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Revised method for the germination test of *Cynara cardunculus* using optimal temperatures [Rules Proposal 2008 item C.5.1]

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Summary

Experiments were carried out to test the germination capacity of *Cynara cardunculus* using different methods with light conditions and media. These experiments where performed with three seed lots at three different ISTA accredited laboratories. Focus was on the temperature used for the different germination methods, because the germination of *C. cardunculus* is negatively affected by high germination temperatures. The results of this peer validation test support the results of previous experiments, either performed by Nunhems Netherlands or independent research groups. The combined results indicate that the germination of *C. cardunculus* is more optimal at 20° C or 15-20° C and should not be performed at 20-30° C.

Introduction

In 2007 there were several artichoke seed lots of Nunhems Netherlands which, according to the results of the Naktuinbouw, did not meet a 80% germination standard that is required for Nunhems Netherlands to export seeds to certain countries. After internal QA testing Nunhems Netherlands found germination figures well above 80% for the same seed lots, using a vitality test with lower temperatures.

Nunhems Netherlands is using a different vigour protocol for *C. cardunculus* as compared to the Naktuinbouw. Nunhems Netherlands is using coconut fibre as medium and 20/15° C light/dark (12/12 hours), where the Naktuinbouw is using an ISTA protocol using sand as medium with 20/30 °C (12/12 hours). Besides these differences in conditions, different evaluation days are used: Nunhems 10/21 days, Naktuinbouw 7/10/15/21 days.

To test whether the growing conditions of the different protocols might explain the differences in germination figures obtained an internal trial was performed. It is known from literature that a high temperature in the vigour test has a negative effect on the germination of *C. cardunculus* seeds (Ierna *et al.*, 2004, Basnizki *et al.*, 1985, Vanella and Damato, 2005). That is why it was decided to test different temperature regimes in the vitality test, In fact three different protocols where tested using three different seed lots. Following the trial performed at Nunhems Netherlands, results can be found in the attachment, the samples were sent to the Naktuinbouw to be tested at three ISTA accredited laboratories for peer validation. This report gives the results of this peer validation to support the request for a change in temperature regime for the germination of *C. cardunculus*.

Material and methods

Seeds

Three seed lots were selected with germination figures around 80% (table 1), the germination figure needed to obtain the ISTA Orange certificate.

Test method

To test the effect of temperature on the germination of the seeds independent of the medium used, six methods were compared at three ISTA certified labs in Germany, USA and Scotland (table 2). For each object (seed lot x laboratory x method) 8 replicates of 50 seeds were sown, after 7, 10, 15 and 21 days normal seedlings, abnormal seedlings and non germinated seeds are recorded. The data was recalculated into germination results per 100 seeds.

Data analysis

General introduction

For each combination of laboratory, method and seed lot the numbers of normal and abnormal plants and non germinated seeds were recorded.

The numbers of normal plants were analyzed using generalized linear modeling facilities of GenStat, a Binomial model (i.e. number of normal plants) (Payne *et al.*, 2003).

Binomial model

The model for the data, number of normal plants, was specified as having a Binomial error distribution with a logit-link function. The effects of laboratories, methods and seed lots were tested against the mean deviance of laboratory x method x seed lot term in the model. The predictions (based on the model) and corresponding standard error were calculated. The standard errors are based on the dispersion factor that was set to the mean deviance of the laboratory x method x seed lot.

Table 1: Results of the average amount of normal plants (N), abnormal plants (Ab) and dead seeds (D) with their statistical differences. Figures are based on the predictions of the GenStat analysis.

Temperature	N	Ab	D	stat. diff.
15/20° C	84	7	9	c
20° C	80	7	12	b
20/30° C	73	8	19	a
Substrate				
S	81	5	15	b
BP	78	10	12	a

When looking at the data as presented in table 1 and figure 1, the temperature effect becomes immediately visible. A temperature of 20-30° C gives the lowest germination. This temperature effect becomes also visible when presenting the results per lab (figure 2). According to the results of lab 3 a higher temperature gives a higher germination, especially using between paper as growing medium.

Table 4 contains the statistical effects for the different factors tested in this peer validation test. The analysis resulted in a significant method effect, which means that more normal plants were recorded using lower temperatures for the germination, alternating 15-20° C and 20° C constant. In addition a laboratory effect was registered and the interactions (laboratory x method; laboratory x seed lot; method x seed lot) were significant, meaning that the results gathered in each laboratory for each lot and method were not always comparable.

Discussion

With regard to total amount of normal seedlings, there is a clear temperature effect visible. The methods using 20/30° C have, on average, a lower amount of normal seedlings as compared to the methods using either 15/20 or 20° C. This is statistically proven with both method 1 and 4 being significantly lower than the other methods, although there is lab effect visible. The results of Ierna *et al*. (2004) show the same temperature dependency over a range of 4 to 32° C, where the optimum germination temperature is between 16 and 20° C. From the results it becomes also visible that the methods using 20-30° C never reach a germination of 80%, while the other methods do. Vanella and Damato (2005) conclude that in order to have the best correlation between laboratory and field germination results, the *C. cardunculus* seeds must be tested at 20° C and no higher than 25° C.

