to obtain enough material for all participating laboratories and an additional pre-test. In the pre-test one set of samples was analysed at Naktuinbouw using the method described prior to the samples being sent to the laboratories participating in the comparative test. In the interim period the samples were stored at 4 ºC in air-sealed plastic bags. After the results were confirmed in the pre-test, the samples were sent by express mail to nine laboratories: Agdia (Elkhart, IN, USA); SNES-GEVES (Beaucouze Cedex, France); Clause Tezier (Valence, France); Naktuinbouw (Roelofarendsveen, the Netherlands); Nunhems NL (Haelen, the Netherlands); Sakata Seeds (Chiba-ken, Japan); National Center for Seeds and Seedlings (Tsukuba, Japan); Universidad Politécnica de Valencia (Valencia, Spain) and Microlab (Rehovot, Israel). Purified CGMMV, MNSV and SqMV (Plant Research International, Wageningen, the Netherlands) were included in each package as positive controls along with the antisera and microtiter plates.

Test method

Antisera (coating and conjugates) for detecting CGMMV, MNSV and SqMV were purchased from PrimeDiagnostics, Wageningen, Netherlands. The CGMMV, MNSV and SqMV polyclonal antisera were raised in rabbits against purified virus of isolates IVT, Cu-18 and M respectively. The test organiser supplied Microtiter plates. Extracts from each sample were tested in two different plates for each virus to minimize microtiter plate effects.

ELISA buffers

Coating buffer 1.59 g Na_2CO_3 plus 2.93 g $NaHCO_3$ were dissolved in 1 litre of de-ionised/distilled water and the solution was checked to determine that the pH was 9.6 and adjusted if necessary.

Extraction buffer (0.5 M PBS): 8.0 g NaCl, 1.0 g KH_2PO_4, 14.5 g $Na_2HPO_4.12H_2O$, 20.0 g PVP (ELISA grade mol. wt. 10,000), and 2.0 g ovalbumine (grade II) were dissolved in 990 ml of de-ionised/distilled water. 10 ml of Tween 20 was added and the solution was checked to determine that the pH was 7.4 and adjusted if necessary.

Conjugate buffer (0.05 M PBS): 8.0 g NaCl, 1.0 g KH_2PO_4, 14.5 g $Na_2HPO_4.12H_2O$, 20.0 g PVP (ELISA grade mol. wt. 10,000), and 5 g BSA (ELISA grade, e.g. BSA fraction 5) were dissolved in 999.5 ml of de-ionised/distilled water. 0.5 ml Tween 20 was added and the solution was checked to determine that the pH was 7.4 and adjusted if necessary.

Substrate buffer: 97 ml diethanolamine plus 15 ml HCl (32%) were mixed with 888 ml of de-ionised/distilled water. The solution was checked to determine that the pH was 9.6 and adjusted if necessary.

Washing buffer PBS/Tween 20 (0.05M): 8.0 g NaCl, 1.0 g KH_2PO_4, and 14.5 g $Na_2HPO_4.12H_2O$ were dissolved in 998.5 ml of de-ionised/distilled water. 1.5 ml of Tween 20 was added and the solution was checked to determine that the pH was 7.4 and adjusted if necessary.

Coating of ELISA plates

The CGMMV, MNSV and SqMV coating sera were diluted 1:1,000 by adding 50 µl of serum to 50 ml coating buffer. Separate plates were coated with one of the three coating solutions of CGMMV, MNSV and SqMV by adding 100 µl per well. Plates were covered with a lid or wrapped with plastic to minimise evaporation and incubated overnight at 4 °C.

Extraction of virus from flour and incubation of extracts

Five ml of extraction buffer was added to each tube containing 0.5 g of flour. Each tube was vortexed for 20 seconds at high speed and then allowed to settle for 5 minutes. The coating buffer was removed from plates and the plates immediately rinsed three times with washing buffer to remove residues using a suitable washing device. Immediately after rinsing 100 µl of each seed extract was added per well. Positive controls, with all three viruses present in the flour, were prepared by diluting the flour in extraction buffer to give positive controls for each virus at a high and low dilution. Plates were then covered and incubated overnight at 4 °C.

Incubation of conjugate

50 µl of each of CGMMV, MNSV and SqMV conjugate antiserum was added to 50 ml conjugate buffer (1:1,000 dilution). The flour extract solution was removed from plates and these were immediately rinsed using washing buffer to remove residues of seed extracts using a suitable washing device. Immediately after rinsing, 100 µl per well of one of the three diluted conjugates (CGMMV, MNSV or SqMV) was added to the appropriate plate. Plates were then covered and incubated at 37 °C for three hours.

Addition of substrate to ELISA plates

Substrate solution was prepared by adding 10 mg para-nitrophenyl phosphate to 20 ml of substrate buffer. The diluted conjugate was removed from plates and these were immediately rinsed using washing buffer to remove residues using a suitable washing device. Immediately after rinsing 100 µl of substrate solution was added per well and plates were covered and incubated for two hours at 20 °C.

Interpretation of data

Based on the origin of the samples and pre-test results from Naktuinbouw, samples were expected to give positive or negative results. An overview of the composition of each sample is given in Table 1. Laboratories were asked to submit the measured extinction values (A_{405}) for all samples (100 and each plate 4 per virus). In addition, they were asked to state whether a sample was positive or negative for each virus. As a guideline it was recommended that a negative-positive threshold of 2.5 times the extinction value (or A_{405}) of healthy samples be used. Evaluation of the threshold was not an objective of this comparative test since there are alternative ways to calculate a threshold (Sutula *et al.*, 1986).

Data analysis

General introduction

The positive and negative samples were analyzed separately using a generalized linear model with a binomial distribution and a logit link function. This resulted in a prediction of the percentage of false positives and false negatives for each participating laboratory. The standard errors of the predictions based on the binomial model were used to test for differences among laboratories.

Repeatability and reproducibility

The repeatability and the reproducibility were computed on the data scale for a nominal proportion π equal to 0.05 using the formulae:

Repeatability: $S_r^2 = \dfrac{\pi(1-\pi)}{\left\lfloor \dfrac{1}{2J}\sum_{i,j} n_{ij} \right\rfloor}$

Reproducibility: $S_R^2 = S_r^2 + \pi^2 (1-\pi)^2 \, \hat{\sigma}^2_{Laboratory}$

Where:

J is the number of laboratories

n_{ij} is the denominator for the false-positive or false-negative proportions in laboratory j

$\hat{\sigma}^2_{Laboratory}$ is an estimate of the laboratory variance after fitting a Generalized Linear Mixed Model (logistic link) (Laffont, 2006).

Results

The pre-test data obtained at Naktuinbouw agreed with the expected results from the prepared samples. However, it appeared that some of the diluted samples with artificially low ELISA values, obtained by

mixing contaminated and healthy flour, were very difficult for the labs in the comparative test to detect because the values were around the threshold level between negative and positive samples.

CGMMV

In total 28 CGMMV positive samples were selected for the comparative test. CGMMV was detected in several samples although the cumulative CGMMV A_{405} values (Figure 1a) were relatively limited in comparison to MNSV and SqMV (Figure 2a and Figure 3a, respectively). The number of false positive CGMMV results was very limited and not significantly different among participating laboratories (Table 2) since just two laboratories found one false positive result (Figure 4). Laboratories 5 and 8 detected CGMMV in many and laboratory 6 in all of the CGMMV infested samples, including those with very low virus loads. However, for quite a lot of laboratories there were several false negative CGMMV results (Figure 5). Especially laboratories 4, 7 and 9 contributed to a large number of false negative results because these laboratories had very low A_{405} values compared to some of the other laboratories (Figure 1b). Statistical analysis did show that there were significant differences in the performance amongst the laboratories (Table 3).

MNSV

For MNSV, 24 positive samples were selected for the comparative test (Figure 2a). The number of false positives was relatively limited (Figure 6) and a statistical analysis revealed no significant differences among laboratories (Table 4). There were several false negative MNSV results for laboratories 3, and again 4 and 7 due to a relatively low sensitivity (Figure 2b and 7). Clearly there were significant differences in the performance of the laboratories (Table 5). Laboratories 4, 7 and 9 contributed to a large number of false negative results because these laboratories had very low A_{405} values compared to some of the other laboratories.

SqMV

In total 30 samples infested with SqMV were tested. The number of false positive SqMV samples was limited and not-significantly different among participating laboratories. Laboratories 4 and 5 had one false positive result each (Table 6, Figure 3a and 8). Laboratories 1, 5, 6 and 8 detected most of the SqMV infested samples (Table 7, Figure 9), but laboratories 2, 4 and 7, on the other hand, contributed to a large number of false negative results. These false negatives occurred mainly in the diluted samples (Figure 3b).

The reproducibility dispersion (between-laboratory variability plus within-laboratory variability) and the repeatability dispersion (within-laboratory variability) for CGMMV, MNSV and SqMV based on the binomial data are presented in Table 8. SqMV gave a better reproducibility and repeatability than MNSV and CGMMV.

Discussion

All the laboratories were able to detect samples with a medium to high virus load. However, several laboratories had problems with samples with relatively low loads of the three viruses since there were a considerable number of false negative results. An important question is whether the proposed method will minimise seed transmission under practical conditions? The ELISA method to detect SqMV has already been in use for a long time (Franken *et al.*, 1990) and no complaints have been filed at Naktuinbouw since the introduction of the assay (Koenraadt, personal communications). Samples with artificially low virus loads were over-represented on purpose in this comparative test but these low virus loads are not representative for naturally contaminated seed lots. In addition, it should be kept in mind that in the proposed method, the final result for a seed lot is not based on one ELISA but on 20 ELISAs as 20 sub-samples of 100 seeds each are tested. Moreover, seed transmission of CGMMV and MNSV in particular is often low since these viruses are not present in the embryo but localised on the seed coat.

There was a clear ‘laboratory effect’ in the comparative test but it is unclear whether this was caused by the proposed method or other factors. The discrepancy in the number of positive and negative data between several laboratories suggests that some factors may have been introduced during transport or in the laboratory since some laboratories under-performed in the detection of all three viruses. A similar

comparative test in the framework of NAL (Naktuinbouw Accredited Laboratories) showed that the overall performance of experienced seed health testing laboratories was much better than in this comparative test (see appendix 1). It is reasonable to assume that the choice of samples with artificially low loads of the viruses and the inclusion of laboratories with limited experience in seed health testing are important factors explaining the laboratory effect, and not the proposed method since that has been used in many laboratories for more than 10 years.

The proposed ELISA method allows for a quick check of whether a seed lot is contaminated with CGMMV, MNSV or SqMV. It is relatively cheap since three viruses can be determined in one extract thus minimising the costs of seed and labour. However, there are some disadvantages associated with ELISA as well. In general, the antisera are rather specific and therefore will not detect other seed-borne viruses that might be present on the seeds. A comparison of antisera revealed that they are rather specific and there was only a faint cross- reaction (Yoon *et al.*, 2002). Other tobamoviruses of cucurbits such as Kyuri green mottle mosaic virus (KGMMV), Zucchini green-mottle mosaic virus (ZGMMV) and cucumber fruit mottle mosaic virus (CFMMV) will likely not be picked up in this test. In addition, the efficacy of seed treatments cannot be evaluated since the proposed method does not discriminate between infectious and non-infectious virus. Alternative assays such as bioassays, ELISA of seedlings, grow out or RT-PCR might be alternatives to detect infectious viruses (Suzuki *et al.*, 2004).

Conclusions and recommendations

Most laboratories were able to detect CGMMV, MNSV and SqMV with the prescribed DAS-ELISA protocol. Therefore this protocol has value as an international reference method. Some laboratories that found high background values in all ELISAs had difficulty detecting the virus in samples with low virus titres. Washing may not have been optimal in these laboratories. The intensity of washing between the different ELISA steps is known to influence the amount of background in the test. Describing washing procedures in more detail may be helpful to solve this problem.

The use of CGMMV-, MNSV- and SqMV-contaminated flour is useful for comparative testing. Determining threshold values was not an objective of this comparative test. In this study the threshold was a function of the background. Background reduction through additional washings or the use of alternative threshold calculations e.g. subtractions of background from all the readings, could be useful in increasing the probability of detecting samples with very low virus load (Sutula *et al.*, 1986).

Acknowledgments

Thanks to all the laboratories that participated in the comparative test. Special thanks are due to Menno Hoekstra and Robin Borst (Naktuinbouw) who prepared all the samples, and to Jean Louis Laffont and Sylvain Gregoire who helped to design this comparative test.

References

Akanda, A.M., Tsuno, K. and Wakimoto, S. (1991) Serological detection of four plant viruses in cucurbitaceous crops from Bangladesh. *Annals of the Phytopathological Society of Japan* 57(4): 499-505.

Alvarez, M. and Campbell, R.N. (1978) Transmission and distribution of squash mosaic virus in seeds of cantaloupe. *Phytopathology* 68: 257-263.

Büttner, C., Marquardt, K. and Schickedanz, F. (1995) Studies on transmission of cucumber mosaic virus (CMV) and cucumber green mottle mosaic virus (CGMMV) by nutrient solutions of ebb and flow irrigation systems. *Gartenbauwissenschaft* 60(3): 109-114.

Campbell, R.N., Wipf-Scheibel, C., and Lecoq, H. (1996) Vector-assisted seed transmission of melon necrotic spot virus in melon. *Phytopathology* **86**: 1294-1298.

Clark, M.F. and Adams, A.N. (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* 34: 475-483.

Dorst, H.J.M. van (1988) Surface water as source in the spread of cucumber green mottle mosaic virus. *Netherlands Journal of Agricultural Science* 36: 291-299.

Faris-Mukhayyish, S. and Makkouk, K.M. (1983) Detection of four seed-borne plant viruses by the enzyme-linked immunosorbent assay (ELISA). *Phytopathologische Zeitschrift* 106: 108-114.

Franken, A.A.J.M., Maat, D.Z. and Kamminga, G.C. (1990) Detection of squash mosaic virus in seeds of melon (Cucumis melo) by enzyme-linked immunosorbent assay (ELISA). *Netherlands Journal of Plant Pathology* **96**: 91-102.

Hamilton, R.I. and Nichols, C. (1978) Serological methods for detection of pea seed-borne mosaic virus in leaves and seeds of *Pisum sativum* L. *Phytopathology* **68**: 539-543.

Hollings, M., Komuro, Y. and Tochihara, H. (1975) Descriptions of Plant Viruses **No. 154**, Wellesbourne, UK, AAB.

Kawai, A., Kimura, S., Nishio, T. and Nagao, N. (1985) Detection for cucumber green mottle mosaic virus in cucumber seeds using enzyme-linked immunosorbent assay. *Research Bulletin of the Plant Protection Service, Japan* 21: 47-53.

Kumari, S.G. and Makkouk, K.M. (1993) Evaluation of different ELISA procedures for the detection of pea seed-borne mosaic potyvirus and broad bean stain comovirus in lentil leaf extracts. *Arab Journal of Plant Protection* **11**: 86-91.

Laffont, J.L. (2006) Estimation of repeatability and reproducibility for 'PSbMV' and 'PEBV' data. 3 pp.

Macias, W. (2000) Methods of disinfecting cucumber seeds that originate from plants infected by cucumber green mottle mosaic tobamovirus (CGMMV). *Vegetable Crops Research Bulletin* 53: 75-82.

Matsuo, K. (1993) Detection of three strains of melon necrotic spot virus by three ELISA procedures and their distribution in Japan. *Annals of the Phytopathological Society of Japan* 59: 26-32.

Nolan, P.A. and Campbell, R.N. (1984) Squash mosaic virus detection in individual seeds and seed lots of cucurbits by enzyme-linked immunosorbent assay. *Plant Disease* 68: 971-975.

Purcifull, D.E., Christie, S.R. and Lima, J.A.A. (1981) Detection of four isometric plant viruses in sodium dodecyl sulfate immunodiffusion tests. *Phytopathology* 71: 1221-1224.

Sutula, C.L., Gillet, J.M., Morrissey, S.M. and Ramsdell, D.C. (1986) Interpreting ELISA data and establishing the positive-negative threshold. *Plant Disease* **70**, 722–726.

Suzuki, K., Okumura, A., Okuno, T., Takeuchi, S., Toyoda, K., Hikichi, Y. and Nasu, Y. (2004) Efficient inactivation of *Pepper mild mottle virus* (PMMoV) in harvested seeds of green pepper (*Capsicum annuum* L.) assessed by a reverse transcription and polymerase chain reaction (RT-PCR)-based amplification. *Scientific Reports of the Faculty of Agriculture*, Okayama University, 2004 (Vol. 93).

Yoon, J.Y., Min, B.E., Choi, J.K. and Ryu, K.H. (2002) Genome structure and production of biologically active in vitro transcripts of cucurbit-infecting *Zucchini green mottle mosaic virus*. *Phytopathology* 92: 156-163.

Table 1. List of CGMMV-, MNSV- and SqMV-contaminated samples in the comparative test

Tube number	Pathogen	SqMV	MNSV	CGMMV	Tube number	Pathogen	SqMV	MNSV	CGMMV
1	SqMV	1	0	0	51	all	1	1	1
2	MNSV	0	1	0	52	SqMV	1	0	0
3	Neg	0	0	0	53	MNSV	0	1	0
4	MNSV	0	1	0	54	CGMMV	0	0	1
5	SqMV	1	0	0	55	MNSV	0	1	0
6	SqMV	1	0	0	56	CGMMV	0	0	1
7	MNSV	0	1	0	57	CGMMV	0	0	1
8	SqMV	1	0	0	58	MNSV	0	1	0
9	MNSV	0	1	0	59	CGMMV	0	0	1
10	Neg	0	0	0	60	MNSV	0	1	0
11	SqMV	1	0	0	61	CGMMV	0	0	1
12	Neg	0	0	0	62	neg	0	0	0
13	all	1	1	1	63	CGMMV	0	0	1
14	SqMV	1	0	0	64	SqMV	1	0	0
15	MNSV	0	1	0	65	CGMMV	0	0	1
16	CGMMV	0	0	1	66	SqMV	1	0	0
17	CGMMV	0	0	1	67	SqMV	1	0	0
18	MNSV	0	1	0	68	CGMMV	0	0	1
19	CGMMV	0	0	1	69	CGMMV	0	0	1
20	Neg	0	0	0	70	SqMV	1	0	0
21	MNSV	0	1	0	71	CGMMV	0	0	1
22	CGMMV	0	0	1	72	SqMV	1	0	0
23	CGMMV	0	0	1	73	SqMV	1	0	0
24	SqMV	1	0	0	74	CGMMV	0	0	1
25	CGMMV	0	0	1	75	SqMV	1	0	0
26	SqMV	1	0	0	76	CGMMV	0	0	1
27	CGMMV	0	0	1	77	neg	0	0	0
28	CGMMV	0	0	1	78	neg	0	0	0
29	SqMV	1	0	0	79	SqMV	1	0	0
30	CGMMV	0	0	1	80	neg	0	0	0
31	Neg	0	0	0	81	SqMV	1	0	0
32	CGMMV	0	0	1	82	neg	0	0	0
33	CGMMV	0	0	1	83	CGMMV	0	0	1

34	MNSV	0	1	0	84	CGMMV	0	0	1
35	SqMV	1	0	0	85	neg	0	0	0
36	SqMV	1	0	0	86	MNSV	0	1	0
37	Neg	0	0	0	87	neg	0	0	0
38	SqMV	1	0	0	88	MNSV	0	1	0
39	MNSV	0	1	0	89	SqMV	1	0	0
40	Neg	0	0	0	90	neg	0	0	0
41	MNSV	0	1	0	91	MNSV	0	1	0
42	SqMV	1	0	0	92	neg	0	0	0
43	SqMV	1	0	0	93	MNSV	0	1	0
44	Neg	0	0	0	94	neg	0	0	0
45	MNSV	0	1	0	95	neg	0	0	0
46	SqMV	1	0	0	96	SqMV	1	0	0
47	MNSV	0	1	0	97	MNSV	0	1	0
48	Neg	0	0	0	98	neg	0	0	0
49	SqMV	1	0	0	99	neg	0	0	0
50	CGMMV	0	0	1	100	MNSV	0	1	0