Conclusions and recommendations

The results of this peer validation test show that seed germination of *C. cardunculus* is substantially controlled by temperature. This observation is consistent with earlier research on the same species (Ierna, *et al*., 2004, Vanella and Damato 2005 and Basnizki *et al*., 1985). The temperature used in the test used by the NAKT, 20/30° C has a negative effect on the total amount of normal seedlings. Therefore it is recommended to change the temperature from 20-30° C to 20° C or 15-20° C (Dark/Light) which are more optimal germination temperatures for *C. cardunculus*.

References

Basnizki, V., Mayer, A. M. (1985) Germination of *Cynara* seeds; effect of light and temperature and function of the endosperm. Agronomie 5 (6) page 529-532.

Ierna, A., Restuccia, G., Mauromical, G. (2004) Effects of Seed Osmopriming on Germination of *Cynara cardunculus* under Low, Optimal and High temperatures. Acta horticultura No. 660 page 333-338.

Payne, R.W., Baird, D.B., Cherry, M., Gilmour, A.R., Harding, S.A., Kane, A.F., Lane, P.W., Murray, D.A., Soutar, D.M., Thompson, R., Todd, A.D., Tunnicliffe Wilson, G., Webster, R. and Welham, S.J. (2003) GenStat Release 7.1 Reference Manual. VSN International, Wilkinson House, Jordan Hill Road, Oxford, UK.

Vanella, S., Damato, G. (2005) Influence of Temperature and Substrate on the Germination of Artichoke Achenes. Acta Horticultura 2005 No. 681 page 361-367.

Tables and figures

Table 2: Overview of the artichoke seed lots used for the peer validation test in which different germination methods were compared.

Sample	Variety	Lot number
370.581	Concerto	4151658003
370.582	Concerto	5051300028
370.583	Opal	6150009007

Table 3: The different methods used for the peer validation test of germination of artichoke. BP = Between Paper, S = Sand.

Method	Medium	Temperature	Light
1	BP	20-30	12/12
2	BP	15-20	12/12
3	BP	20	12/12
4	S	20-30	12/12
5	S	15-20	12/12
6	S	20	12/12

Table 4: Determination of statistical significant differences for lot-, laboratory- and method-effects and their interaction for number of normal plants of artichoke. *** statistical significant differences compared to the F-value**

Factor	Df	*Number of normal plants*
Laboratory effect	2/162	18.1*
Seed lot effect	2/162	0.3
Method effect	5/162	54.9*
Laboratory.seed lot effect	4/162	19.5*
Laboratory.method effect	10/162	8.3*
Method.seed lot effect	10/162	8.9*
Laboratory.method.seed lot effect	20/162	1.6

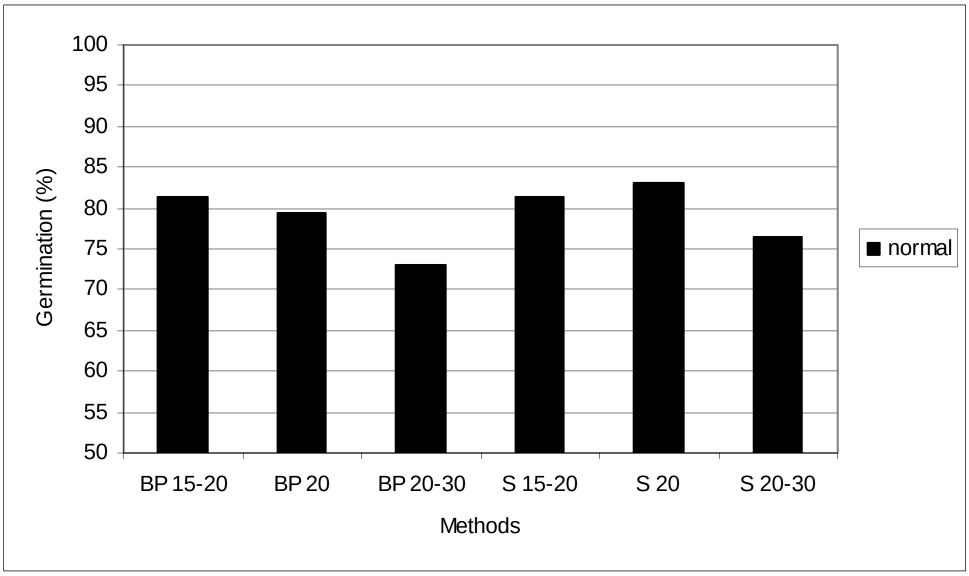

Figure 1: Overall germination results of artichoke (three seed lots tested by three laboratories) per method.

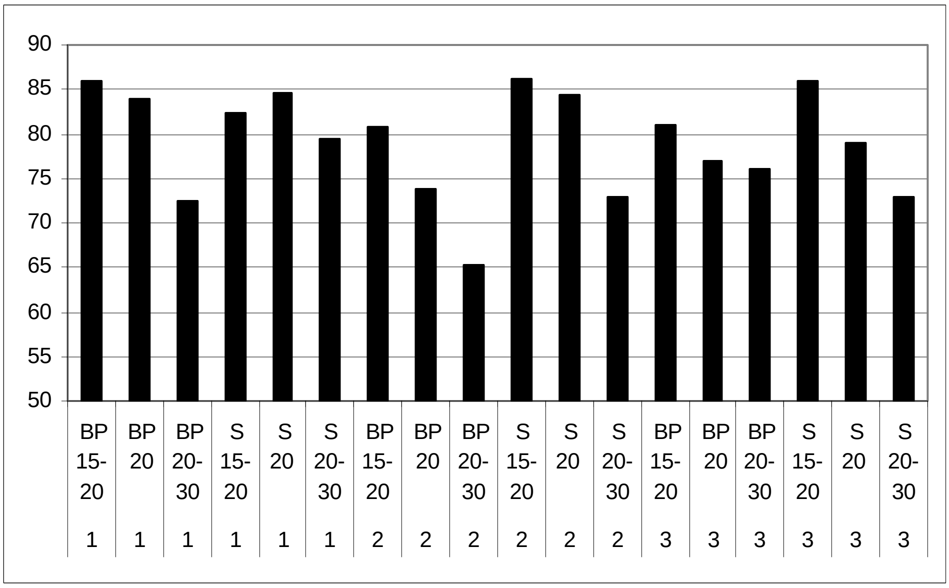

Figure 2: Germination results of artichoke per lab per method.

MVR-01-002
Evaluation of crepe cellulose paper (Versa-Pak®) covered with sand as an ISTA medium [Rules Proposal 2008 item C.5.2]

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Introduction

A significant germination medium 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*. This difference is the option of conducting germination tests on crepe cellulose paper (Versa-Pak®) (TCP) and creped cellulose paper covered with S (TCPS) on the following species: *Pisum sativum s.l.*, *Glycine max, Phaseolus vulgaris, Zea mays subsp. mays, Gossypium spp. and Helianthus annuus.* A number of laboratories utilizing AOSA methods have adopted the TCPS method because it allows precise calibration of moisture levels through the use of dry sand and volume calibrated water sprayer tables (Figure 1).

Figure 1. Germination tray covered with crepe cellulose paper (Versa-Pak®) moving through a water sprayer table.

Typically, TCPS tests are conducted by moistening a sheet of crepe cellulose paper (Versa-Pa ®), placing seeds on the wet media and covering the seed with 2 cm of dry sand. Initial discussions with the ISTA Germination committee chairperson suggested the TCP method is already covered as paper (P); however the TCPS method (combination of paper and S as a medium) would require comparative testing before consideration for inclusion in the *International Rules for Seed Testing*.

Pisum sativum was selected as the test crop for evaluating the TCPS method comparison to the ISTA approved methods of BP and S. Peas were selected since no GMO events are known to occur, thus easing international movement of the seed.

Material and methods

A comparative test was designed to compare germination results of *Pisum sativum* on three media types. The study included four *Pisum sativum* seed lots donated by Seminis, Inc., Oxnard, California, USA. Creped cellulose paper (Versa-Pak®) was donated and shipped to participants by National Packaging Service Corporation (NPS) Green Bay Wisconsin, USA. Participants included: Sue Alvarez, Seminis Inc., Oxnard CA, USA,(AOSA); Ronald Don, Official Seed Testing Station, Edinburgh, Scotland,(ISTA); Kari Fiedler, Mid-West Seed Services, Inc., Brookings, SD, USA,(AOSA/ISTA); Dr. Norbert Leist, Landwirtschaftliches Technologie Zentrum Augustenberg und Forschungsanstah, Karlsruhe, Germany,(ISTA); Lea Mazor, Official Seed Testing Laboratory, Bet-Dagan, Israel,(ISTA); Dr. Günter Müeller, Thüringer Landesanstalt für Landwirtschaft TLL, Jena, Germany,(ISTA); Kim North, Seed Science Center, Ames, IA USA,(AOSA); and Victor Vankus, National Tree Seed Laboratory, Dry Branch, GA USA, (AOSA/ISTA).

Germination testing was conducted on BP, S and TCPS using four lots of *Pisum sativum* at 20ºC for eight days. Each germination medium was evaluated utilizing four, 100 seed replications or eight, 50 seed replications per sample (Note: 50 seed replicates were combined to obtain four 100 seed replicates for data analysis). All data was analyzed using four, 100 seed replications.

The results were analysed in a number of ways:

Z-scores were calculated and results assessed. For each sample tested at each laboratory the z-score was calculated as:

$$z = \frac{(x - \mu)}{\sigma}$$

where:

x is a the result of an individual laboratory testing an individual sample;

σ is the standard deviation of all of the results obtained on that sample by the participant laboratories; and

μ is the mean of the results obtained on that sample by the participant laboratories.

The z-score for an item indicates how far and in what direction it differs from the mean with a z-score of ± 1.96 indicates a significant difference from the mean value (p=0.05).

z-scores are used by ISTA for evaluation of proficiency tests. In ISTA proficiency tests the absolute sum of the z-scores gained by a laboratory when performing a germination, moisture content, purity or tetrazolium test on the 3 samples within a proficiency round is used to confer a rating or score to the laboratory :

Rating/Score	Sum of absolute z-scores
A	≤3.5
B	≤5.3
C	≤7.0
BMP	>7.0

However, in this comparative test there were 4 samples and the ISTA rating system could not be applied. Instead other assessments of the z-scores were made in accordance with ISO/IEC Guide 43-1 "Proficiency Testing by Interlaboratory Comparison - Part 1: *Development and Operation of Proficiency Testing Schemes*".