Table 2. Predictions, standard errors and significant differences of each laboratory scored for false-positive samples of CGMMV

Laboratory	Prediction	Standard Error	Significant differences
1	0.0	0.0	a
2	0.0	0.0	a
3	0.0	0.0	a
4	0.01	0.01	a
5	0.0	0.0	a
6	0.0	0.0	a
7	0.01	0.01	a
8	0.0	0.0	a
9	0.0	0.0	a

Table 3. Predictions, standard errors and significant differences of each laboratory scored for false-negative samples of CGMMV

Laboratory	Prediction	Standard Error	Significant differences
1	0.61	0.09	b
2	0.43	0.09	ab
3	0.54	0.09	b
4	0.71	0.09	b
5	0.14	0.07	a
6	0.0	0.0	a
7	0.68	0.09	b
8	0.11	0.06	a
9	0.86	0.07	b

Table 4. Predictions, standard errors and significant differences of each laboratory scored for false-positive samples of MNSV

Laboratory	Prediction	Standard Error	Significant differences
1	0.01	0.01	a
2	0.0	0.0	a
3	0.0	0.0	a
4	0.03	0.02	a
5	0.03	0.02	a
6	0.0	0.0	a
7	0.0	0.0	a
8	0.0	0.0	a
9	0.0	0.0	a

Table 5. Predictions, standard errors and significant differences of each laboratory scored for false-negative samples of MNSV

Laboratory	Prediction	Standard Error	Significant differences
1	0.46	0.10	b
2	0.38	0.10	b
3	0.54	0.10	bc
4	0.79	0.08	bc
5	0.0	0.0	d
6	0.08	0.06	a
7	0.54	0.10	bc
8	0.0	0.0	d
9	0.29	0.09	ab

Table 6. Predictions, standard errors and significant differences of each laboratory scored for false-positive samples of SqMV

Laboratory	Prediction	Standard Error	Significant differences
1	0.0	0.0	a
2	0.0	0.0	a
3	0.0	0.0	a
4	0.01	0.01	a
5	0.01	0.01	a
6	0.0	0.0	a
7	0.0	0.0	a
8	0.0	0.0	a
9	0.0	0.0	a

Table 7. Predictions, standard errors and significant differences of each laboratory scored for false-negative samples of SqMV

Laboratory	Prediction	Standard Error	Significant differences
1	0.10	0.05	ab
2	0.43	0.09	cd
3	0.27	0.08	bc
4	0.57	0.09	d
5	0.03	0.03	a
6	0.03	0.03	a
7	0.50	0.09	cd
8	0.03	0.03	a
9	0.27	0.08	bc

Table 8: Reproducibility dispersion and repeatability dispersion for CGMMV, MNSV and SqMV (based on the binomial data, $\pi = 0.05$) detected in flour of cucumber seeds for all laboratories and samples

Pathogen	Reproducibility dispersion	Repeatability dispersion
CGMMV	0.0064	0.0004
MNSV	0.0058	0.0013
SqMV	0.0039	0.0006

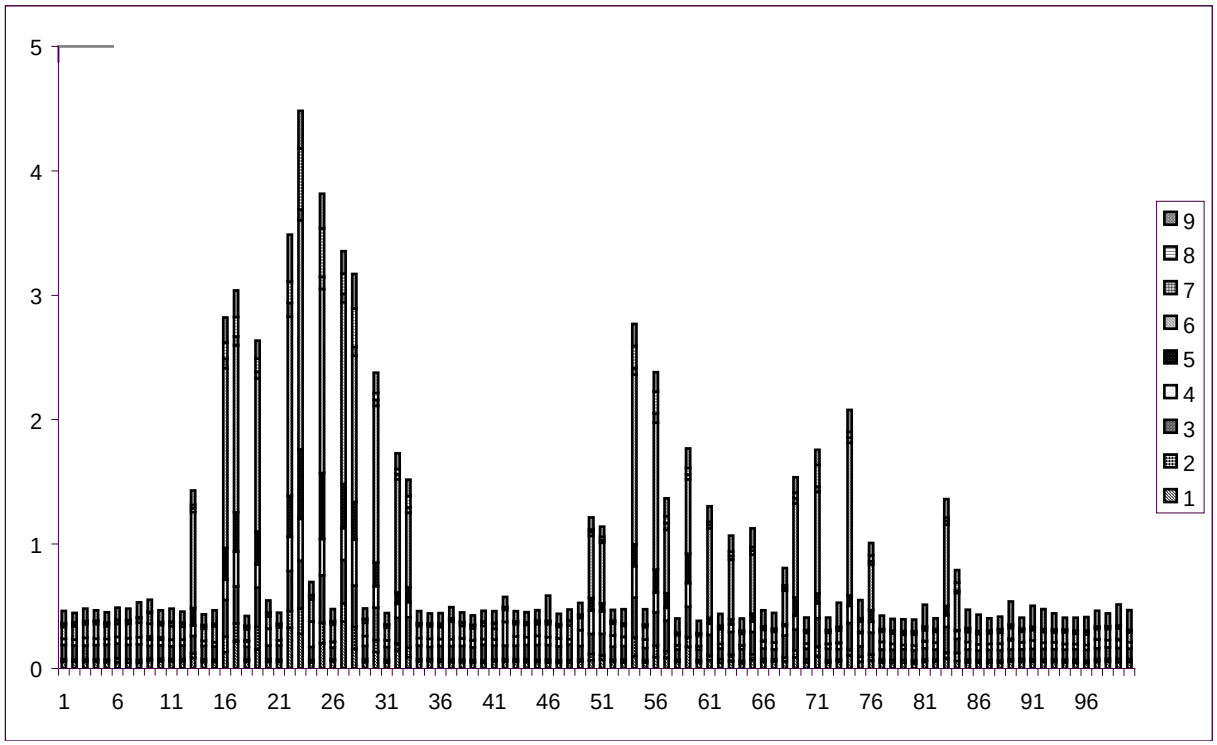


Figure 1a. Cumulative A_{405} ELISA values per sample of the nine laboratories in the comparative test for the detection of CGMMV

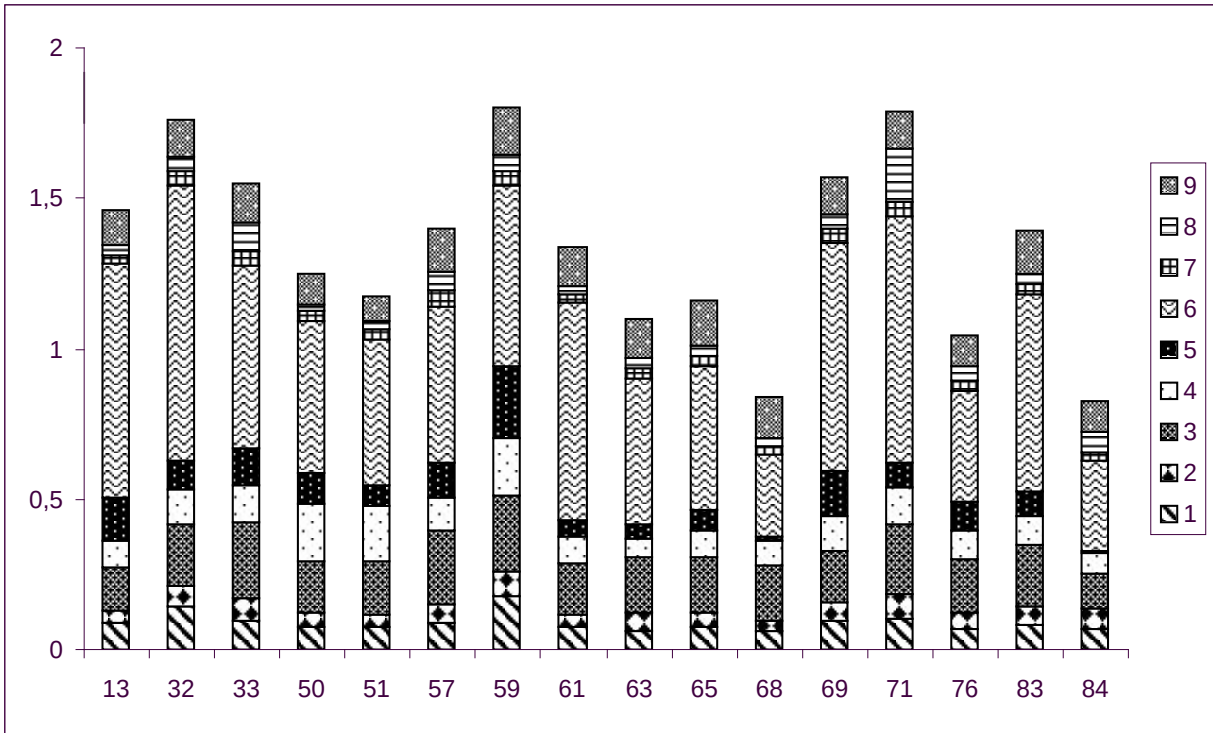


Figure 1b. Detail of CGMMV-positive samples with relatively low cumulative A_{405} values (<2) for each laboratory

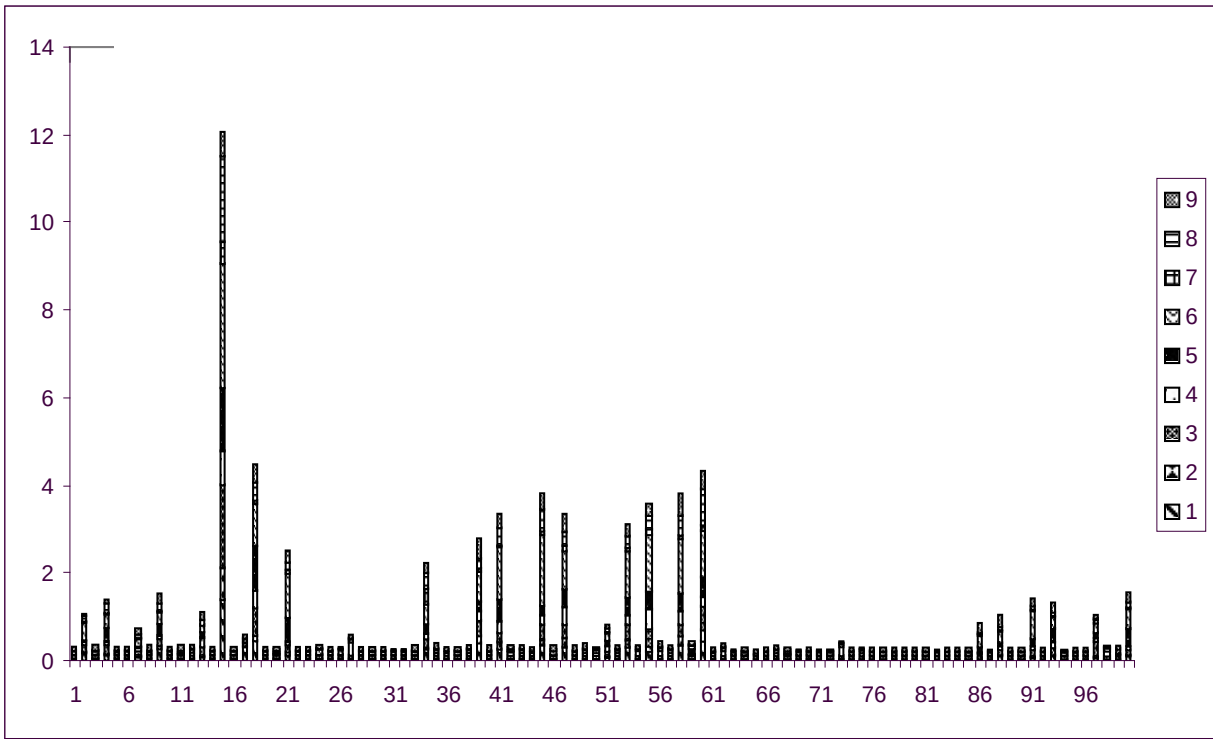


Figure 2a. Cumulative A_{405} ELISA values per sample of the nine laboratories in the comparative test for the detection of MNSV

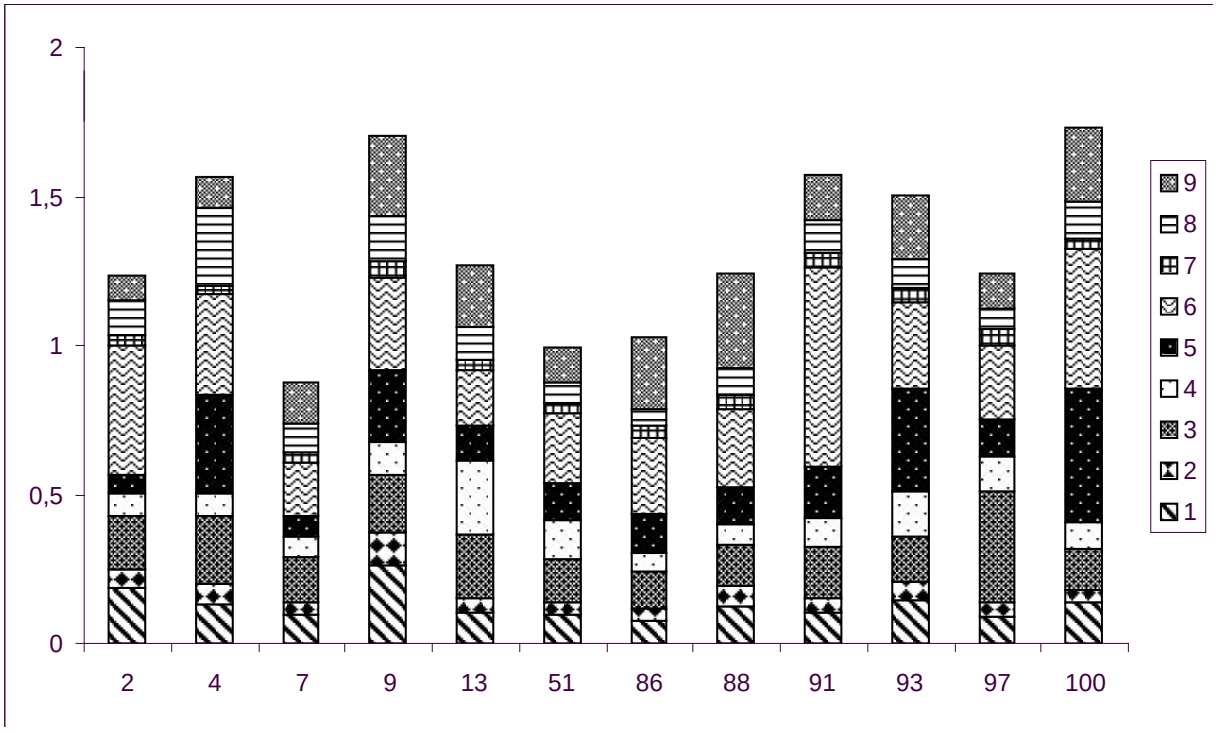


Figure 2b. Detail of MNSV-positive samples with relatively low cumulative A_{405} values (<2) for each laboratory

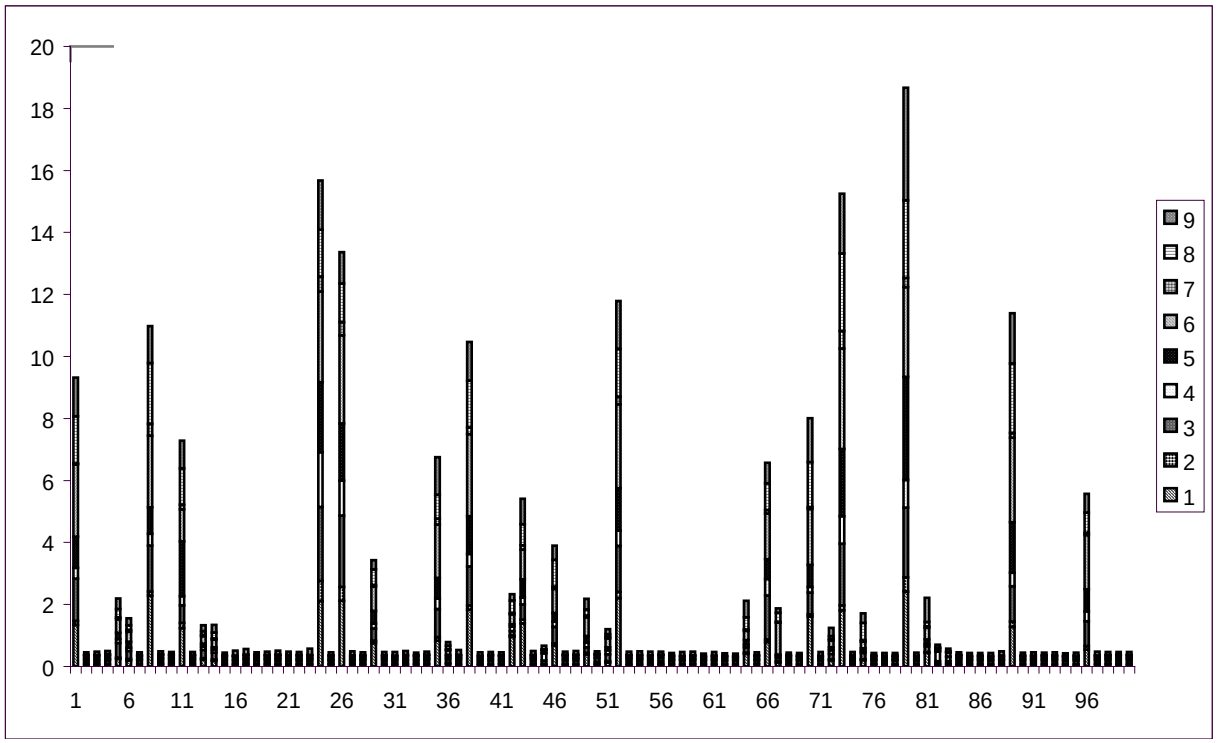


Figure 3a. Cumulative A_{405} ELISA values per sample of the nine laboratories in the comparative test for the detection of SqMV

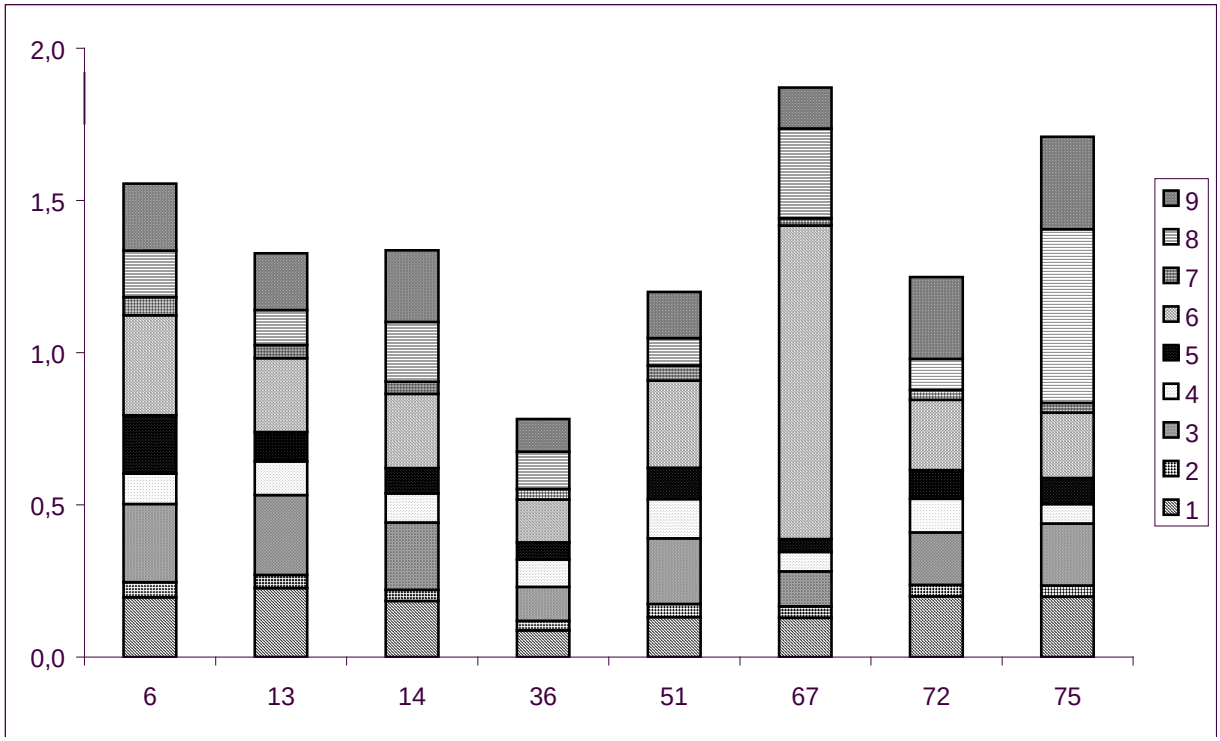


Figure 3b. Detail of SqMV-positive samples with relatively low cumulative A_{405} values (<2) for each laboratory