Four statistical tests using z-scores were used to assess proficiency of laboratories taking part in this comparative test. They were also used to assign participating laboratories with performance ratings for the germination tests they carried out.

the individual z-score were used to assess individual results;

the behaviour of the absolute values of a set of z-scores were used to assess groups of results;

the Re-scaled Sum of z-scores (RSZ) were used to detect consistent bias in an analytical system; and

The Squared Sums of z-scores (SSZ) were used to detect if there were abnormally high deviations in the overall performance of a laboratory.

A Assessment of individual z-scores

Ratings for individual z-scores were assigned as follows:

z-Score Range	Rating
$\lvert z_i \rvert \leq 2$	Satisfactory
$2 < \lvert z_i \rvert < 3$	Questionable
$\lvert z_i \rvert \geq 3$	Unsatisfactory

B Combining z-scores: distribution of absolute values

The distribution of the absolute z-scores for a participating laboratory were used to assign an assessment of a laboratories performance, based on the analyses for a given germination attribute (i.e. normal seedlings, abnormal seedlings, dead seed):

Absolute z-Scores	Rating
All z-scores < 2	Good
All z-scores < 3	Satisfactory
Only one z-score ≥ 3	Questionable
Two or more z-scores ≥ 3	Unsatisfactory

C Combining z-scores: re-scaled sum of z-scores

The Re-scaled Sums of z-Scores (RSZ) were calculated as $\Sigma z_i/\sqrt{n}$. RSZ uses the signs of the z-scores to detect consistent bias in an analytical system Ratings based on RSZ were assigned as follows:

|RSZ| Range	Rating
$\lvert RSZ \rvert \leq 2$	Good
$2 > \lvert RSZ \rvert \leq 3$	Satisfactory
$3 > \lvert RSZ \rvert < 4$	Questionable
$\lvert RSZ \rvert \geq 4$	Unsatisfactory

D Combining z-scores: squared sum of z-scores

The Squared Sum of z-Scores (SSZ) was calculated as Σz_i^2. SSZ does not take into account the signs of the z-scores and was used to detect abnormally high deviations. SSZ is compared with the value in the χ^2 (chi-squared) distribution table at the agreed confidence levels with n degrees of freedom, where n = the number of samples. Ratings were assigned according to confidence intervals as follows:

Confidence Limit	Rating
95%	Satisfactory
99%	Questionable
> 99%	Unsatisfactory

Table of χ^2 versus confidence interval

	χ^2				
Conf. Int.	**n = 2**	**n = 3**	**n = 4**	**n = 5**	**n = 6**
95%	≤ 5.99	≤ 7.82	≤ 9.49	≤ 11.07	≤ 12.59
99%	> 5.99 ≤ 9.21	> 7.82 ≤ 11.34	> 9.49 ≤ 13.28	> 11.07 ≤ 15.09	> 12.59 ≤ 16.81
> 99%	> 9.21	> 11.34	> 13.28	> 15.09	> 16.81

Repeatability and reproducibility were analysed with the statistical tool developed by S. Grégoire according to ISO 5725-2 and available for download at the ISTA website within the stats tool box:

http://www.seedtest.org/upload/cms/user/ISO572511.zip

Effect of the different factors (laboratory, sample, test) were analysed by variance analysis. ANOVA and LSD mean separations were calculated according to MSUSTAT (1991).

Results

The mean results of germination tests obtained by the participant laboratories on the four *Pisum sativum* seed lots using 3 different germination media are illustrated in figures 2, 3 and 4.

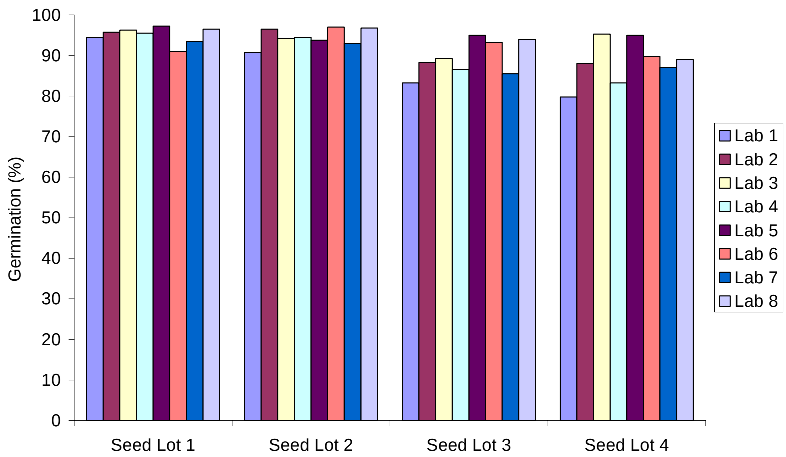

Figure 2. The results of germination tests carried out **BP** at the eight participating laboratories