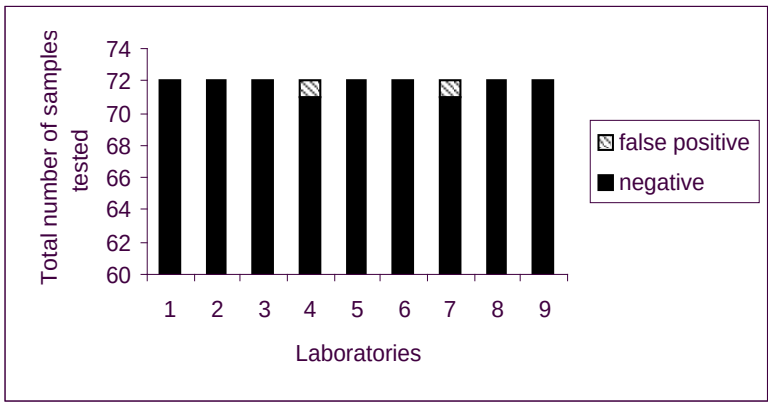


Figure 4. Number of negative samples in relation to the number of false positives scored by each laboratory for CGMMV

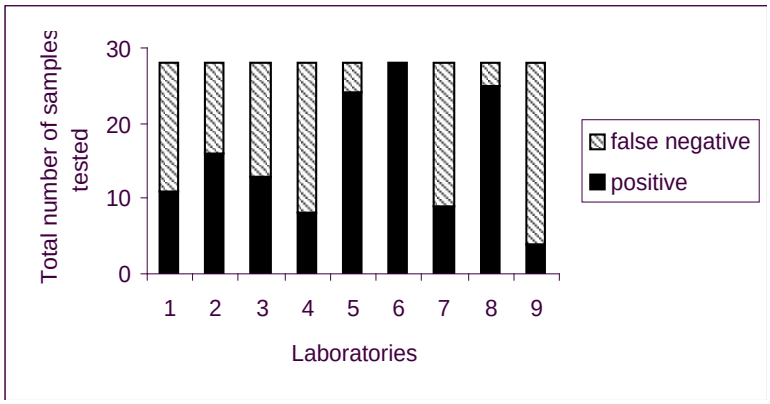


Figure 5. Number of positive samples in relation to the number of false negatives scored by each laboratory for CGMMV

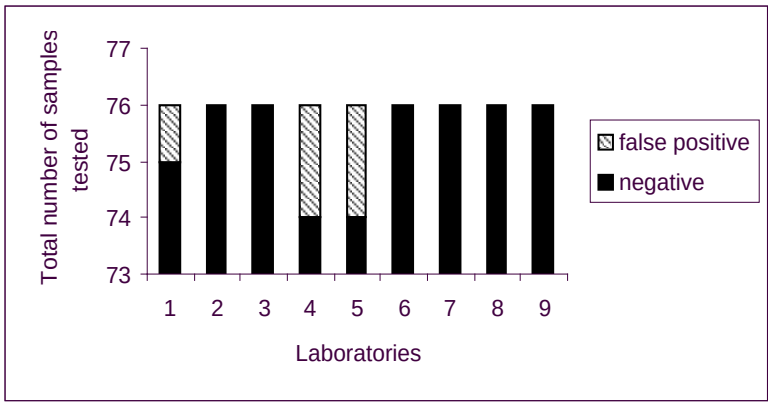


Figure 6. Number of negative samples in relation to the number of false positives scored by each laboratory for MNSV

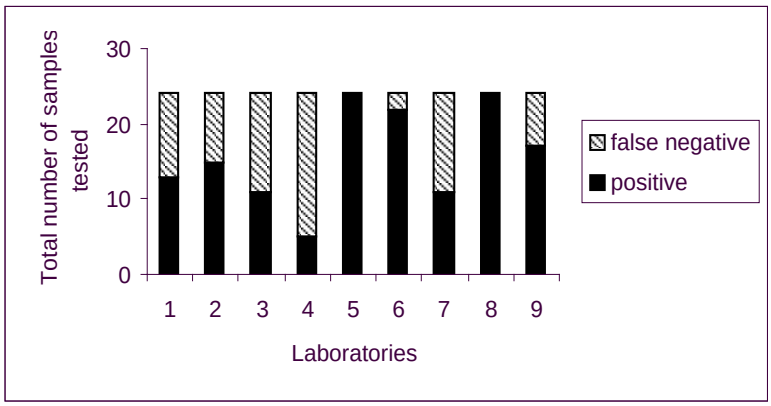


Figure 7. Number of positive samples in relation to the number of false negatives scored by each laboratory for MNSV

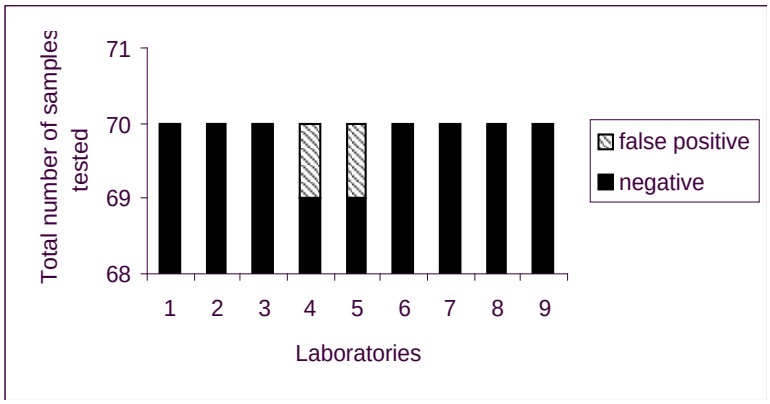


Figure 8. Number of negative samples in relation to the number of false positives scored by each laboratory for SqMV

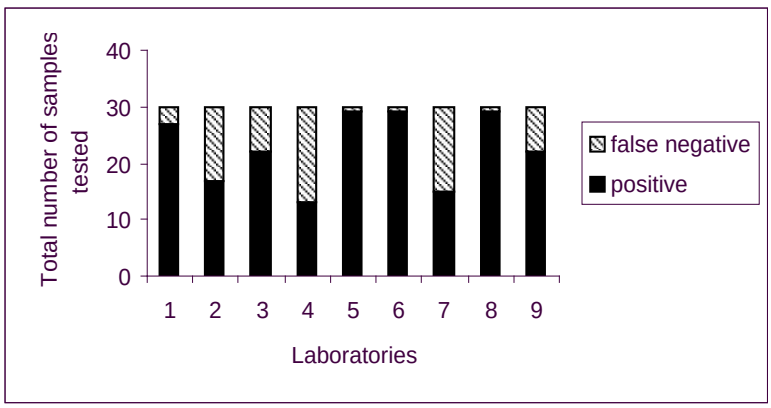


Figure 9. Number of positive samples in relation to the number of false negatives scored by each laboratory for SqMV

Proposal for the addition of *Phaseolus vulgaris* as a species to which the conductivity test for seed vigour can be applied [Rules Proposal 2010 C.15.1.]

Alison A Powell

School of Biological Sciences, University of Aberdeen, 23, St Machar Drive, Aberdeen, AB24 3UU, UK

a.a.powell@abdn.ac.uk

Summary

Six seed lots of *Phaseolus vulgaris*, all having a laboratory germination of >80%, were tested by five laboratories using the electrical conductivity test, as described in the ISTA Rules for *Pisum sativum*, in each of three runs of the test (i.e. 6 lots x 5 labs x 3 test runs = 100 tests). All laboratories consistently identified the same significant differences in the seed lot conductivity and the data was repeatable within laboratories and reproducible between laboratories. The results of all tests, with one exception, gave a z-score between +2.00 and -2.00 and all data fell within the tolerance levels established for peas in the ISTA Rules. This provides evidence in support of the inclusion of *Phaseolus vulgaris* within the ISTA Rules as a species to which the conductivity test can be applied.

Introduction

The conductivity test is currently validated in the ISTA Rules as a test that can be applied to *Pisum sativum.* Differences in solute leakage from seed lots of peas in the conductivity test can be attributed to impaired membrane integrity and the development of dead tissue on the living cotyledons as the result of seed ageing or imbibition damage (Mathews and Powell, 2006), both of which occur in many grain legumes (Powell, Matthews and Oliveira, 1984). It is therefore not surprising that measurements of solute leakage using the conductivity test have also been related to vigour in other species. Differences in solute leakage in *Phaseolus vulgaris* were first shown by Matthews and Bradnock (1968) who also demonstrated that conductivity related to field emergence, i.e. it predicted seed vigour. The test was subsequently shown to relate to field emergence of 30 seed lots of *P. vulgaris* in the UK on two sowing dates (Powell, Oliveira and Matthews, 1986) and 39 lots sown in Poland on three sowing dates in each of three years (Kolasinka, Szyrmer and Dul, 2000). The aim of this study was to demonstrate that the conductivity test as applied to *Phaseolus vulgaris* is both repeatable within laboratories and reproducible between laboratories.

Materials and Methods

Samples of six seed lots of *Phaseolus vulgaris*, having standard laboratory germinations above 80% (minimum acceptable germination), were supplied by the Processors and Growers Research Organisation, Peterborough, UK. Samples of the seed lots were sent from Aberdeen UK to the participating laboratories, namely SNES, GEVES, Angers, France; LaRAS, Bologna, Italy, OSTS, SASA, Edinburgh, UK, Queensland Seed Technology Lab, Australia; and Agriquality, Christchurch, New Zealand.

Each laboratory completed the conductivity test using the same method as that described for peas in the ISTA Rules (ISTA, 2007) i.e. 4 replicates of 50 seeds, each soaked in 250ml deionised /distilled water for 24 h at 20^{o}C. The test was run on three separate occasions in each lab.

The data was analysed using (a) Analysis of Variance, (b) calculation of z-scores and (c) the statistical tool developed by S. Grégoire according to ISO 5725-2 and available for download at the ISTA website: http://www.seedtest.org/upload/cms/user/ISO572511.zip

Results

The mean conductivity values for the six lots in the three repeat runs of the test (Table 1) ranged from 25.7 µS cm $^{-1}$ g $^{-1}$ to 38.3 µS cm $^{-1}$ g $^{-1}$ in Run 1, 25.5 to 40.2 µS cm $^{-1}$ g $^{-1}$ in run 2 and 25.3 to 39.0 µS cm $^{-1}$ g $^{-1}$ in run 3. There were clear significant differences between the seed lots in each run (Table 1). Lot C had the highest conductivity, indicative of the lowest vigour. This was followed by lot D, lots E and F (similar conductivity readings), lot A, then B with the lowest conductivity and highest vigour.

In each run there were small, but significant differences in the overall mean conductivity readings obtained in the five laboratories (Table 2), with 2 and 3 tending to have slightly higher values than the other three laboratories. This was confirmed by the analysis of the overall means (Table 3)

Comparison of the means of three runs for each seed lot from each laboratory (Table 3) further confirmed the differences in readings between the lots (conductivity of lot C>D>E and F>A>B). The ranking of the seed lots from low to high vigour was completely consistent between laboratories (Table 3).

Calculation of the Coefficient of Variation (CV) for the whole experiment gave a value of 4.3%, indicating little residual variability. The significance of the small differences in conductivity between laboratories can indeed be explained by the good repeatability of the data within laboratories and between runs.

Low vigour seed typically show greater variability within the lot. This was reflected in the higher standard deviations of low vigour seed lots (e.g. lots C and D) than found in the high vigour lots such as lots B and A (Tables 4a and 5a). Z scores, calculated both for individual test runs (Table 4b) and for the combined test runs (Table 5b) were, with only one exception, all within the range +2.00 to -2.00.

Repeatability and reproducibility were analysed with the statistical tool developed by S. Grégoire, based on ISO 5725-2; this allows the calculation of h- and k-values. The h-values show the tendency for a laboratory to give over-estimations or under-estimations compared to the mean of all the results available whereas the k-values give a measure of the variability of the repeats. Higher values indicate greater under- or over-estimations (h-values) or greater variability between replicates (k-values).

There were no significant h-values for all the lots and labs in runs 1 and 3 (Figure 1a,c) and only one in run 2 (lab 3, lot C, Figure 1b). Thus there was only one occasion when a lab tended to significantly under- or under-estimate the conductivity. Out of 90 k-values, only seven were significant, indicating that there was greater variability between the replicates in the test. These were: in run 1, for lab 3, lots A and C ($p< 0.01$); run 2, lab 2, lots A ($p<0.01$), D ($p<0.05$) and F ($p<0.01$); run 3 lab 2, lots E and F ($p<0.05$).

The values of repeatability and reproducibility depend on the scale and unit of measurement. Thus it is difficult to compare the current values for repeatability and reproducibility from a conductivity test with the previous validation data on germination. However, the values obtained from the ISO analysis for repeatability and reproducibility (Table 6) were almost all less than those reported for previous germination test data (Fiedler *et al* 2008; Ducournau *et al*, 2007).

Discussion

The conductivity test consistently identified differences between seed lots in each of five laboratories. The test was both repeatable within laboratories and reproducible in different laboratories. In addition, the replicates within the laboratories and the mean values obtained for each lot in different laboratories all fell within tolerance, using the tolerance tables in the ISTA Rules (ISTA, 2007). This provides evidence in support of the addition of *Phaseolus vulgaris* to the ISTA Rules as a species for which the conductivity test can be applied.

Acknowledgements:

I am grateful to Dr Anthony Biddle and Mark White, PGRO, UK for supplying the *Phaseolus* seeds and to Barbara Brunton, Emanuela Casarini, Karen Hill, Gillian McLaren and Marie-Hélène Wagner for participating in this comparative test.

References

Ducournau, S. Garreau, P. and Léchappé, J. (2008). Effect of temperature and growing media on sunflower germination. *Seed Testing International*, **135**, 34-36.

Fiedler, K.A, Gutormson, T.J., Brix-Davis, K.A. and Carlson, L.N. (2008). Evaluation of crepe cellulose paper covered with sand as an ISTA medium. *ISTA Method Validation Reports*, **2008**, 9-42.

ISTA 2007. ISTA Rules 2007. International Seed Testing Association, Bassersdorf, Switzerland.

Kolasinka, K., Szyrmer J. and Dul, S. 2000. Relationship between seed quality tests and field emergence of common bean seed. *Crop Science*, **40**, 470-475

Matthews, S. and Powell, AA. 2006. Electrical conductivity test: Physiological basis and use. *Seed Testing International* , **131**, 32-35 International Seed Testing Association, Zurich, Switzerland.

Matthews, S. and Bradnock, W.T. 1968. Relationship between seed exudation and field emergence in peas and French beans. *Horticultural Research*, **9**, 89-93.

Powell AA, Matthews S, Oliveira M de A. 1984**.** Seed quality in grain legumes. *Advances in Applied Biology* **10**, 217-285.

Powell AA, Oliveira M de A, Matthews S. 1986**.** Seed vigour in cultivars of dwarf French bean (*Phaseolus vulgaris*) in relation to the colour of the testa. *Journal of Agricultural Science, Cambridge*, **106**, 419-425.

Table 1: Comparison of seed lot means in each of three test runs

Each piece of data is the mean of the results from five laboratories

Lot	Run		
	1	2	3
A	27.0[d]	27.2[d]	26.7[d]
B	25.7[e]	25.5[e]	25.3[e]
C	38.3[a]	40.2[a]	39.0[a]
D	32.9[b]	34.9[b]	34.4[b]
E	29.9[c]	29.9[c]	30.4[c]
F	29.7[c]	30.0[c]	29.7[c]

In each column, values followed by different letters are significantly different using LSD at the 5% level

Table 2: Comparison of mean conductivity from five laboratories in each of three test runs

Each piece of data is the mean of the results for 6 seed lots

Laboratory	Run		
	1	2	3
1	29.2[d]	29.8[cd]	28.3[c]
2	31.8[b]	32.4[b]	33.3[a]
3	33.0[a]	34.7[a]	33.6[a]
4	28.6[e]	29.2[d]	29.1[c]
5	30.4[c]	30.4[c]	30.4[b]

In each column, values followed by different letters are significantly different using LSD at the 5% level

Table 3: Comparison of seed lots and laboratory mean conductivity readings.

For each lot and lab, the data is the mean of three test runs.

In each column the number in parentheses is the rank order of the seed lot as determined by that laboratory, with 1 = highest conductivity reading (lowest vigour) and 5 = lowest conductivity reading (highest vigour)

Lot	Laboratory					
	1	2	3	4	5	Mean
A	25.1 (5)	28.8 (5)	28.9 (5)	25.6 (5)	26.5 (5)	27.0[d]
B	24.2 (6)	26.9 (6)	27.2 (6)	24.1 (6)	25.1 (6)	25.5[e]
C	36.2 (1)	40.7 (1)	42.5 (1)	37.3 (1)	38.8 (1)	39.1[a]
D	32.2 (2)	35.5 (2)	38.4 (2)	31.6 (2)	32.8 (2)	34.1[b]
E	28.1 (4)	31.5 (3)	33.1 (3)	27.9 (3)	29.7 (3)	30.1[c]
F	28.4 (3)	31.4 (4)	32.6 (4)	27.3 (4)	29.2 (4)	29.8[c]
Mean	29.1[D]	32.5[B]	33.8[A]	29.0[D]	30.1[C]	

In a column, values followed by different letters are significantly different using LSD at the 5% level

In a row, values followed by different upper case letters are significantly different using LSD at the 5% level.

Table 4: Comparisons of means, standard deviations (SD) and z-scores for each lot tested in five laboratories, calculated for each of three individual test runs.

Lab	Run	A	B	C	D	E	F
(a) Means and SD for each run							
1	1	25.3	25.1	35.6	32.4	28.5	28.1
2	1	28.3	26.2	40.5	32.6	31.2	31.7
3	1	29.6	27.8	40.4	36.7	31.9	31.7
4	1	25.5	24.2	36.9	29.6	28.2	27.2
5	1	26.2	25.1	38.1	33.3	29.7	29.7
Mean		26.98	25.68	38.30	32.92	29.90	29.68
SD		1.8860	1.3809	2.1529	2.5411	1.6264	2.0499
1	2	26.0	24.4	37.9	33.0	28.0	29.4
2	2	28.5	27.1	39.9	36.7	31.0	31.3
3	2	29.4	26.9	46.7	38.5	33.6	33.1
4	2	25.5	24.3	37.5	32.6	27.2	27.9
5	2	26.5	24.9	39.0	33.9	29.5	28.3
Mean		27.18	25.52	40.30	34.94	29.86	30.00
SD		1.6843	1.3719	3.7537	2.554	2.5472	2.1772

1	3	24.1	23.1	35.9	31.3	27.8	27.7
2	3	29.5	27.5	41.8	37.1	32.3	31.3
3	3	27.6	26.9	40.4	40.0	33.7	33.0
4	3	25.7	23.8	37.5	32.6	28.3	26.9
5	3	26.8	25.3	39.4	31.2	29.8	29.6
Mean		26.74	25.32	39.00	34.44	30.38	29.7
SD		2.0256	1.9032	2.3356	3.9285	2.5509	2.5150
(b) z-scores for lots in each of three runs							
Lab	Run	A	B	C	D	E	F
1	1	-0 8910	-0.4200	-1.2541	-0.2046	-0.8608	-0.7708
2	1	0.6998	0.3766	1.0219	-0.1259	0.7993	0.9854
3	1	1.3890	1.5352	0.9754	1.4875	1.2297	0.9854
4	1	-0.7850	-1.0718	-0.6503	-1.3065	-1.0452	-1.2098
5	1	-0.414	-0.4200	-0.0929	0.1495	-0.1229	0.0098
1	2	-0.7006	-0.8163	-0.6127	-0.7596	-0.7302	-0.2756
2	2	0.7837	1.1517	-0.0799	0.6891	0.4476	0.5971
3	2	1.3181	1.0059	1.7316	1.3939	1.4683	1.4238
4	2	-0.9974	-0.8893	-0.7193	-0.9162	-1.0443	-0.9645
5	2	-0.4037	-0.4519	-0.3197	-0.4072	-0.1413	-0.7808
1	3	-1.3033	-1.1665	-1.3273	-0.7993	-1.0114	-0.7952
2	3	1.3626	1.1454	1.1988	0.6771	0.7527	0.6362
3	3	0.4246	0.8302	0.5994	1.4153	1.3015	1.3121
4	3	-0.5134	-0.7987	-0.6422	-0.4684	-0.8154	-1.1133
5	3	0.0296	-0.0105	0.1713	-0.8247	-0.2273	-0.0398

Table 5: Comparisons of means, standard deviations (SD) and z-scores for each lot tested, calculated from the overall data for three test runs

Data in bold indicates a z-score that is outwith the range of +2.00 to -2.00

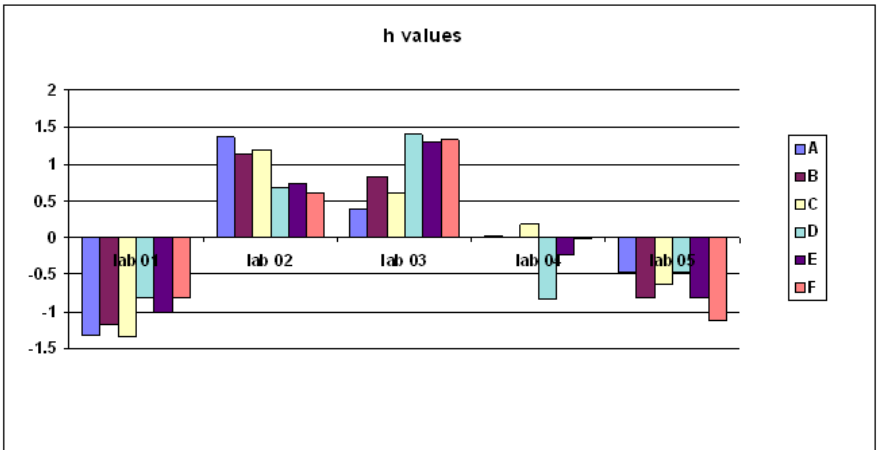
Lab	Run	A	B	C	D	E	F
(a) Means and SD for each run							
1	1	25.3	25.1	35.6	32.4	28.5	28.1
	2	26.0	24.4	37.9	33.0	28.0	29.4
	3	24.1	23.1	35.9	31.3	27.8	27.7
2	1	28.3	26.2	40.5	32.6	31.2	31.7
	2	28.5	27.1	39.9	36.7	31.0	31.3
	3	29.5	27.5	41.8	37.1	32.3	31.3
3	1	29.6	27.8	40.7	36.7	31.9	31.7
	2	29.4	26.9	46.7	38.5	33.6	33.1
	3	27.6	26.9	40.4	40.0	33.7	33.0
4	1	25.5	24.2	36.9	29.6	28.2	27.2
	2	25.5	24.3	37.5	32.6	27.2	27.9
	3	25.7	23.8	37.5	32.6	28.3	26.9
5	1	26.2	25.1	38.1	33.3	29.7	29.7
	2	26.5	24.9	39.0	33.9	29.5	28.3
	3	26.8	25.3	39.4	31.2	29.8	29.7
Mean		26.97	25.50	39.19	34.10	30.05	29.80
SD		1.7418	1.4631	2.7617	2.9847	2.128	2.0935
(b) z-scores for lots in each of three runs							
1	1	-0.9588	-0.2802	-1.2673	-0.5696	- 0.7284	-0.8120
	2	-0.5569	- 0.7587	-0.4671	-0.3685	-0.9633	-0.1911
	3	-1.6477	-1.6472	-1.1913	-0.9381	-1.0573	-1.0031
2	1	0.7636	0.4716	0.4743	-0.5026	0.5404	0.9076
	2	0.8784	1.0867	0.2571	0.8711	0.4464	0.7165
	3	1.4525	1.3601	0.9451	1.0051	1.0573	0.7165
3	1	1.5099	1.5652	0.5468	0.8711	0.8694	0.9076
	2	1.3951	0.9500	2.7193	1.4742	1.6682	1.5763
	3	0.3617	0.9500	0.4381	1.9767	1.7152	1.5285
4	1	-0.8440	- 0.8954	-0.8292	-1.5077	-0.8694	-1.2419
	2	-0.8440	-0.8270	-0.6119	-0.5026	-1.3393	-0.9076
	3	-0.7291	-1.1688	-0.6119	-0.5026	-0.8224	-1.3852
5	1	-0.4421	-0.2802	-0.3947	-0.2680	-0.1645	-0.0478

	2	-0.2698	-0.4169	-0.0688	-0.0670	-0.2585	-0.7165
	3	-0.0976	-0.1435	0.0760	-1.0186	-0.1175	-0.0478

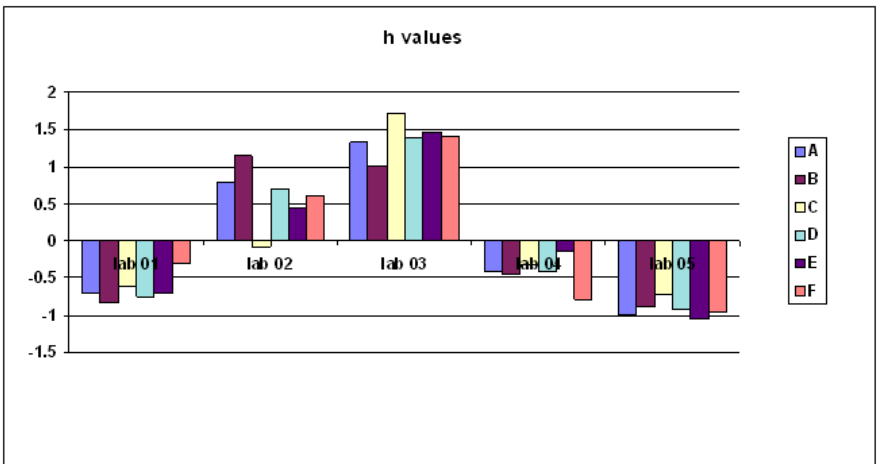
Table 6: Values for repeatability and reproducibility of results from the conductivity test on *Phaseolus vulgaris* in each of three repeat runs of the test

Lot	Run		
	1	2	3
a) Repeatability			
A	0.9511	0.9870	1.2313
B	0.9941	1.1774	0.7343
C	1.3355	2.2287	1.5391
D	1.5723	1.8247	1.8058
E	0.9823	1.1073	1.6753
F	1.4130	1.1719	1.0245
b) Reproducibility			
A	2.0759	1.8856	2.2814
B	1.6850	1.7185	2.0234
C	2.4377	4.2475	2.6868
D	2.8810	3.0104	4.2581
E	1.8515	2.7382	2.9488
F	2.3930	2.4105	2.6782

Run 1



Run 2



Run 3

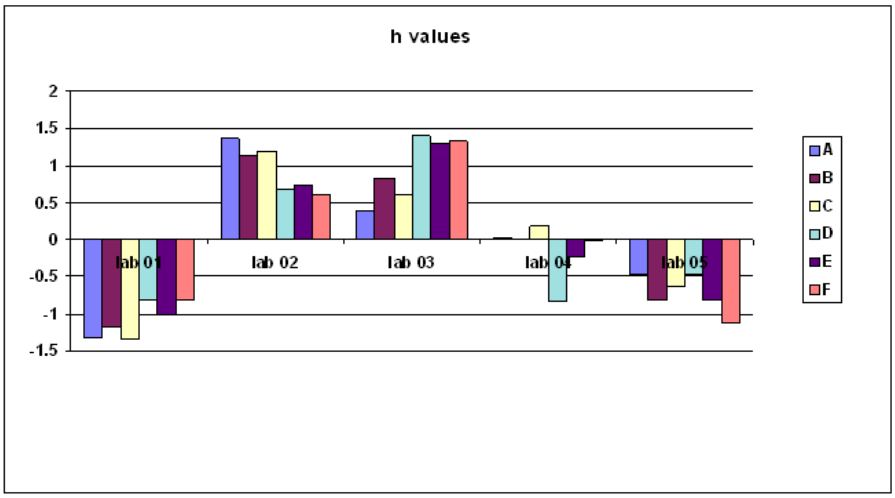
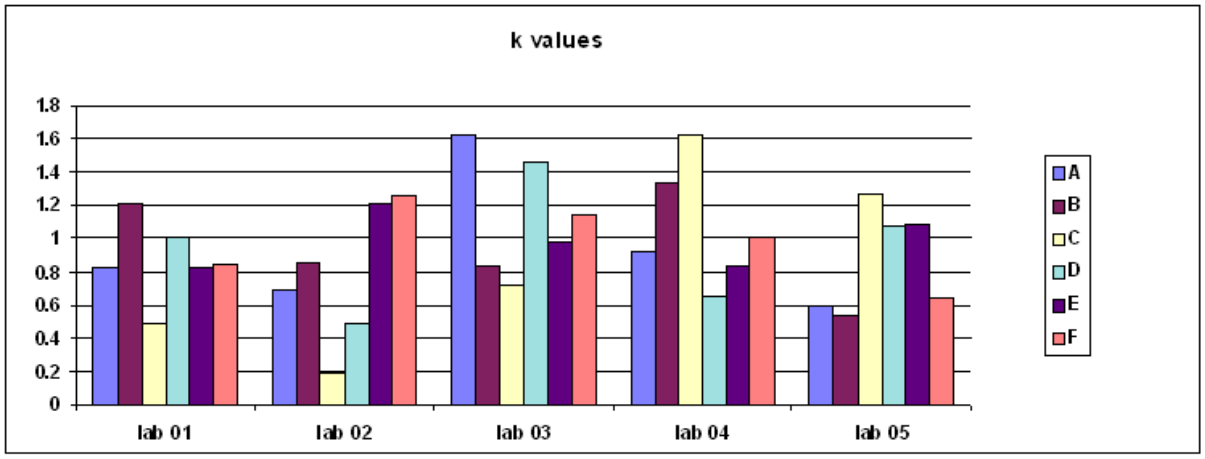
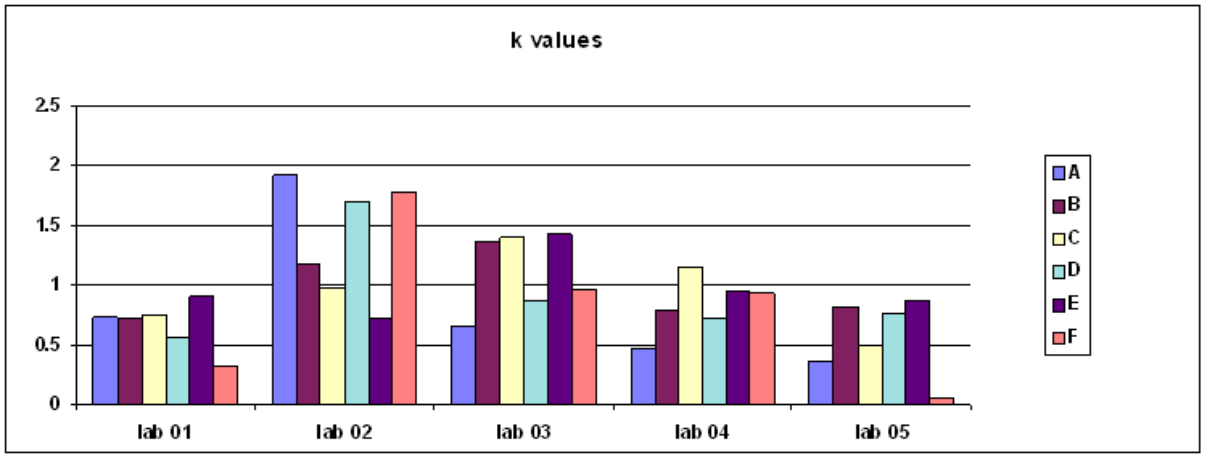


Figure 1: h-values for six seed lots of *Phaseolus vulgaris* following three test runs of the conductivity test in five laboratories

Run 1



Run 2



Run 3

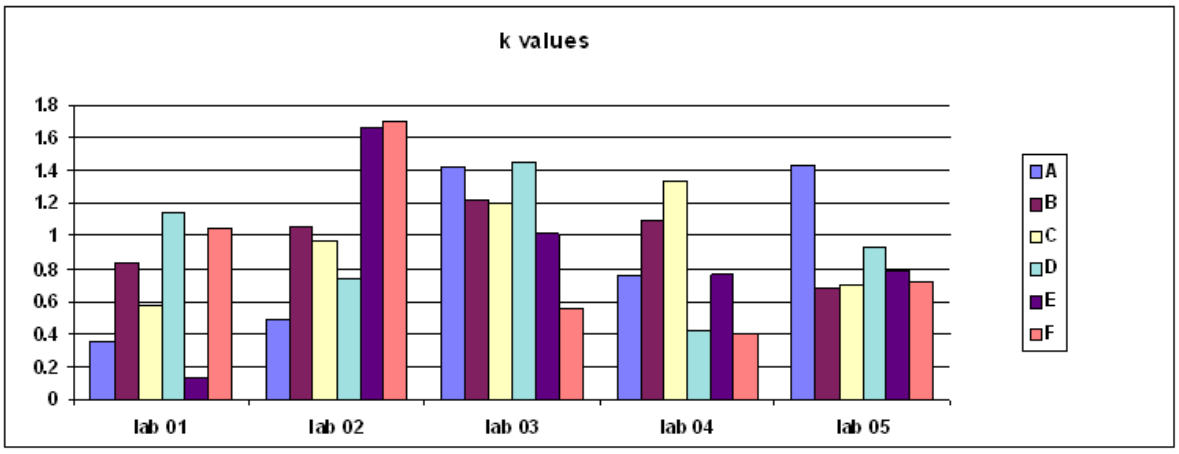


Figure 2: k-values for six seed lots of *Phaseolus vulgaris* following three test runs of the conductivity test in five laboratories

Evaluation of the controlled deterioration test as a repeatable and reproducible vigour test for *Brassica* species [Rules Proposal 2010 C.15.3.]

Alison A Powell

School of Biological Sciences, University of Aberdeen, 23 St Machar Drive, Aberdeen AB24 3UU, UK

a.a.powell@abdn.ac.uk

Summary

Five or six seed lots of Brassica species having standard laboratory germinations above 90% were used in three comparative tests of the Controlled Deterioration (CD) vigour test in 1995-1998, 1998-2001 and 2001-2004. Five lots of swede (*Brassica napus* var *napobrassica*) were used in the first two tests (different lots in each test) and six lots of oil seed rape (*Brassica napus* subsp *oleifera)* in the third test. Three repeat runs of the test were completed in 1995-1998 and 1998-2001 and two runs in 2001-2004. The CD test was carried out at 20% seed moisture content and 45°C for 24h before seeds were set to germinate at 20°C. CD germination was assessed as total germination (normal plus abnormal seedlings) in the first comparative test and as both total and normal germination in the other two tests. The CD test consistently identified differences between seed lots within laboratories and there were few significant differences in the results of different test runs within each laboratory. There were small, but sometimes significant differences in the overall seed lot mean from different laboratories, but each laboratory consistently identified the same lots as having low (lower CD germination) or high vigour (higher CD germination). The results support the validation of the CD test as a vigour test to be included within the ISTA Rules.

Introduction

There are currently two validated vigour tests, the accelerated ageing test and the conductivity test, both of which are applied to species of the grain legumes. There are no tests validated for small seeded vegetable species. However, vigour is also a problem for vegetable species, which is evident in both the emergence and storage potential of seeds. When low vigour seed lots are sown directly into the field or in glasshouses for transplant production, emergence is slower and the final emergence is often lower. In addition, the transplants produced from low vigour seeds are variable in size as a result of the slow and asynchronous emergence. Low vigour lots also show a more rapid decline in seed quality during storage than do high vigour lots.

The Controlled Deterioration (CD) vigour test was developed as a test to identify differences in the vigour of small seeded vegetable species (Matthews, 1980; Powell and Matthews, 1981). The principle of the test is that of seed ageing, whereby the rate of ageing is increased at high temperature and moisture content. This is the same principle on which the accelerated ageing (AA) test is based. However, CD differs from AA, by raising the seed moisture content to a pre-determined level before the period of deterioration begins. All seed lots therefore have the same moisture content during the subsequent period of deterioration at high temperature (45°C) for 24 hours and hence undergo a pre-determined degree of deterioration. Following the period of deterioration, the seeds are germinated and the total germination (normal plus abnormal seedlings) counted (Matthews, 1980; Powell and Matthews, 1981). Deterioration moves the sample of the seed lot along the seed survival curve (Figure 1). A high vigour lot (e.g. lot A, Figure 1), retains a high germination after CD (CD germination), whereas that of lots having lower vigour decreases (e.g. lots B and C, Figure 1)

The results of the CD test, expressed as the total germination, have been shown to relate to the emergence and storage potential of many species. CD results were statistically significant indicators of the field emergence of nine crops in each of two years (between 11 and 30 lots per crop) (Matthews, 1980). The crops included, small seeded vegetable species (turnip [*Brassica campestris* var *rapa*], swede [*Brassica napus* var

napobrassica], kale [*Brassica oleracea* var *acephala*], Brussels sprouts [*Brassica oleracea* var *gemmifera*], carrot [*Daucus carota*], lettuce [*Lactuca sativa*] and onion [*Allium cepa*]). The relationship between field emergence and CD in Brassicas was supported by the work of the ISTA CD working group in 1999 (Powell and Matthews, 2005) and by Powell and Dutton (1984). In addition, the CD Working Group noted a correlation between the CD test results and the rate of emergence of swede (Powell and Matthews, 2005), as did Larsen *et al*. (1998) for oilseed rape (*Brassica napus* subsp *oleifera*). Correlations between CD results (total germination %) and field emergence have also been seen in vining peas (Bustamente *et al*., 1984), combining peas (Powell *et al*., 1997), Italian ryegrass (Marshall and Naylor, 1985), pepper (Kavak *et al*., 2008), and in watermelon (*Cucumis melo*) at high and low temperatures and under mechanical stress (Mavi and Demir, 2007). Mavi and Demir (2007) also showed a correlation between total CD germination and compost emergence under salinity stress. Wang *et al*. (1994) reported that normal, as well as total CD germination correlated with the emergence of six lots of red clover at eight sowing dates in one year, with similar correlations found for both methods of germination assessment.

Emergence of vegetable species under controlled glasshouse production has also been predicted by total germination after CD. Thus the total CD germination of commercially acceptable lots of several Brassica crops (cauliflower [*B. oleracea* var *botrytis*], Brussels sprouts [*B. olearacea* var *gemmifera*], cabbage [B. *oleracea* var *capitata*] and calabrese [*B. oleracea* var *italica*]) was correlated with seedling performance (emergence, rate and spread of emergence and variation in seedling size) in modules (Powell *et al*., 1991). Similar findings have been reported recently for aubergine (Demir *et al*., 2005) and peppers (Basak *et al*., 2006).

The CD test also predicts seed storage potential, with a clear relationship seen between the total germination after the CD test and germination after commercial storage of 29 seed lots of Brussels sprouts (Powell and Matthews, 1984b), 15 lots of onions (Powell and Matthews, 1984a), 13 lots of peppers (Basak *et al*., 2006) and watermelon (9 lots), melon (12 lots) and cucumber (7 lots) (Demir and Mavi, 2008). The storage potential of rye during 80 days natural storage was also predicted by CD (Steiner and Stahl, 2002).