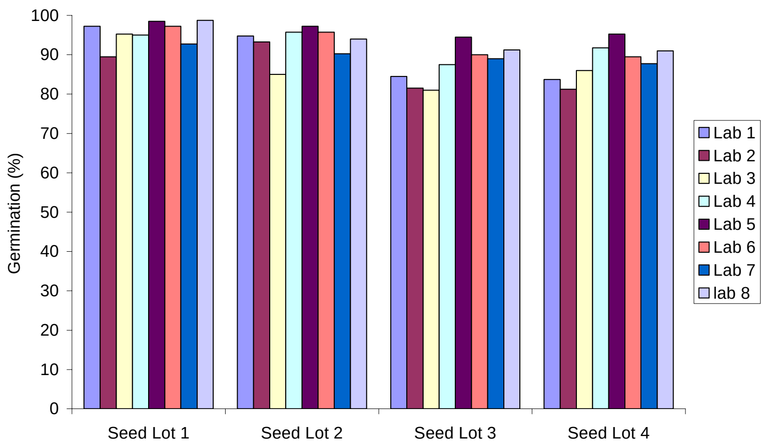

Figure 3 The results of germination tests carried out using **S** at the eight participating laboratories

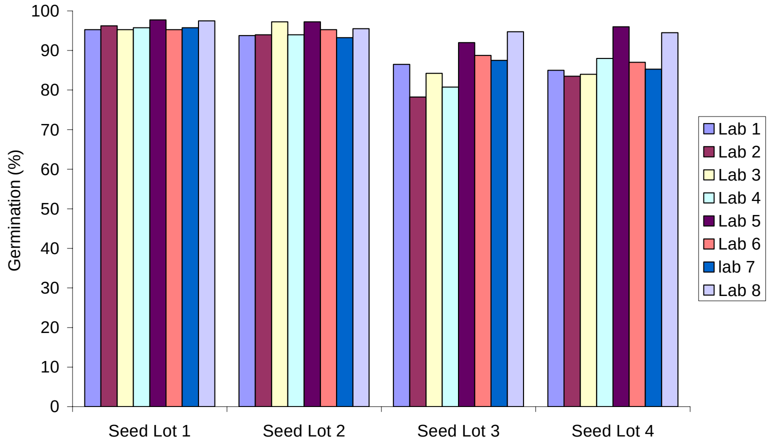

Figure 4 The results of germination tests carried out using **TCPS** at the eight participating laboratories

A Assessment of individual z-scores

Normal seedlings

In terms of individual z-scores satisfactory ratings were obtained for all but seed lot 1 tested in laboratory 5 using BP as the germination media (questionable rating) and seed lot 2 tested in laboratory 3 using S as the germination media (questionable rating) (Tables 1, 2 and 3).

Table 1. Normal seedling z-scores obtained in comparative trial of germination media for Pisum seed – BP Media

z-scores	Germination Media: BP			
Lab	Sample 1	Sample 2	Sample 3	Sample 4
1	-0.2648	-1.7771	-1.4206	-1.6295
2	0.3582	0.9031	-0.2609	-0.0708
3	0.6074	-0.1457	-0.0290	1.2988
4	0.2336	-0.0291	-0.6668	-0.9682
5	1.1058	-0.3787	1.3046	1.2516
6	-2.0092	1.1362	0.8988	0.2598
7	-0.7632	-0.7283	-0.8988	-0.2598
8	0.7320	1.0196	1.0727	0.1181

Table 2. Normal seedling z-scores obtained in comparative trial of germination media for Pisum seed – S Media

z-scores	Germination Media: S			
Lab	Sample 1	Sample 2	Sample 3	Sample 4
1	0.5453	0.3812	-0.6103	-0.9958
2	-1.9133	0.0000	-1.2403	-1.5453
3	-0.0892	-2.0967	-1.3453	-0.5014
4	-0.1685	0.6354	0.0197	0.7623
5	0.9418	1.0166	1.4896	1.5315
6	0.5453	0.6354	0.5447	0.2678
7	-0.8823	-0.7624	0.3347	-0.1168
8	1.0211	0.1906	0.8072	0.5975

Table 3. Normal seedling z-scores obtained in comparative trial of germination media for Pisum seed – TCPS Media

z-scores	Germination Media: TCPS			
Lab	Sample 1	Sample 2	Sample 3	Sample 4
1	-0.8368	-0.8208	-0.0171	-0.6079
2	0.1550	-0.6607	-1.5216	-0.9217
3	-0.8368	1.4215	-0.4274	-0.8171
4	-0.3409	-0.6607	-1.0657	0.0196
5	1.6426	1.4215	0.9859	1.6930
6	-0.8368	0.1401	0.3932	-0.1896
7	-0.3409	-1.1412	0.1653	-0.5556
8	1.3946	0.3003	1.4874	1.3792

Abnormal seedlings

For Abnormal Seedlings Satisfactory ratings were obtained by all labs for all samples using BP and TCPS as the germination medium. For S, two questionable ratings were obtained (Lab 2: Seed Lot 1 and Lab 3: Seed Lot 2) (Tables 4, 6 and 6)