The CD test has therefore been shown to identify differences in the vigour of many vegetable species. Previous work has shown the repeatability of the test within and between six laboratories in the UK (Powell *et* al., 1984). The aim of the comparative tests reported here was to demonstrate the repeatability and reproducibility of the test, as applied to Brassica species, in ISTA laboratories in several countries.

Materials and methods

Three comparative tests of the controlled deterioration (CD) vigour test were carried out, one in each of the periods 1995-1998, 1998-2001 and 2001-2004. Five seed lots were tested in each of three test runs in 1995-1998 and 1998-2001 and six seed lots were tested in two test runs in 2001-2004. Six laboratories participated in the tests, namely, (1) Official Seed Testing Station for England and Wales, Cambridge, UK; (2) SNES - GEVES, Angers, France; (3) LaRAS, University of Bologna, Italy; (4) Danish Plant Directorate, Lyngby, Denmark; (5) National Institute for Agricultural Quality Control, Budapest, Hungary; and (6) the University of Aberdeen, UK. Not all laboratories were able to participate in all three comparative tests. However, three laboratories from these were able to participate in all the tests.

Seed material

Seed lots of swede (*Brassica napus* var *napobrassica*) were obtained from Sharpes International, Sleaford, Lincolnshire, UK in 1995-1998 and 1998-2001. Seed lots of oil seed rape (*Brassica napus* subsp *oleifera)* were provided by SNES-GEVES in 2001-2004. All seed lots had standard germination values of greater than 93%. These species were used as typical examples of the Brassicaceae, since it was difficult to source and finance the supply of expensive F_1 hybrid vegetable Brassica seeds for the comparative tests. Previous work (Matthews, 1980) has shown that members of the Brassicaceae respond in a similar way to CD, and correlations between the results of the CD test and expressions of vigour have been shown for many species from this family (see above). The seed of each lot was packed in individual aluminium foil packets and sent to each laboratory from Aberdeen, UK. When laboratories received the seed, it was held in a refrigerator at 5-10^{o}C. The first tests took place within 8 weeks of receiving the seed and the time between test runs did not exceed 6 weeks.

The test procedure was conducted as follows:

Adjustment of seed moisture content :

The initial moisture content (mc) of the submitted sample was determined using the constant temperature oven method (17 hours at 103°C) according to Chapter 9 of the ISTA Rules (ISTA, 2008). This is subsequently referred to as the initial seed mc. To adjust the seed moisture content, the fraction of pure seed was mixed thoroughly and four replicates of at least 100 seeds drawn randomly. Each sub-sample was weighed to four decimal places. The seed mc of each replicate was then raised to 20%. The weight of seed at this mc is calculated as:

Weight of replicate at 20% mc = initial seed weight x $\frac{(100\text{-initial seed mc})}{(100 - \text{desired mc})^*}$

*i.e 80

The required weight of seed was calculated correct to 3 decimal places

Each of the 4 replicates was set to imbibe on a moist germination paper. Each laboratory used their normal germination papers. They were advised that there should be no free water on the surface of the paper. Depending on the germination papers being used, 3-4 ml water per paper usually gives a moist but not wet paper. The same amount of water per paper was added on each test occasion.

The seeds were weighed regularly to determine when they reached the required moisture content. Weighing was accurate, correct to 3 decimal places. The time taken to reach the required mc varies depending on the seed lot, laboratory temperature etc, but is usually around 1.5h.

Once each replicate had reached the required weight, it was placed immediately into an aluminium foil packet, which was flattened with the edge of the hand to remove air and heat sealed approximately 3cm above the level of the seeds. The sealed packets were placed at 7 ±2°C for 24h to allow equilibration of the moisture within the seeds. Comparisons of seed mc before and after equilibration in 1998-2001 confirmed that there is no change in the seed mc during the equilibration period.

Deteriorating the seed

The four replicate packets of each seed lot were placed into a water bath at 45° ± 0.5 °C for 24h ± 15 minutes. When the packets were removed from the water bath, the seeds within the packets were cooled by placing the packets under cold running water for 5 minutes. Comparisons of seed mc before and after deterioration in 1998-2001 confirmed that there is no change in the seed mc during the period of deterioration.

Testing for germination

A germination test was set up using the deteriorated seed within 30 minutes of removing the seeds from the water bath, using 100 seeds from each replicate packet. The germination conditions used for the CD germination test for Brassica species were those outlined for the standard germination test in Chapter 5 of the ISTA Rules (ISTA, 2008) using 20°C as the germination temperature.

Calculation and expression of results

The results were expressed as the percentage total germination (i.e. percentage normal plus abnormal seedlings) in 1995-1998 and as both percentage total germination and percentage normal germination (normal seedlings only) in 1998-2001 and 2001-2004.

Statistical analysis

The effect of the different factors (laboratory, seed lot, test run) were analysed by variance analysis. ANOVA of arc sine transformed data was used to compare means using the LSD method at the 5% significance level.

Repeatability and reproducibility were analysed with the statistical tool developed by S. Grégoire (2007) according to ISO 5725-2 (ISO/IEC Guide 43-1, 1997) and available for download at the ISTA website: http://www.seedtest.org/upload/cms/user/ISO572511.zip

Results

Initial explorations of the data were conducted using side-by-side boxplots. This revealed that in 2001-2004, Lab 1 exhibited particularly low values for both total germination and normal germination. Consultation with the laboratory revealed that in this comparative test, an analyst having little experience with the CD test had carried out the test. Lab 1 was therefore excluded from the analysis of the data in 2001-2004.

ANOVA

Details of the ANOVA analysis can be found in Appendix 1.

1995-1998

The data described for 1995-1998 is that for the total germination after CD only.

Comparisons of the overall means for each lot (3 runs per lot per laboratory) revealed that lot 1 had the lowest vigour (82% CD germination, significant at $p < 0.05$), followed by lots 2, 3, and then lots 4 and 5 (both high vigour) (Table 1). The differences in vigour were consistently identified by all laboratories (Table 1). Comparisons of overall laboratory means (Table 1) showed only small differences between laboratories although lab 3 had a significantly higher overall mean at 97% (5 lots x 3 runs) (Table 1), followed by lab 4, with labs 1 and 2 having the lowest mean CD results. Comparisons within each lab (Table 2) revealed only one instance (lab 2) where the mean of any one run differed from other runs (Table 2). In addition, in each of the three runs (Appendix 2a), each lab picked out lot 1 as having the lowest CD germination (lowest vigour). Lots 3, 4 and 5 consistently had the highest CD germination and lot 1, the lowest. Thus each laboratory performed the test consistently.

1998-2001

Both total germination and normal germination were recorded after the CD germination test.

Total germination

The overall means for the five lots (Table 3) identified lots 3 and 4 as having the highest CD germination (high vigour), followed (in decreasing order) by lots 2, 1 and 5 (low vigour). The same differences in vigour were identified in each laboratory (Table 3). There were small, but significant differences in the overall CD germination results from the laboratories (Table 3). However, as seen in the previous comparative test (1995-1998), comparisons of the runs within the laboratories revealed only small differences in the means of runs (Table 4), although these were significant in laboratories 2 and 3. In each of the three runs, all three laboratories consistently identified lots 3 and 4 as having the highest CD germination (high vigour), with lot 5 the lowest i.e. low vigour (Appendix 2b).

Normal germination

The normal germination after CD (both the overall lot means and the means within each lab) revealed the same ranking of the lots as did the total germination, with the highest normal CD germination recorded for lots 3 and 4, followed by lots 2, 1 and 5 (Table 5). Laboratory 2 had a slightly, and significantly, higher overall mean than laboratories 1 and 3 (Table 5). Within each laboratory there were small differences in the means of the three runs (Table 6), although these were significant only for one run in laboratory 3. The three laboratories consistently ranked the same lots as high and low vigour in each of the three runs (Appendix 2b).

2001-2004

Both total germination and normal germination were recorded after the CD germination test.

Total germination

Comparison of the overall seed lot means (5 laboratories x 2 runs, Table 7) identified lots 1 and 5 as having significantly lower CD germinations than the other four seed lots. These lots were also identified as low vigour lots in each laboratory. There were only small, although significant, differences in the mean overall

CD germination of the laboratories (Table 7). Comparison of the runs within each laboratory (Table 8) revealed no significant differences in the results of the runs.

Normal germination

As seen for the total CD germination, lots 1 and 5 were identified as low vigour (Table 9), but in this case, lot 2 also had a similar normal CD germination. Lot 3 had the highest normal germination and hence the highest vigour (Table 9). There were significant differences in the overall means of the laboratories, with lab 2 having a lower overall mean than the other laboratories (Table 9). However, the only significant difference between the runs was seen in laboratory 3 (Table 10) and the differences between lots were consistent in each run and laboratory (Appendix 2c).

Reproducibility and repeatability analysis

The statistical tool developed by S. Grégoire, based on ISO 5725-2, allows the calculation of h- and k-values. The h-values show the tendency for a laboratory to give over-estimations or under-estimations compared to the mean of all the results available whereas the k-values give a measure of the variability of the repeats. Higher values indicate greater under- or over-estimations (h-values) or greater variability between replicates (k-values)

1995-1998

Out of 60 CD germination test results (4 labs x 5 lots x 3 test runs) there were only four instances in which the h-values (Figure 2a-c) were significant, indicating an over- or underestimation of the CD germination. These occurred in laboratory 3 where the h-values for lots 2, 3 and 5 were significant ($p<0.05$) in run 2 (Fig 2b) and for lot 5 in run 1 (Figure 2a).

Similarly, significant k-values (Figure 3a-c), indicative of greater variability of the repeat tests, were observed for only four germination test results (out of 60), namely for lots 1 and 2 (both $p<0.01$) in run 1, laboratory 1 (Figure 3a), and lots 4 and 5, laboratory 2 ($p<0.05$) in run 2 (Figure 3b).

1998-2001

Total germination

There were no significant h-values in the three labs (Figure 4a-c) indicating that no laboratory tended to under or over estimate the CD germination.

In general, the k-values (Figure 5a) revealed that no laboratory showed consistently higher variability between replicates than any other in the three test runs. In each run there were instances where the k-value was significant indicating greater variability but these were not associated with individual labs or lots. Thus significant k-values were found in run 1 for lot 3, lab 1 and lot 4, lab 2 (Figure 5a), in run 2 for lot 2, lab 1 (Figure 5b) and in run 3 for lot 5, lab 2 and lot 2, lab 3 (Figure 5c).

Normal germination

As seen for the total germination data, the h-values (Figure 6a-c) revealed no significant under- or over-estimation of the CD germination.

Again, no laboratory showed consistently higher variability between replicates than any other in the three test runs. Significant k-values (greater variability) were found only in run 1 for lot 3, lab 1 (Figure 7a), in run 2 for lot 5, lab 1 (Figure 7b) and in run 3 for lot 5, lab 2 (Figure 7c).

2001-2004

Total germination

There were only three significant h-values, indicating under or over estimation of the CD germination. These occurred in run 1 for single lots in different labs, namely lab 3, lot 4; lab 4, lot 3; lab 5, lot 5 (Figure 8a), where CD germination was underestimated.

Significant k- values, indicating variability between replicates in the CD germination test, were also seen only for single lots in different laboratories, namely, in run 1, lab 2, lot 6, lab 3, lot 4; lab 5, lot 5 (Figure 9a) and in run 2, lab 2, lot 5 (Figure 9b).

Thus there was no evidence of consistent variation associated with the different laboratories.

Normal germination

As seen for the total germination data, there were few significant h-values. These occurred only in lab 2 (run 1, lots 1 and 5, Figure 10a; run 2, lots 1 and 4, Figure 10b). Consistently higher variability between replicates (h-value) was found only in run 1 for lots 1, 4 and 6 in lab 2 (Figure 11a). There were no significant h values in run 2 (Figure 11b).

Repeatability and reproducibility values

Repeatability expresses the evaluation of the variability of the results obtained in different repeats of the same sample in one laboratory where all elements of the test are the same for each repeat. The higher the repeatability value, the less repeatable is the test. Reproducibility is the sum of the repeatability plus the variability observed between different laboratories analysing the same sample. Again, higher reproducibility data indicate lower reproducibility.

The repeatability and reproducibility data for all comparative tests of CD are shown in Table 11. In general lower germination results will lead to higher values of both repeatability and reproducibility, indicating that both aspects of the test are poorer. Thus, in the Controlled Deterioration test, the low vigour lots, with lower CD germination values would be expected to have higher values. This was indeed the case for the seed lots identified as having markedly lower vigour, namely lot 1 in 1995-1998, and lot 5 in 1998-2001.

There has been no previous analysis of vigour test data using ISO 5725-2 to which the current data can be compared, although data from standard germination tests have been analysed by Fiedler *et al*. (2008) for pea and by Ducournau *et al* (2007) for sunflower. However, it is difficult to compare values for repeatability and reproducibility from previous trials since both values depend on the scale and unit of measurement. Bearing this limitation in mind the current data were compared with those from the previous analyses. The values for repeatability (0.9789 -11.9443; Table 11a) and reproducibility (1.1134 – 18.2301; Table 11b) in the CD test were largely comparable with those observed by Ducournau *et al* (2007) in a standard germination test (Appendix 3a), even though the CD germination data came from seeds that had been aged in the CD test. Where the CD germination was high (i.e. high vigour seed), the repeatability and reproducibility values were also comparable with those obtained for pea by Fiedler *et al* (2008) (Appendix 3b).

Discussion

Differences in seed lot germination after the Controlled Deterioration test were consistently identified in repeat test runs of the test during comparative tests in each of three periods of work for the Vigour Committee (1995-1998, 1998-2001, 2001-2004). Assessment of the total germination after CD has shown that in a wide range of species, seed lots having low total germination after CD are low vigour lots that show poor emergence characteristics and storage potential, while high germination after CD is typical of high vigour lots (see Introduction). Assessment of normal germination after CD, and its relation to vigour has only been illustrated in red clover (Wang *et al*, 1994), where both normal and total CD germination predicted emergence differences equally well. Nevertheless, the assessment of normal germination after CD was also consistent in all the comparative tests. This suggests that where total germination is high, differences in normal germination could be used to give guidance regarding vigour differences.

The CD germination data represents germination after a period of ageing and aged seed lots are well known to show greater variability. Nevertheless, the levels of repeatability and reproducibility observed for CD germination were comparable with those for standard germination tests in other species (Ducournau *et al* 2007; Fiedler *et al*, 2008).

Application of the AA tolerance tables to the CD data revealed few instances where data were out of tolerance. Comparisons of the repeat runs of the test for each lab and lot (75 comparisons) revealed only two occasions (Appendix 2) where the data for the three runs were out of tolerance. Similarly, comparison of the seed lot means for total and normal germination in the different laboratories (27 comparisons) found only three occasions (Appendix 2) when the data for the laboratories were out of tolerance.

Exclusion of laboratory 1 in 2001-2004 served to emphasise the importance of analyst experience in completion of the test. In previous comparative tests, the data from laboratory 1 were in tolerance with the other laboratories, but in 2001-2004, a less experienced analyst had carried out the tests. Even so, the repeatability between runs within this lab was good (data not presented), which suggested that there was likely to be a consistent error by the analyst. Consultation with the laboratory revealed that the error was associated with recognition of abnormal seedlings and not with the completion of the test.

In conclusion, the results of this study reveal that the Controlled Deterioration test can be applied consistently both within and between laboratories to seeds of Brassica species. This supports the inclusion of the Controlled Deterioration test in the ISTA Rules as a vigour test for Brassica species.

Acknowledgements

I am grateful to all of those who have contributed to the preparation and completion of these comparative tests and in particular Louise Yule (University of Aberdeen, UK), Anders Lomholt (Plant Directorate, Denmark), Katalin Ertsey (National Institute for Agricultural Quality Control, Hungary), Marie-Hélène Wagner (SNES, France), Emanuela Casarini (LaRAS, Italy) and Steve Jones (OSTS for England and Wales, UK).

I also thank all the Vigour Committee for their review of this manuscript

References

Basak, O., Demir, I., Mavi, K. and Matthews, S. (2006). Controlled deterioration test for predicting seedling emergence and longevity of pepper (*Capsicum annuum* L.) seed lots. *Seed Science and Technology*, **34**, 723-734.

Bustamente, L, Seddon, R.D., Don, R. and Rennie, W.J. (1984). Pea seed quality and seedling emergence in the field. *Seed Science and Technology*, **12**, 551-557.)

Demir, I., Ermis, S., Okcu, G. And Matthews, S. (2005). Vigour tests for predicting seedling emergence of aubergine seed lots. *Seed Science and Technology*, **33**, 481-484.

Demir, I. and Mavi, K. (2008). Controlled deterioration and accelerated aging tests to estimate the relative storage potential of cucurbit seed lots. *HortScience*, **45**, 1544-1548.

Ducournau, S. Garreau, P. and Léchappé, J. (2008). Effect of temperature and growing media on sunflower germination. *Seed Testing International*, **135**, 34-36.

Fiedler, K.A, Gutormson, T.J., Brix-Davis, K.A. and Carlson, L.N. (2008). Evaluation of crepe cellulose paper covered with sand as an ISTA medium. *ISTA Method Validation Reports*, **2008**, 9-42.

Gregoire, S. (2007). A tool to assess repeatability and reproducibility in inter-laboratory tests according to ISO 5725-2 definitions. ISTA, Statistical Tools for Seed Testing: Interlaboratory testing using ISO 5725 URL:http://www.seedtest.org/en/content---1--1143.html

ISO/IEC Guide 43-1 (1997) Proficiency Testing by Interlaboratory Comparison – Part 1: *Development and Operation of Proficiency Testing Schemes.* International Organisation for Standardisation, Geneva, Switzerland.

ISTA (2008). International Rules for Seed Testing. International Seed Testing Association, Zurich.

Kavak, S, Ilbi, H and Eser, B. (2008). Controlled deterioration test predicts vigour and field emergence in pepper seed lots. *Seed Science and Technology*, **36**, 456-461.

Larsen, S. U., Poulsen, F. U., Erikson, E. N. and Pedersen, H. (1998). The influence of seed vigour on field performance and the evaluation of the applicability of the controlled deterioration vigour test in oil seed rape (*Brassica napus*) and pea (*Pisum sativum L*). *Seed Science and Technology*, **26**, 627-641.

Marshall, A.H. and Naylor, R.E.L. (1985). Seed vigour and field establishment in Italian ryegrass *Lolium multiflorum* Lam.). *Seed Science and Technology*, **13**, 781-794.

Matthews, S. (1980) Controlled deterioration: a new vigour test for crop seeds. In *Seed Production* (ed. P. Hebblethwaite.) pp. 647-660. Butterworths, London.

Mavi, K. and Demir, I. (2007). Controlled deterioration and accelerated ageing tests to predict seedling emergence of watermelon under stressful conditions and seed longevity. *Seed Science and Technology*, **35**, 445-459.

Powell, A.A., Don, R., Haigh, P., Phillips, G., Tonkin, J.H.B. and Wheaton, O.E. (1984). Assessment of the repeatability of the controlled deterioration vigour test both within and between laboratories. *Seed Science and Technology*, **12**, 421-427.

Powell A.A. and Dutton, K.J. (1984). Assessment of the quality of swede sown in NE Scotland and possible causes of poor seed quality. In *Proceedings of Better Brassicas '84*, St Andrews 1984, 166-170.

Powell, A.A. and Matthews, S. (1981). Evaluation of controlled deterioration, a new vigour test for small seeded vegetables. *Seed Science and Technology*, **9**, 633-640.

Powell, A.A. and Matthews, S. (1984a). Prediction of the storage potential of onion seed under commercial storage conditions. *Seed Science and Technology*, **12**, 641-647.

Powell, A.A. and Matthews, S. (1984b). Use of the controlled deterioration test to predict the retention of vigour and viability during storage of Brussels sprouts seeds. *Seed Science and Technology*, **12**, 649-657.

Powell, A.A. and Matthews, S. 2005. Controlled deterioration vigour test. *Seed Testing International*, **129**, 21-24. International Seed Testing Association, Zurich Switzerland.

Powell, A.A,, Ferguson, A.J. and Matthews, S. (1997). Identification of vigour differences among combining pea (*Pisum sativum*) seed lots. *Seed Science and Technology*, **25**, 443-464

Powell, A.A., Thornton, J.M. and Mitchell, J.A. (1991)**.** Vigour differences in Brassica seed and their significance to emergence and seedling variability. *Journal of Agricultural Science, Cambridge*, **116**, 369-373.

Steiner, A.M. and Stahl, M. (2002). Vigour rating of rye varietal categories (*Secale cereale* L.) using controlled deterioration testing. *Seed Science and Technology*, **14**, 705-714.

Wang, Y.R., Hampton, J.G. and Hill, M.J. (1994). Red clover vigour testing-effects of three test variables. *Seed Science and Technology*, **22,** 99-105.

Table 1: Comparison of laboratories and lots: total CD germination 1995-1998

Laboratory	Seed lot					Mean
	1	2	3	4	5	
1	*75.75[a]	92.42[b]	94.25[bc]	97.25[d]	96.08[cd]	90.95[ab]
2	*78.42[a]	89.17[b]	93.08[c]	96.58[d]	95.58[d]	90.57[a]
3	*92.50[a]	96.25[b]	99.25[c]	99.25[c]	99.33[c]	97.32[c]
4	*82.58[a]	89.33[b]	95.42[c]	96.25[c]	94.67[c]	91.65[b]
Mean	82.06[a]	91.79[b]	95.50[c]	97.33[d]	96.42[d]	

Lab x Lot means that share common letters indicate groups of means are not significantly different at the 5% level using the LSD method

The comparisons are made for a given laboratory

* Indicates that the data for the four laboratories are not in tolerance, based on the tolerance tables for the accelerated ageing test

Table 2 Comparison of run means within laboratories: total CD germination 1995-1998.

ANOVA completed for each laboratory separately

Laboratory	Run		
	1	2	3
1	90[a]	92[a]	91[a]
2	92[b]	91[a]	91[a]
3	98[a]	97[a]	97[a]
4	95[a]	92[a]	92[a]

Table 3 Comparison of laboratories and lots: total CD germination 1998-2001

Laboratory	Seed lot					Mean
	1	2	3	4	5	
1	80.75[b]	92.92[c]	97.25[cd]	97.92[d]	66.33[a]	87.03[a]
2	89.00[b]	95.67[c]	97.25[c]	97.00[c]	73.33[a]	90.45[b]
3	89.83[b]	95.58[c]	98.25[c]	98.75[c]	82.42[a]	92.97[c]
Mean	86.53[b]	94.72[c]	97.58[d]	97.89[d]	74.03[a]	

Lab x Lot means that share common letters indicate groups of means are not significantly different at the 5% level using the LSD method

The comparisons are made for a given laboratory

Table 4 Comparison of runs within laboratories: total CD germination 1998-2001

ANOVA completed for each laboratory separately

Laboratory	Run		
	1	2	3
1	87[a]	86[a]	85[a]
2	91[a]	93[b]	89[a]
3	92[a]	93[a]	96[b]

Table 5 Comparison of laboratories and lots: Normal CD germination 1998-2001

Laboratory	Seed lot					Mean
	1	2	3	4	5	
1	73.50[b]	86.17[c]	92.75[d]	94.08[d]	44.92[a]	78.28[a]
2	78.92[b]	91.08[c]	96.17[c]	94.83[c]	44.08[a]	81.02[b]
3	76.83[b]	88.33[c]	92.42[d]	95.67[d]	37.58[a]	78.17[a]
Mean	76.42[b]	88.53[c]	93.78[d]	94.86[d]	42.19[a]	

Lab x Lot means that share common letters indicate groups of means are not significantly different at the 5% level using the LSD method

The comparisons are made for a given laboratory

Table 6 Comparison of runs within laboratories: Normal CD germination 1998-2001

ANOVA completed for each laboratory separately

Laboratory	Run		
	1	2	3
1	80a	77a	78a
2	82a	81a	81a
3	75a	79b	81b

Table 7 Comparison of laboratories and lots: Total CD germination 2001-2004

Laboratory 1 excluded from analysis

Laboratory	Seed lot						Mean
	1	2	3	4	5	6	
2	84.13[a]	97.13[c]	98.38[c]	97.13[c]	91.00[b]	95.25[c]	93.83[a]
3	92.75[a]	96.25[ab]	97.38[b]	94.75[ab]	93.25[a]	95.13[ab]	94.92[ab]
4	94.75[a]	94.13[a]	96.38[a]	97.38[a]	97.75[a]	97.50[a]	96.31[bc]
5	98.00[ab]	99.13[b]	97.75[b]	98.38[ab]	92.25[a]	97.88[b]	97.23[c]
Mean	92.41[a]	96.66[b]	97.47[b]	96.91[b]	93.56[a]	96.44[b]	

Lab x Lot means that share common letters indicate groups of means are not significantly different at the 5% level using the LSD method

The comparisons are made for a given laboratory

Table 8 Comparison of runs within laboratories: Total CD germination 2001-2004

ANOVA completed for each laboratory separately

Laboratory	Run	
	1	2
2	94[a]	95[a]
3	95[a]	95[a]
4	95[a]	98[a]
5	97[a]	98[a]

Table 9 Comparison of laboratories and lots: Normal CD germination 2001-2004

Laboratory 1 excluded from analysis

Laboratory	Seed lot						Mean
	1	2	3	4	5	6	
2	59.75[a]	66.25[b]	77.00[c]	73.88[c]	56.63[a]	63.13[b]	66.10[a]
3	71.13[ab]	68.88[a]	86.25[c]	76.25[b]	*72.25[ab]	*73.00[ab]	74.63[b]
4	70.25[a]	71.63[a]	81.13[b]	82.63[b]	*78.63[b]	*82.25[b]	77.75[c]
5	73.13[a]	74.88[a]	87.25[b]	87.50[b]	*77.25[a]	*77.38[a]	79.56[c]
Mean	68.56[a]	70.41[a]	82.91[d]	80.06[c]	71.19[a]	73.94[b]	

Lab x Lot means that share common letters indicate groups of means are not significantly different at the 5% level using the LSD method

The comparisons are made for a given laboratory

* Indicates that the data from the different laboratories are not in tolerance, based on the tolerance tables for the accelerated ageing test

Table 10 Comparison of runs within laboratories: Normal CD germination 2001-2004

Laboratory	Run	
	1	2
2	65[a]	68[a]
3	73[b]	77[a]
4	78[a]	78[a]
5	80[a]	80[a]

Table 11 Values for a) repeatability (sr2) and b) reproducibility (sR2) for total (T) and normal (N) CD germination data in each of three sets of comparative tests

a) Repeatability (sr2)

Year	Run	Lot											
		1		2		3		4		5		6	
		T	N	T	N	T	N	T	N	T	N	T	N
1995-1998	1	4.3084		2.8723		1.5069		1.6266		1.0607			
	2	4.4064		2.1985		1.4216		1.8819		2.1937			
	3	5.1414		2.8099		2.0052		1.7440		1.7017			
1998-2001	1	3.4278	4.0757	1.9649	3.2072	1.6159	3.6286	1.4337	2.3921	8.7860	8.4738		
	2	4.6993	4.4033	2.6405	4.0414	1.6499	0.7993	0.7993	2.7285	6.6249	6.8516		
	3	3.2361	4.1999	3.6892	3.0459	1.2583	2.1538	0.8333	1.8929	11.9443	9.6652		
2001-2001	1	2.3004	5.5696	2.3761	5.7897	2.0666	3.8784	1.6956	5.1761	3.0104	4.6030	2.0104	5.2559
	2	2.0867	4.4230	0.9789	4.0466	1.1726	2.1360	1.4930	3.4339	3.7722	4.3036	2.1115	5.3968

b) Reproducibility (sR2)

Year	Run	Lot											
		1		2		3		4		5		6	
		T	N	T	N	T	N	T	N	T	N	T	N
1995-1998	1	13.6740		4.6480		1.6987		1.8930		1.6739			
	2	7.6349		3.1689		2.5638		2.7291		2.9861			
	3	8.6657		4.2205		4.4597		2.2523		2.3139			
1998-2001	1	5.9547	4.8883	2.2173	3.2178	2.3496	3.7777	3.4791	2.9119	11.8286	16.3307		
	2	6.1644	4.2353	3.8729	5.8166	1.9948	3.0619	1.0206	2.8137	12.7083	6.7345		
	3	6.6285	7.3143	3.3509	3.1192	1.1989	2.207	1.0508	1.7139	18.2301	8.5634		
2001-2001	1	9.8240	7.9967	4.1281	9.4113	2.2845	7.6587	2.9492	9.9724	5.0970	12.2130	2.6398	8.7375
	2	3.0190	7.0489	1.1134	5.2331	1.1411	3.3198	1.6394	6.0527	7.1662	10.5589	2.2845	10.0763

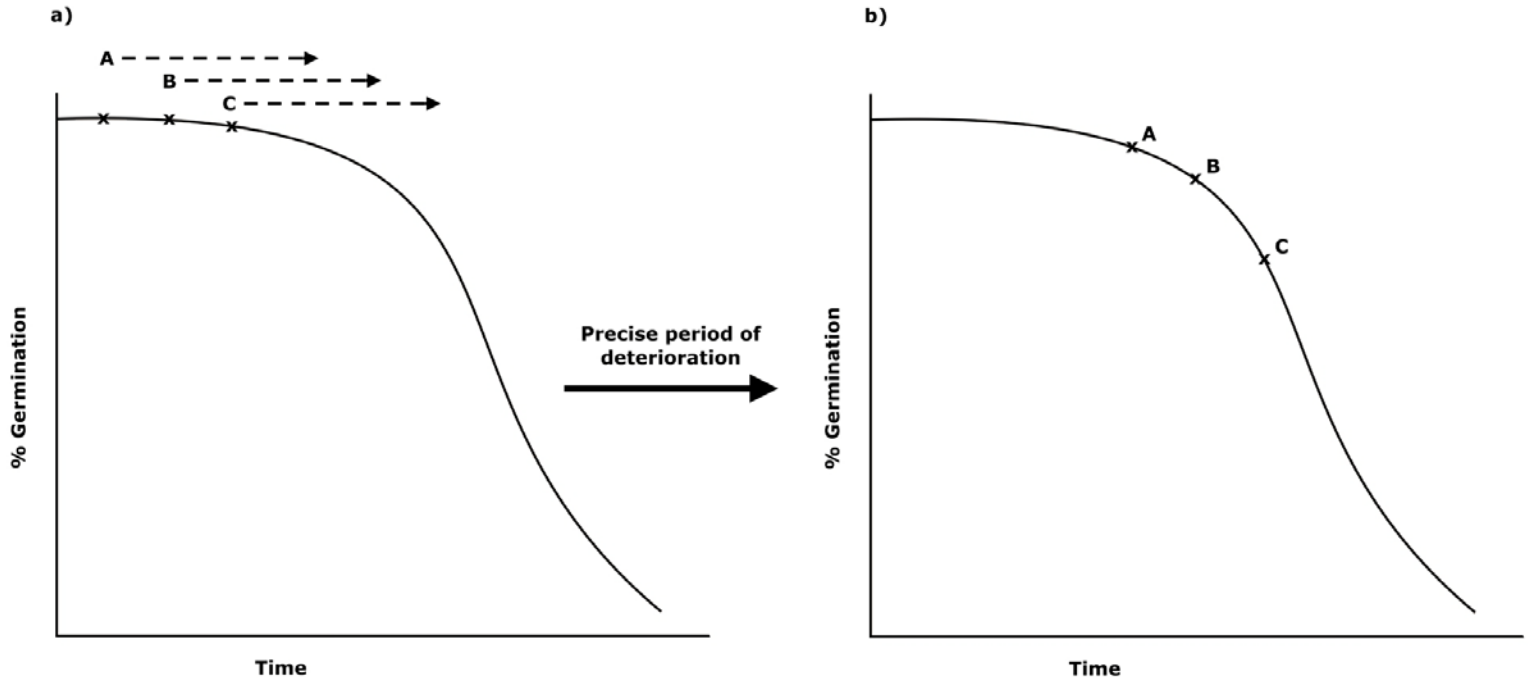
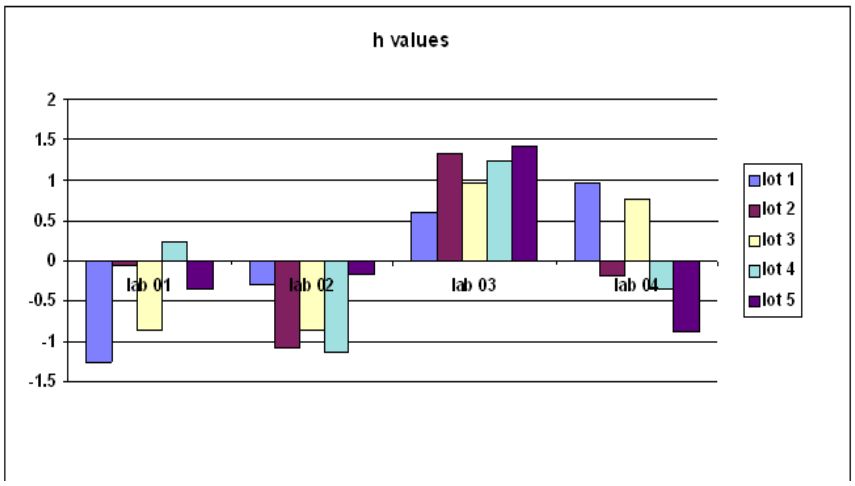
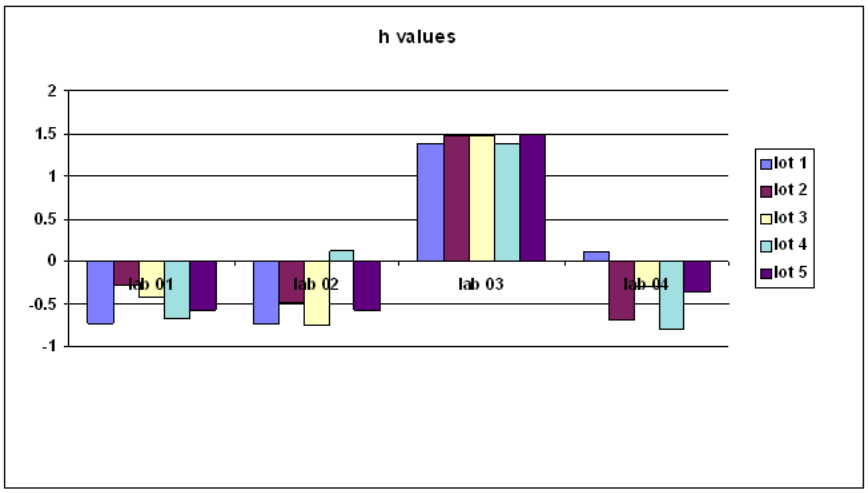


Figure 1: The effect of a predetermined period of deterioration during the Controlled Deterioration test on the germination of samples of three lots (A, B and C) having high germinations before CD

a) Run 1



b) Run 2



c) Run 3

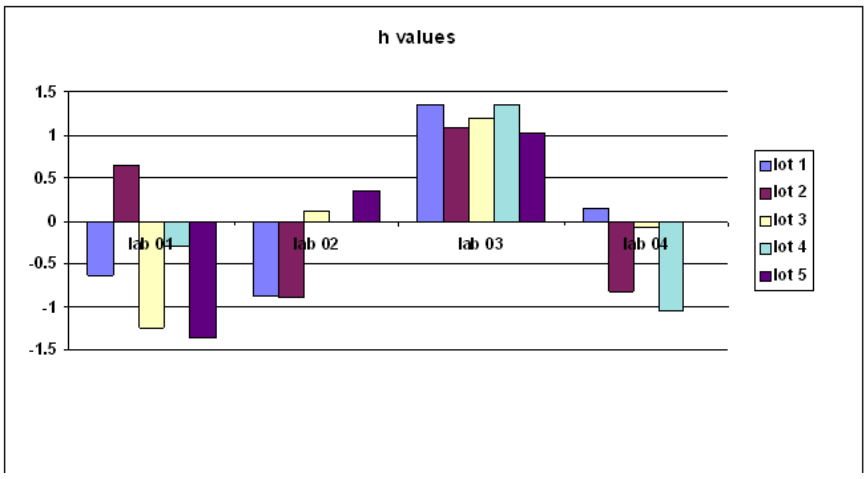
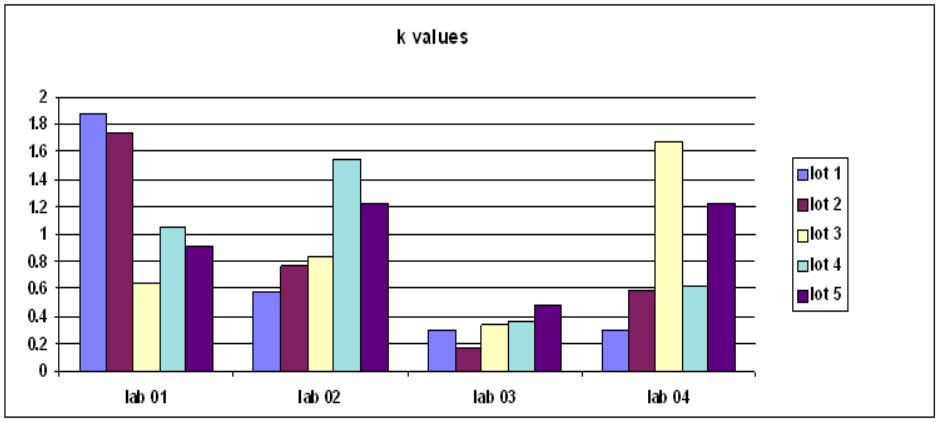
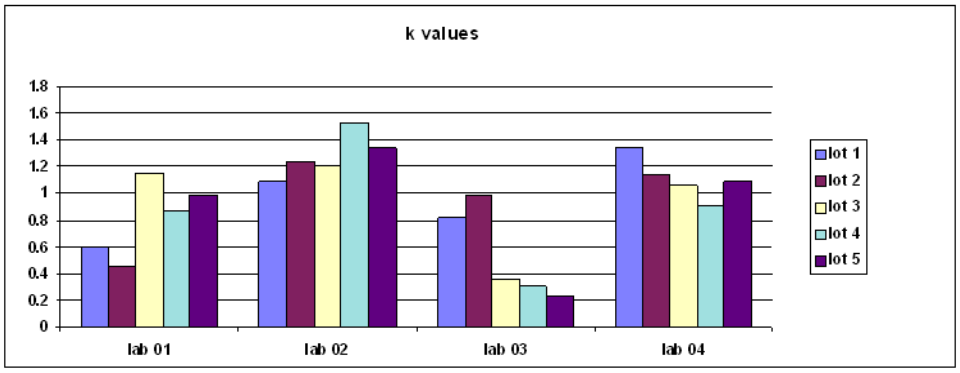