Table 4. Abnormal seedling z-scores obtained in comparative trial of germination media for Pisum seed – BP Media

z-scores	Germination Media: BP			
Lab	Sample 1	Sample 2	Sample 3	Sample 4
1	0.0309	1.7984	1.0187	0.9676
2	-0.2165	-0.0580	0.2530	0.0954
3	-0.5876	0.5221	0.3077	-0.9949
4	0.2783	0.4061	0.9093	1.4585
5	-1.4534	-1.5663	-1.5520	-1.6491
6	1.7627	-0.7542	-1.0597	-0.2862
7	0.8968	0.1740	0.9093	0.3680
8	-0.7113	-0.5221	-0.7863	0.0409

Table 5. Abnormal seedling z-scores obtained in comparative trial of germination media for Pisum seed – S Media

z-scores	Germination Media: S			
Lab	Sample 1	Sample 2	Sample 3	Sample 4
1	-0.5844	-0.4505	0.2080	1.1760
2	1.9658	0.2975	1.6426	1.6801
3	-0.1594	2.1335	0.8392	-0.3360
4	0.3719	-0.1105	0.5523	0.0000
5	-0.7969	-1.0625	-1.3413	-1.4281
6	-0.5844	-0.4505	-0.2511	-0.2520
7	0.7969	0.4335	-0.7101	-0.0840
8	-1.0094	-0.7905	-0.9396	-0.7560

Table 6. Abnormal seedling z-scores obtained in comparative trial of germination media for Pisum seed – TCPS Media

z-scores	Germination media: TCPS			
Lab	Sample 1	Sample 2	Sample 3	Sample 4
1	-0.6861	-0.1631	-0.9330	0.2905
2	0.4695	0.8520	1.5257	1.6335
3	0.4695	-0.5989	0.0329	-0.2776
4	1.0472	0.9970	1.3062	0.4455
5	-0.6861	-1.6133	-1.0208	-1.3106
6	0.1806	0.7069	-0.1866	-0.3809
7	1.0472	0.8520	0.2525	0.8070
8	-1.8417	-1.0332	-0.9769	-1.2073

Dead seed

For Dead Seed Satisfactory ratings were obtained by all labs for all samples using S as the germination medium. For BP two questionable ratings were obtained (Lab 1: Seed Lot 4 and Lab 5: Seed Lot 2) and one questionable rating was obtained with TCPS as the germination medium (Lab 1: Seed Lot 3) (Tables 7, 8 and 9)

Table 7. Dead Seed z-scores obtained in comparative trial of germination media for Pisum seed – BP Media

z-scores	Germination Media: BP			
Lab	Sample 1	Sample 2	Sample 3	Sample 4
1	0.6124	-0.0609	1.2490	2.1466
2	-0.2041	-1.0344	-0.0266	-0.0484
3	-1.0206	-0.5476	-1.089	-1.2105
4	-1.4289	-0.3854	-1.0896	-0.6940
5	1.4289	2.0485	1.2490	0.4681
6	1.0206	-0.3854	0.8238	-0.0484
7	-0.6124	0.9127	-0.2391	-0.1775
8	0.2041	-0.5476	-0.8770	-0.4358

Table 8. Dead Seed z-scores obtained in comparative trial of germination media for Pisum seed – S Media

z-scores	Germination Media: S			
Lab	Sample 1	Sample 2	Sample 3	Sample 4
1	0.1110	0.0473	0.7552	0.0127
2	-1.3692	0.0473	1.1176	1.3347
3	1.2952	0.0474	1.1176	1.3347
4	-0.1850	-1.6557	-1.4197	-1.4110
5	-0.7771	-0.1419	-0.8156	-1.1059
6	-0.1850	-0.8988	-0.9364	-0.1907
7	1.5912	0.9934	0.5135	0.3178
8	-0.4811	1.5611	-0.3323	-0.2924

Table 9. Dead Seed z-scores obtained in comparative trial of germination media for Pisum seed – TCPS Media

z-scores	Germination Media: TCPS			
Lab	Sample 1	Sample 2	Sample 3	Sample 4
1	1.7930	1.6009	2.0205	0.5129
2	-0.4138	-0.3323	-0.1285	-1.1967
3	0.4138	-1.0572	0.8059	1.7951
4	-0.9654	-0.5739	-0.5956	-0.7693
5	-0.9654	0.6343	0.1518	-0.5984
6	0.6896	-1.2989	-0.4088	0.9403
7	-0.9654	0.1510	-0.8759	-0.4274
8	0.4138	0.8760	-0.9694	-0.2564

B Combining z-scores: distribution of absolute values

In this Comparative test the majority of laboratories obtained a “Good” rating for their assessment of normal and abnormal seedlings and dead seeds using all three germination media. There were no “Questionable” or “Unsatisfactory” ratings (Table 10) All laboratories obtained a “Good” rating in their assessment of normal seedlings when using TCPS as the germination medium (all absolute z-scores being less than 2). With BP as the germination medium one laboratory had a “Satisfactory” rating for normal seedlings (Laboratory 6) as did one using S as the germination medium (Laboratory 3).

Table 10. Ratings[1] for the assessment of normal and abnormal seedlings and dead seeds using different germination media.

	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Normal Seedlings								
BP	Good	Good	Good	Good	Good	Satisfactory	Good	Good
S	Good	Good	Satisfactory	Good	Good	Good	Good	Good
TCPS	Good	Good	Good	Good	Good	Good	Good	Good
Abnormal Seedlings								
BP	Good	Good	Good	Good	Good	Good	Good	Good
S	Good	Satisfactory	Satisfactory	Good	Good	Good	Good	Good
TCPS	Good	Good	Good	Good	Good	Good	Good	Good
Dead Seed								
BP	Satisfactory	Good	Good	Good	Satisfactory	Good	Good	Good
S	Good	Good	Good	Good	Good	Good	Good	Good
TCPS	Satisfactory	Good	Good	Good	Good	Good	Good	Good

C Combining z-scores: re-scaled sum of z-scores

Examination of the re-scaled sum of z-scores (RSZ) shows that for the assessment of normal and abnormal seedlings and dead seeds the majority of laboratories obtained a “Good” (| RSZ | ≤ 2) or “Satisfactory (| RSZ | >2 ≤ 3) rating. Only one “Questionable” rating was obtained (Lab 5 for abnormal seedling using S as the germination medium). No “Unsatisfactory” ratings were obtained. Looking at the signs of the z-scores and the ratings it would appear that lab 5 has a consistent bias in its analysis with fewer abnormal seedling and more normal seedlings than other laboratories taking part in this comparative trial.

[1] Ratings based on combined absolute z-scores for the germination attribute using a particular medium

Table 11. The re-scaled sum of z-scores for normal seedlings obtained by participating laboratories using the 3 different germination media

Lab	BP	S	TCPS
1	-2.5460	-0.3398	-1.1413
2	0.4648	-2.3494	-1.4745
3	0.8658	-2.0163	-0.3299
4	-0.7153	0.6244	-1.0238
5	1.6417	2.4898	2.8714
6	0.1427	0.9966	-0.2465
7	-1.3250	-0.7134	-0.9362
8	1.4712	1.3082	2.2808

Table 12 The Re-scaled sum of z-scores for abnormal seedlings obtained by participating laboratories using the 3 different germination media

Lab	BP	S	TCPS
1	1.9078	0.1746	-0.7458
2	0.0369	2.7929	2.2403
3	-0.3763	1.2387	-0.1867
4	1.5260	0.4069	1.8979
5	-3.1104	-2.3144	-2.3154
6	-0.1687	-0.7690	0.1600
7	1.1741	0.2182	1.4793
8	-0.9894	-1.7478	-2.5296

Table 13 The Re-scaled sum of z-scores for dead seeds obtained by participating laboratories using the 3 different germination media

Lab	BP	S	TCPS
1	1.9736	0.4631	2.9636
2	-0.6568	0.5653	-1.0356
3	-1.9341	1.8974	0.9787
4	-1.7989	-2.3357	-1.4522
5	2.5972	-1.4202	-0.3888
6	0.7053	-1.1055	-0.0389
7	-0.0582	1.7080	-1.0589
8	-0.8281	0.2277	0.0320

D Combining z-scores: squared sum of z-scores

The Squared Sum of z-scores (SSZ) for the different germination attributes obtained using the different media by the participating laboratories are shown in tables 14, 15 and 16.

Table 14. The squared sum of z-scores for normal seedlings obtained by participating laboratories using the 3 different germination media

Lab	BP	S	TCPS
1	7.9013	1.8068	1.7438
2	1.0170	7.5870	3.6251
3	2.0780	6.4652	3.5710
4	1.4375	1.0136	1.6888
5	4.6349	6.4850	8.5567
6	6.2029	1.0694	0.9104
7	1.9881	1.4854	1.7545
8	2.7402	2.0875	6.1497

Table 15. The squared sum of z-scores for abnormal seedlings obtained by participating laboratories using the 3 different germination media

Lab	BP	S	TCPS
1	5.2091	1.9708	1.4522
2	0.1233	9.4733	5.9421
3	1.7023	5.3943	0.6564
4	3.1957	0.4556	3.9951
5	9.6938	5.6024	5.8332
6	4.8806	0.6710	0.7123
7	1.7968	1.3343	2.5376
8	1.3983	3.0983	6.8713

Table 16. The squared sum of z-scores for dead seeds obtained by participating laboratories using the 3 different germination media

Lab	BP	S	TCPS
1	6.5466	0.5850	10.1230
2	1.1147	4.9074	1.7303
3	3.9940	4.7103	5.1606
4	3.8590	6.7820	2.2081
5	8.0172	2.5122	1.7155
6	1.8712	1.7553	3.2138
7	1.2968	3.8835	1.9048
8	1.3005	2.8643	1.9439

The SSZ is compared with the value in the χ^2 distribution table with degrees of freedom equal to the number of samples tested by participating laboratories in the different germination media. For 4 samples a χ^2 of ≤ 9.49 gives a confidence interval of 95% which is considered “Satisfactory”, a χ^2 of > 9.49 ≤ 13.38 gives a confidence interval of 99% which is considered “Questionable” and a χ^2 of > 13.28 gives a confidence interval of >99% which is considered “Unsatisfactory”.