Figure 2: h values for total CD germination in each of 3 test runs in 1995-1998

a) Run 1



b) Run 2



c) Run 3

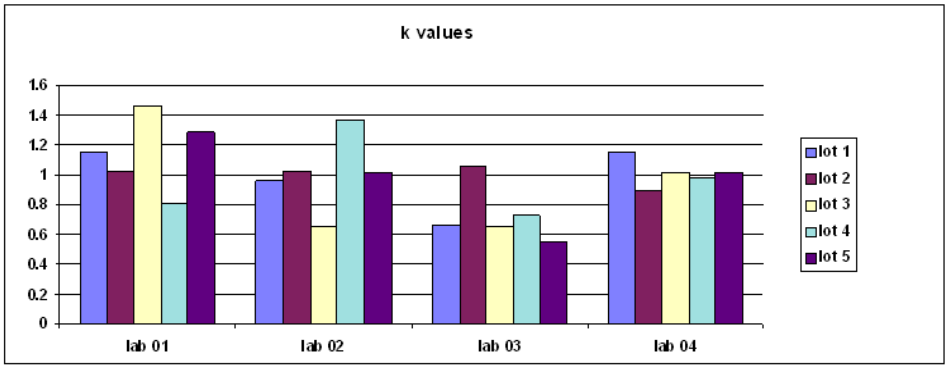
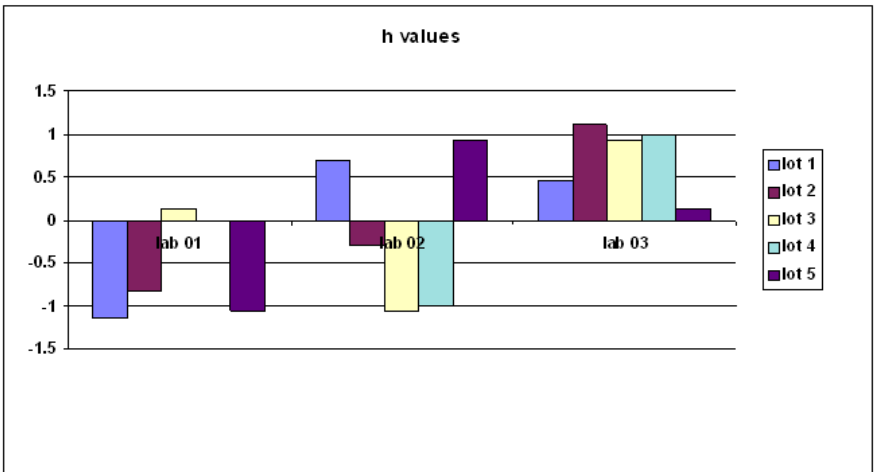
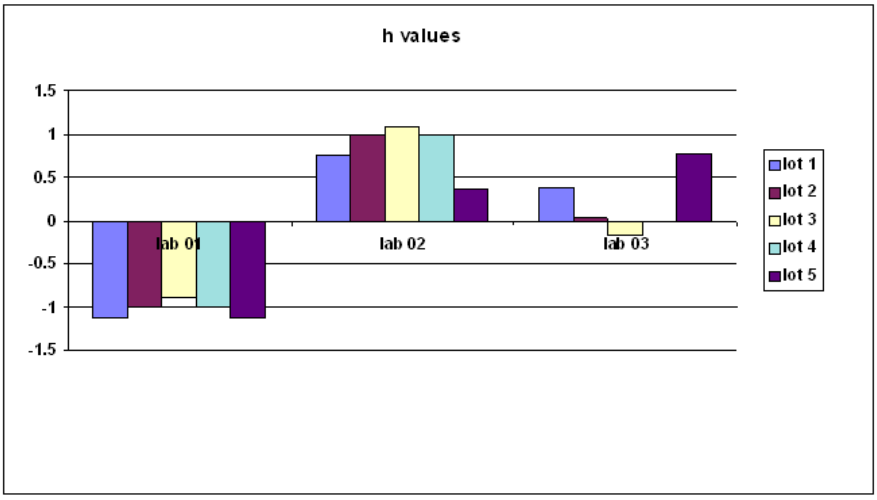


Figure 3: k values for total CD germination in each of 3 test runs in 1995-1998

a) Run 1



b) Run 2



c) Run 3

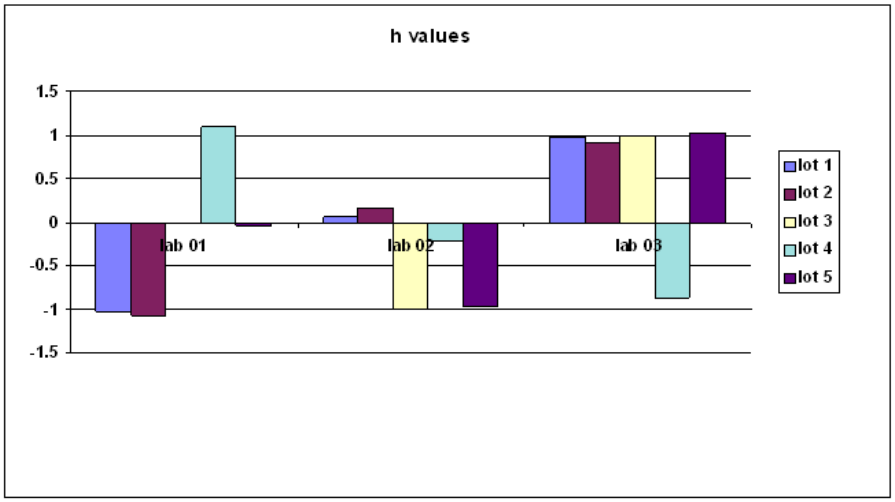
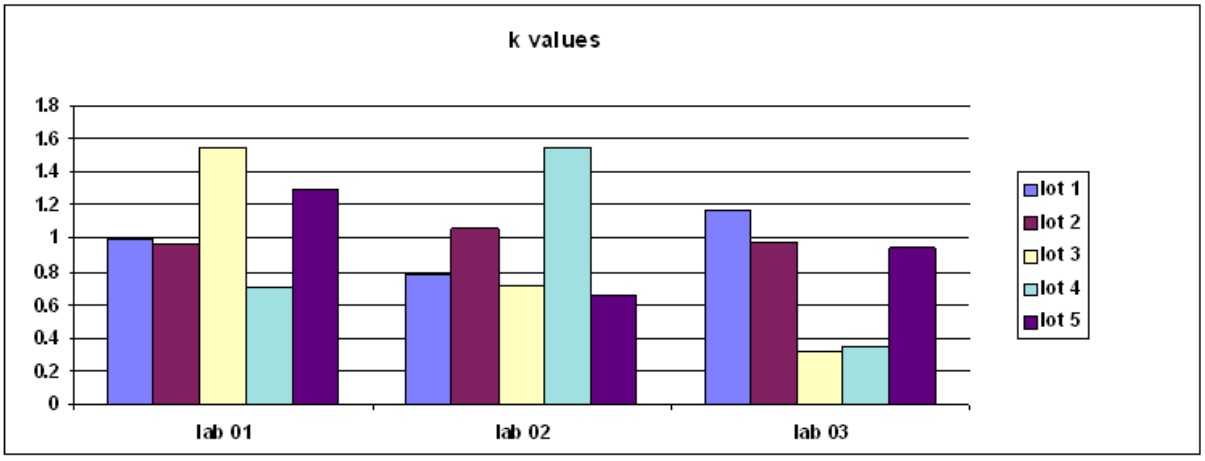
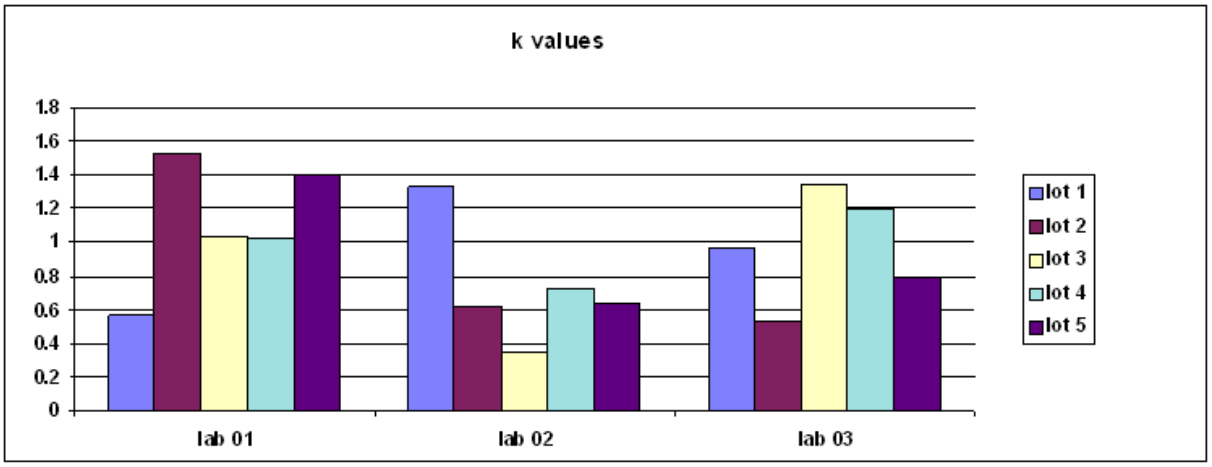


Figure 4: h values for total CD germination in each of 3 test runs in 1998-2001

a) Run 1



b) Run 2



c) Run 3

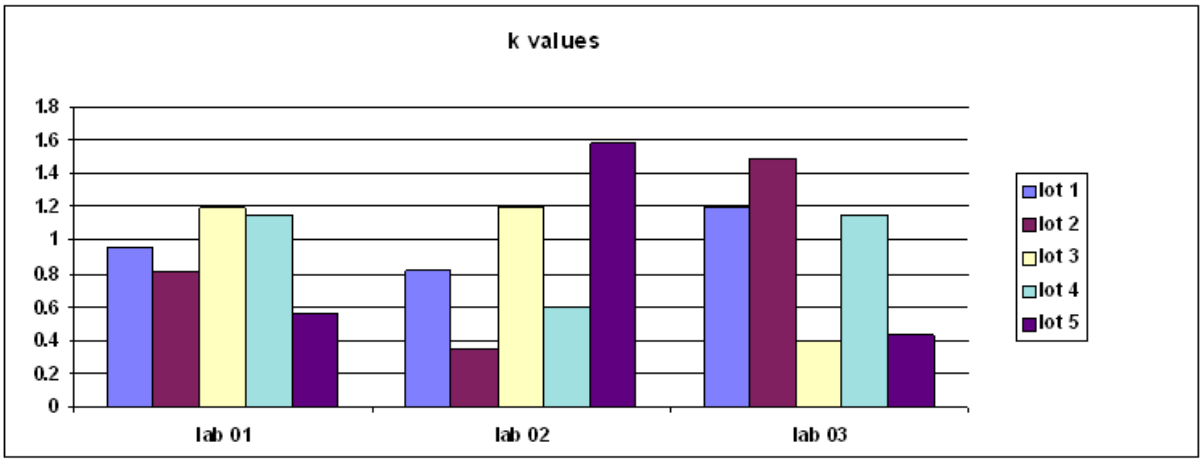
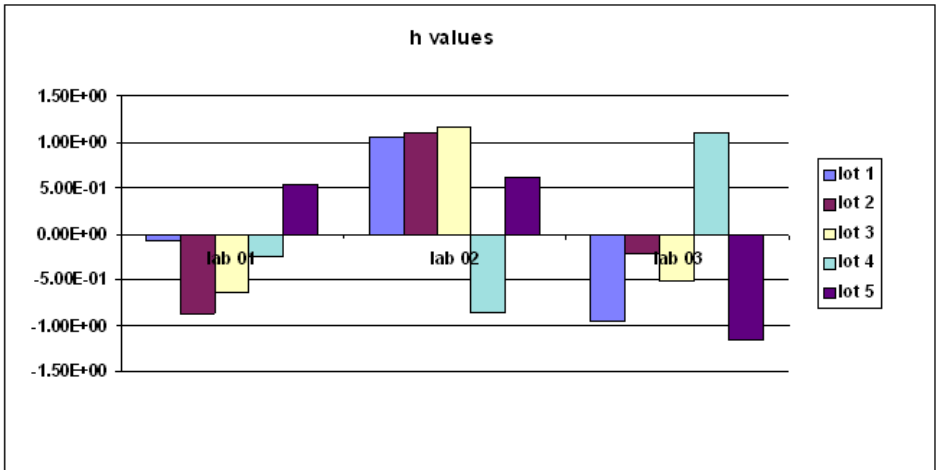
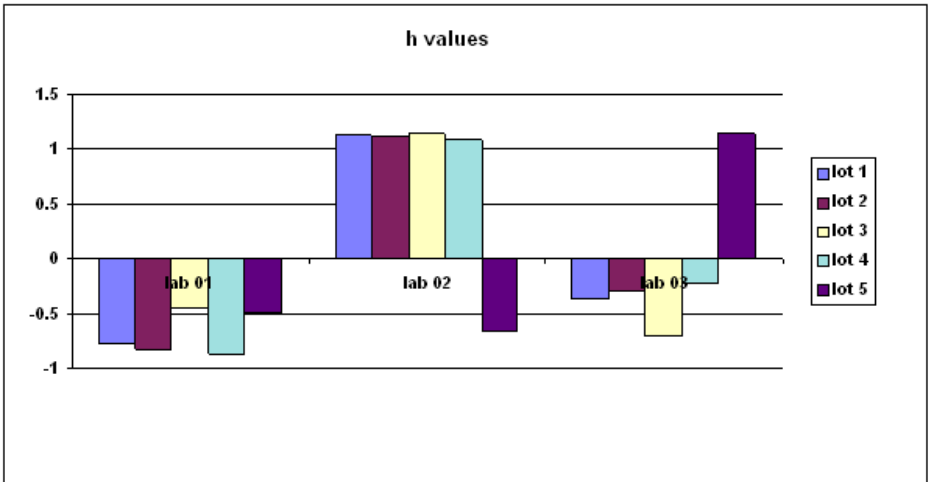


Figure 5: k values for total CD germination in each of 3 test runs in 1998-2001

a) Run 1



b) Run 2



c) Run 3

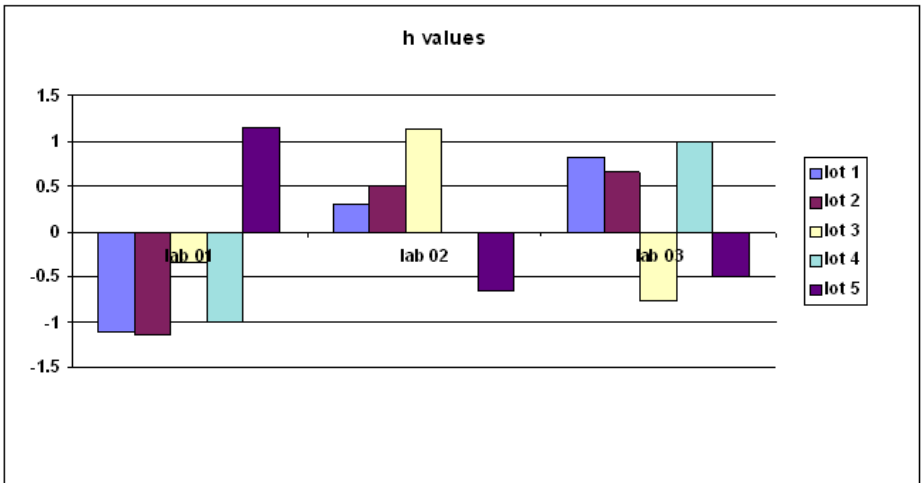
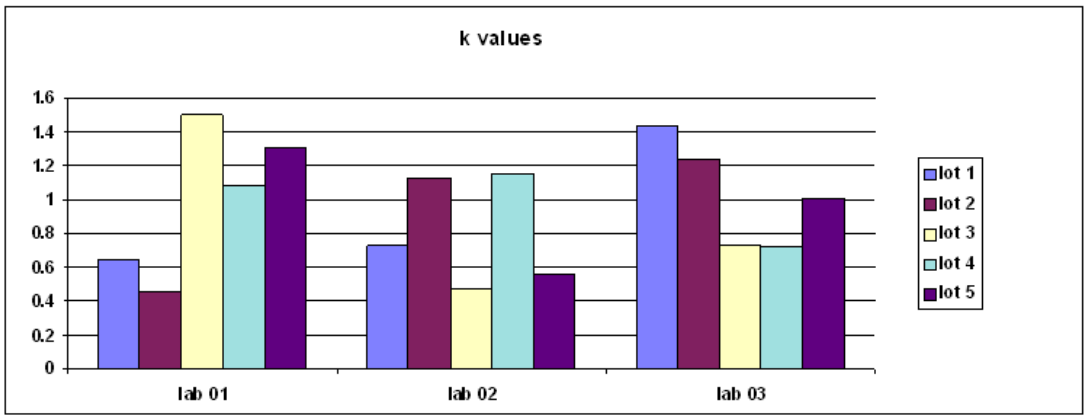
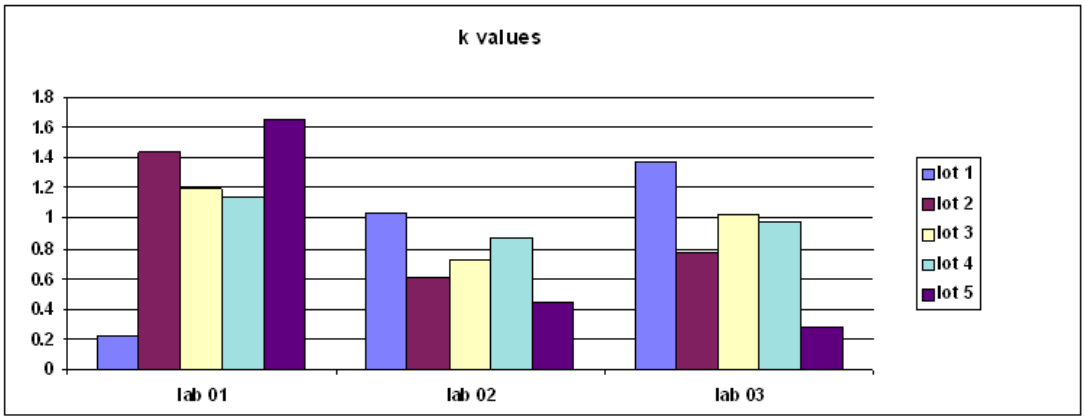


Figure 6: h values for normal CD germination in each of 3 test runs in 1998-2001

a) Run 1



b) Run 2



c) Run 3

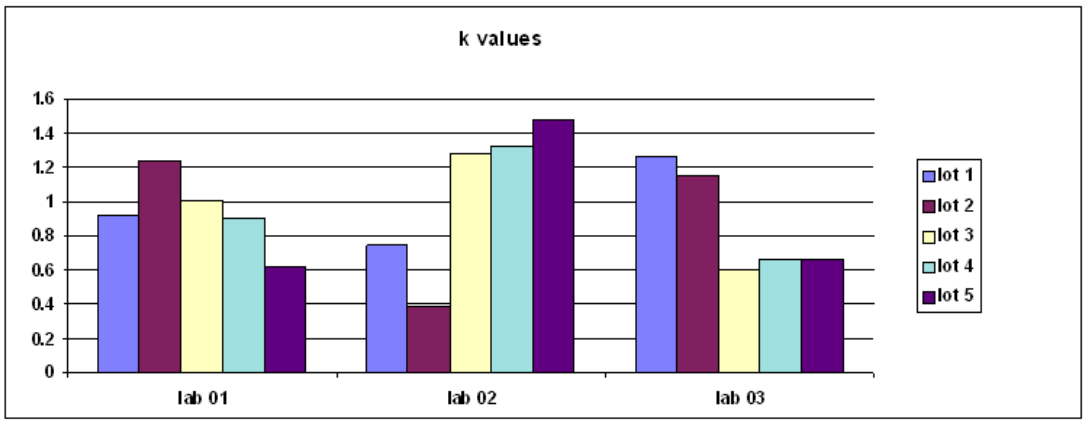
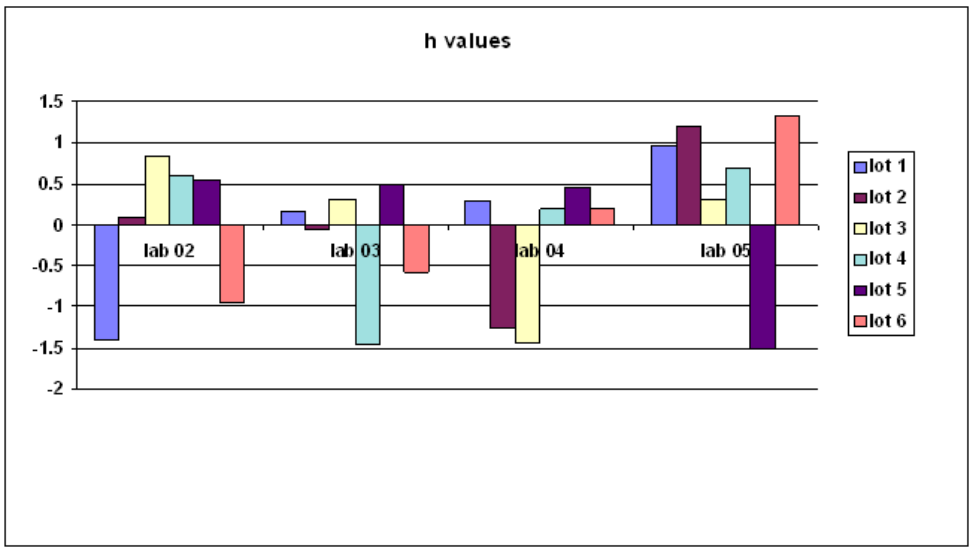


Figure 7: k values for normal CD germination in each of 3 test runs on 1998-2001

a) Run 1



b) Run 2

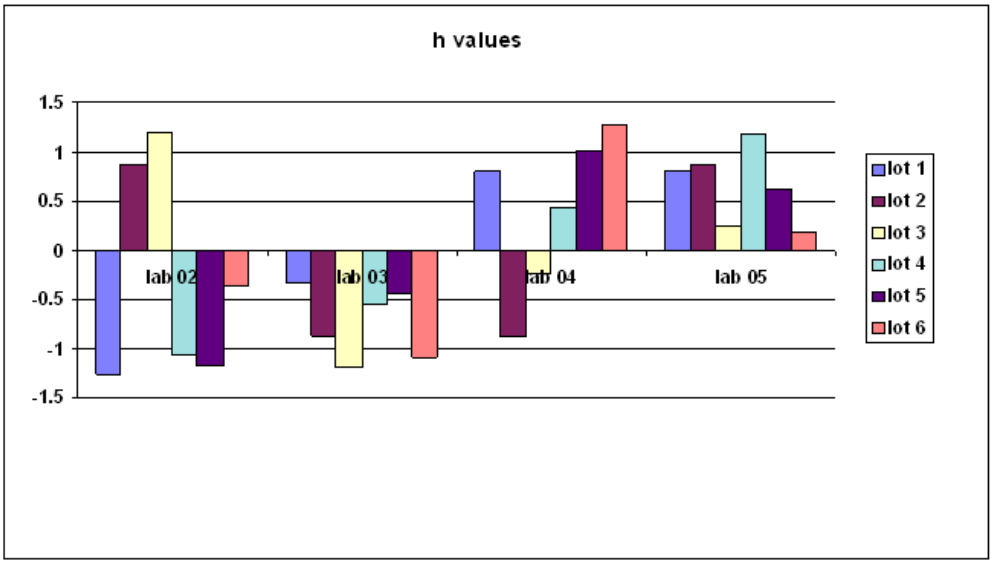
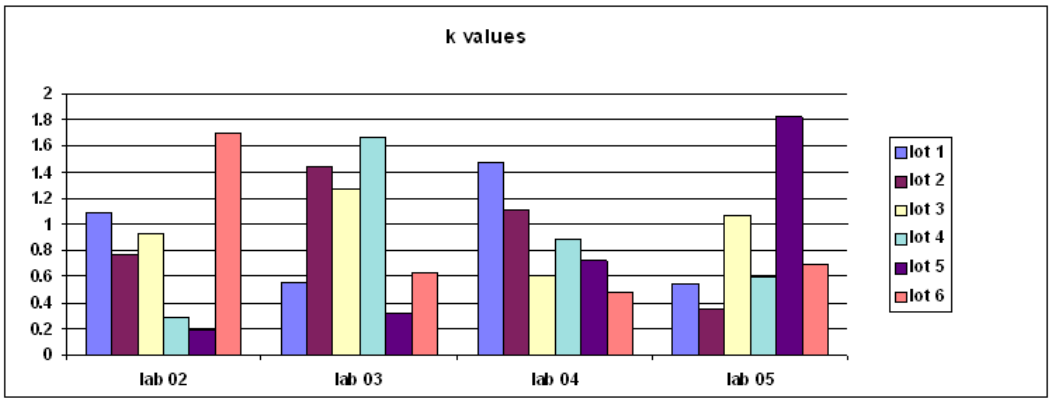


Figure 8: h values for total CD germination in each of two test runs in 2001-2004

a) Run 1



b) Run 2

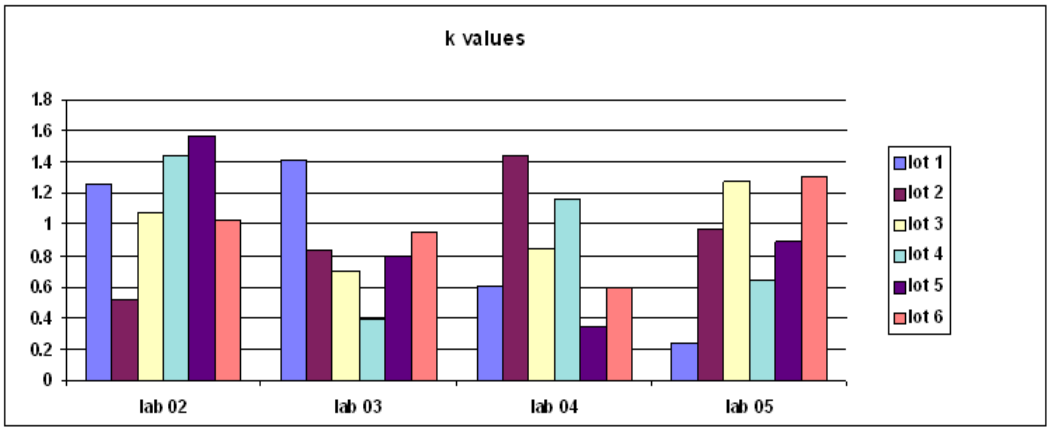
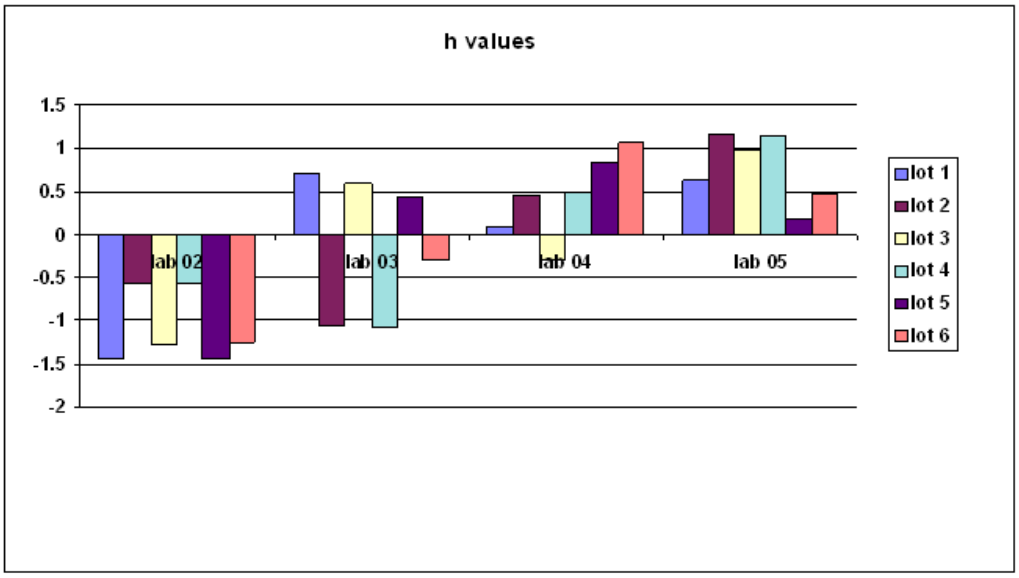


Figure 9: k values for total CD germination in each of two test runs in 2001-2004

a) Run 1



b) Run 2

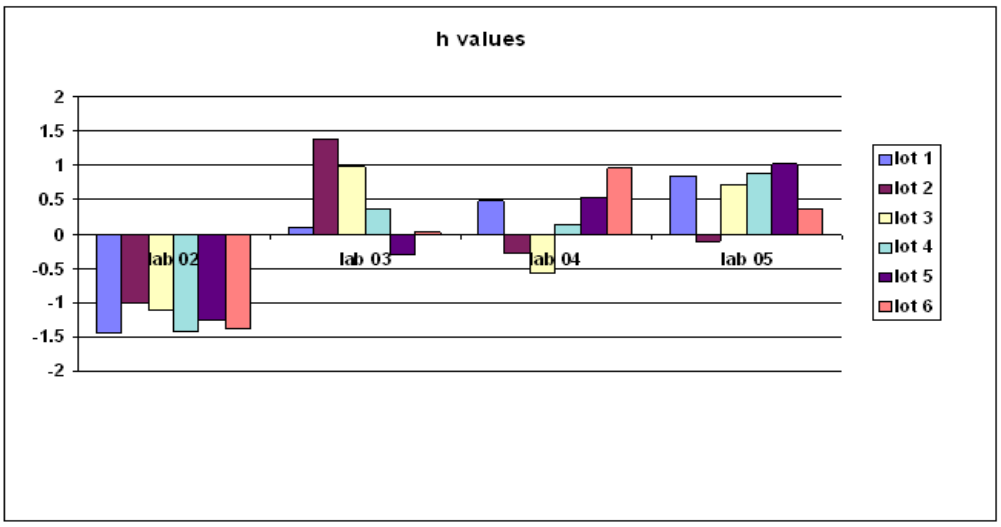
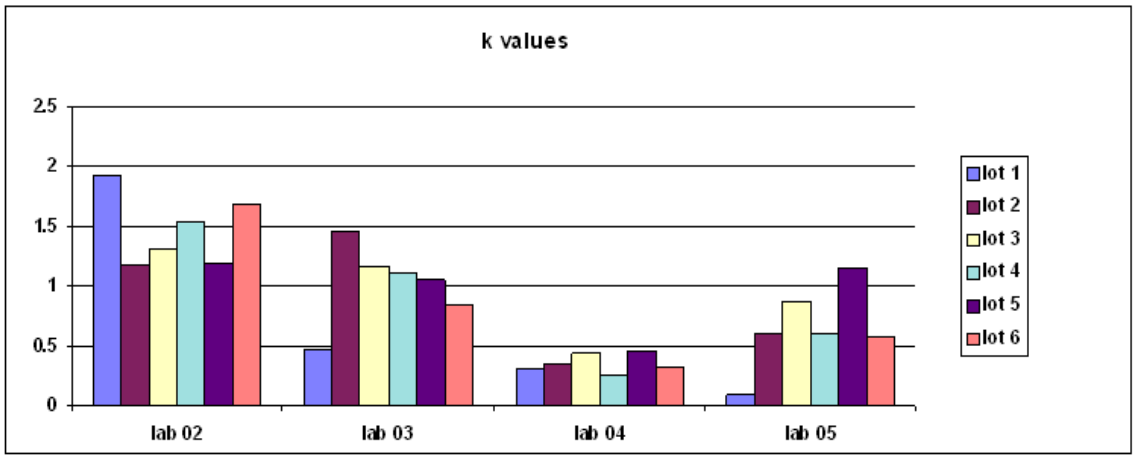


Figure 10: h values for normal CD germination in each of two test runs in 2001-2004

a) Run 1



b) Run 2

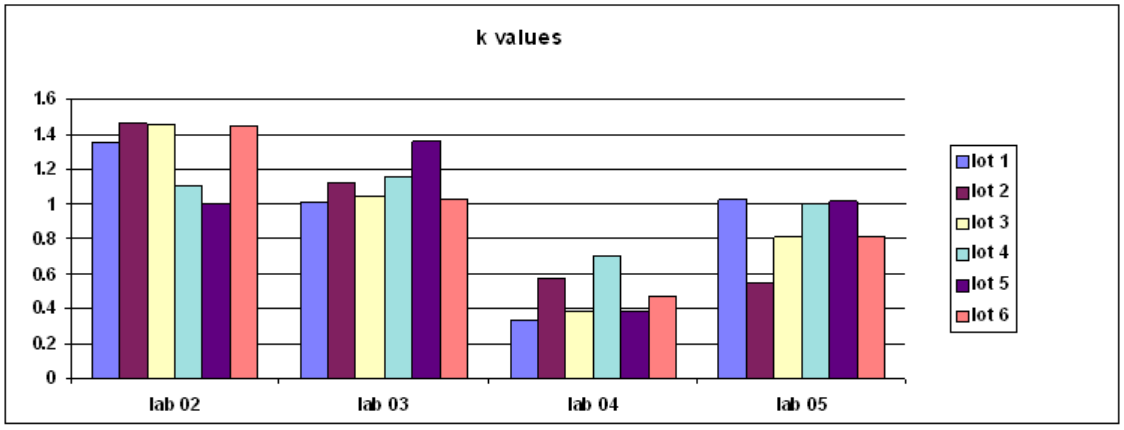


Figure 11: k values for normal CD germination in each of two test runs in 2001-2004

Appendix 1: ANOVA Tables

1995-1998: Total CD germination

Source	DF	Type III SS	Mean Square	F Value	Pr > F
LOT	4	7539.43	1884.86	208.10	<.0001
LAB	3	1800.28	600.09	66.25	<.0001
LAB*LOT	12	1181.03	98.42	10.87	<.0001
RUN(LAB)	8	163.57	20.45	2.26	0.0247
Residuals	212	1920.18	9.06		

1998-2001: Total CD germination

Source	DF	Type III SS	Mean Square	F Value	Pr > F
LOT	4	14727.48	3681.87	121.17	<.0001
LAB	2	1064.23	532.12	17.51	<.0001
LAB*LOT	8	1186.66	148.33	4.88	<.0001
RUN(LAB)	6	537.07	89.51	2.95	0.0094
Residuals	159	4831.52	30.39		

1998-2001: Normal CD germination

Source	DF	Type III SS	Mean Square	F Value	Pr > F
LOT	4	69189.76	17297.44	573.51	<.0001
LAB	2	312.14	156.07	5.17	0.0066
LAB*LOT	8	518.08	64.76	2.15	0.0344
RUN(LAB)	6	428.10	71.35	2.37	0.0324
Residuals	159	4795.57	30.16		

2001-2004: Total CD germination

Source	DF	Type III SS	Mean Square	F Value	Pr > F
LOT	5	683.60	136.72	9.08	<.0001
LAB	3	323.85	107.95	7.17	0.0001
LAB*LOT	15	954.52	63.63	4.23	<.0001
RUN(LAB)	4	151.88	37.97	2.52	0.0431
Residuals	164	2469.13	15.06		

2001-2004: Normal CD germination

Source	DF	Type III SS	Mean Square	F Value	Pr > F
LOT	5	5277.04	1055.41	36.28	<.0001
LAB	3	5121.44	1707.15	58.68	<.0001
LAB*LOT	15	1564.50	104.30	3.58	<.0001
RUN(LAB)	4	351.54	87.89	3.02	0.0195
Residuals	164	4771.46	29.09		

Appendix 2: Comparison of run means within laboratories

1995-1998: Total CD germination

Laboratory	Run	Seed lot					Mean
		1	2	3	4	5	
1	1	69	92	96	98	97	90[a]
	2	78	91	95	98	98	92[a]
	3	78	95	92	96	92	91[a]
	Mean	75[a]	93[b]	94[bc]	97[d]	95[cd]	
2	1	82	88	95	96	98	92[b]
	2	78	90	94	97	95	91[a]
	3	75	89	95	97	97	91[a]
	Mean	78[a]	89[b]	95[b]	97[b]	97[b]	
3	1	94	98	98	100	100	98[a]
	2	92	95	99	100	100	97[a]
	3	92	96	99	98	99	97[a]
	Mean	93[a]	96[b]	99[c]	99[c]	99[c]	
4	1	87	92	98	98	97	95[a]
	2	83	90	95	95	96	92[a]
	3	83	90	95	95	96	92[a]
	Mean	84[a]	91[b]	96[c]	96[c]	96[c]	

1998-2001: Total CD germination

Laboratory	Run	Seed lot					Mean
		1	2	3	4	5	
1	1	80	93	98	97	66	87[a]
	2	82	92	95	99	61	86[a]
	3	81	95	98	100	72	85[a]
	Mean	81[b]	93[c]	98[cd]	98d	66[a]	
2	1	89	93	95	94	84[*]	91[a]
	2	90	98	100	100	78[*]	93[b]
	3	90	95	98	98	58[*]	89[a]
	Mean	90[b]	95[c]	97[c]	97[c]	73[a]	
3	1	88	95	99	100	77	92[a]
	2	89	96	99	99	84	93[a]
	3	94	99	99	98	88	96[b]
	Mean	90[b]	96[c]	99[c]	99[c]	83[a]	

Normal CD germination

Laboratory	Run	Seed lot					Mean
		1	2	3	4	5	
1	1	75	87	91	93	52	80[a]
	2	74	93	94	95	41	77[a]
	3	72	88	94	95	42	78[a]
	Mean	74[b]	86[c]	93[d]	94[d]	45[a]	
2	1	79	90	94	92	53	82[a]
	2	77	92	99	98	41	81[a]
	3	83	91	96	95	40	81[a]
	Mean	80[b]	91[c]	96[d]	95[d]	42[a]	
3	1	72	88	91	96	27[*]	75[a]
	2	72	86	93	96	47[*]	79[b]
	3	84	91	94	96	39[*]	81[b]
	Mean	77[b]	88[c]	93[d]	96[e]	38[a]	

2001-2004: Total CD germination

Laboratory	Run	Seed lot	Mean

		1	2	3	4	5	6	
2	1	74	95	98	99	98	95	94[a]
	2	94	99	98	96	84	96	95[a]
	Mean	84[a]	97[c]	98[c]	98[c]	91[b]	96[c]	
3	1	90	95	98	93	98	95	95[a]
	2	96	98	97	97	88	95	95[a]
	Mean	93[a]	97[ab]	98[b]	95[ab]	93[a]	95[ab]	
4	1	91	90	95	97	98	97	95[a]
	2	99	98	98	98	98	98	98[a]
	Mean	95[a]	94[a]	97[a]	98[a]	98[a]	98[a]	
5	1	97	99	98	99	90	99	97[a]
	2	99	99	98	98	95	97	98[a]
	Mean	98[a]	99[b]	98[b]	99[b]	93[a]	98[b]	

Normal CD germination

Laboratory	Run	Seed lot						Mean
		1	2	3	4	5	7	
2	1	60	63	75	73	53	65	65[a]
	2	60	69	80	75	60	62	68[a]
	Mean	60[a]	66[b]	78[c]	74[c]	57[a]	64[b]	
3	1	74	59	87	68	75	72	73[b]
	2	69	79	85	85	70	75	77[a]
	Mean	72[ab]	69[a]	86[c]	77[b]	73[ab]	74[ab]	
4	1	70	71	81	82	79	82	78[a]
	2	71	72	81	84	78	83	78[a]
	Mean	71[a]	72[a]	81[b]	83[b]	79[b]	83[b]	
5	1	73	77	90	88	72	77	80[a]
	2	73	73	85	88	83	78	80[a]
	Mean	73[a]	75[a]	88[b]	88[b]	78[b]	78[b]	

Appendix 3: Repeatability and reproducibility data obtained following ISO analysis of standard germination tests

mean values of repeatability and reproducibility obtained in a comparative study on the effect of temperature and germination media on the germination of sunflower seed (data extracted from Table 21 in Fiedler *et al* 2008, taken from original paper by Ducournau *et al*, 2007)

Media and temperature regime	Repeatability	Reproducibility
BP 20°C	2.97	8.21
BP 25°C	4.35	10.88
BP 20 – 30° C	3.76	8.34
S 20°C	3.69	8.22
S 25°C	3.37	18.74
S 20 – 30°C	3.21	5.01
O 20°C	3.53	4.57
O 25°C	2.96	3.72
O 20 – 30°C	2.99	4.24

mean values of repeatability and reproducibility for germination tests conducted on 4 lots of *Pisum sativum* using different germination media (from Fielder *et al*, 2008)

Germination media	Seed lot	Mean germination (%)	Repeatability (sr^2)	Reproducibility (sR^2)
BP	1	95.03	2.2197	2.7787
	2	94.56	2.1311	2.8300
	3	89.38	3.0208	5.0431
	4	88.38	2.9791	5.8885
S	1	95.53	1.9552	3.5782
	2	93.25	3.2048	4.8151
	3	87.41	3.1007	5.4670
	4	88.28	3.5045	5.4695
TCS	1	96.53	1.4470	1.6084
	2	95.03	1.9659	2.3097
	3	86.59	3.0805	6.0982
	4	87.91	3.6558	5.7341