In terms of normal seedlings this analysis of SSZ indicates that all results are “Satisfactory”. For abnormal seedlings all the results are “Satisfactory” apart from the BP result of Lab 5 with is “Questionable”. For dead seed results all the results are “Satisfactory” apart from the TCPS result of Lab 1 which is “Questionable”. None of results are “Unsatisfactory”.

Results of 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.

Plots of h-value results for the 3 different media are given in figures 5, 6 and 7.

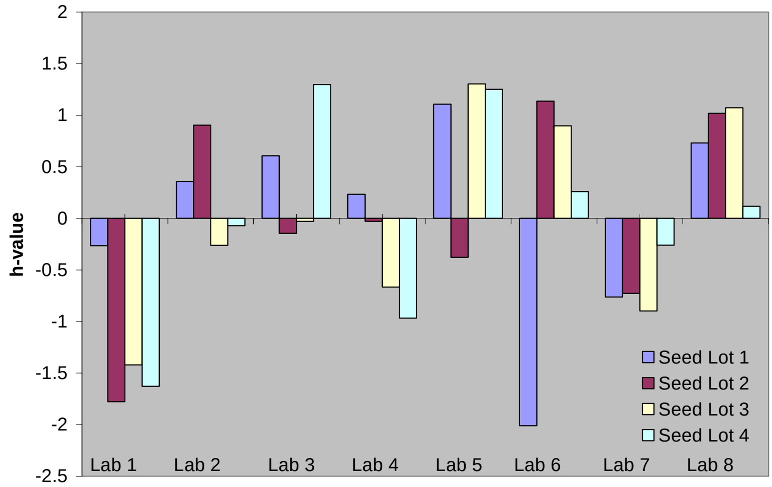

Figure 5. The h-values obtained by participating laboratories carrying out germination tests **BP** on the 4 *Pisum* seed lots used in this comparative test

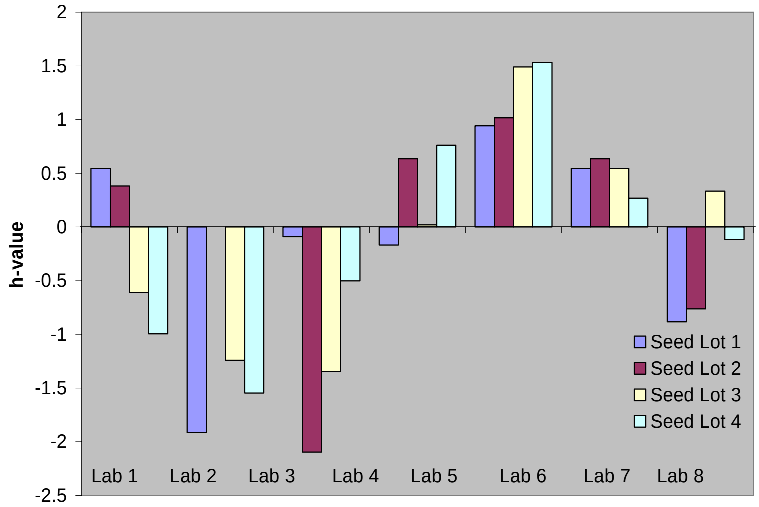

Figure 6. The h-values obtained by participating laboratories carrying out germination tests using **S** on the 4 *Pisum* seed lots used in this comparative test

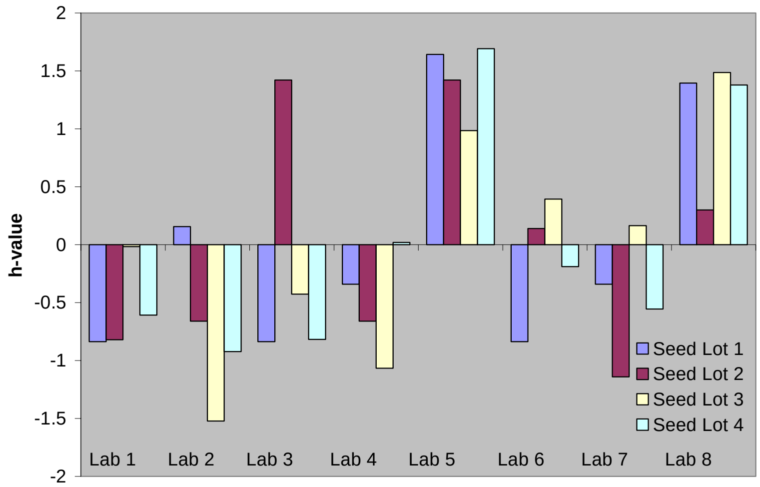

Figure 7. The h-values obtained by participating laboratories carrying out germination tests using **TCPS** on the 4 *Pisum* seed lots used in this comparative test

For this comparative test an h value of 1.75 is significant at 5% while a value of 2.06 is significant at 1%. None of the samples tested by any of the labs showed significant differences when tested using TCPS whereas Lots 1 and 2 gave significantly lower results when tested using BP in Labs 1 and 6 (both $p<0.05>0.01$) and when tested in S in labs 2 and 3 ($p<0.05>0.01$ and $p<0.01$ respectively).

Plots of k-value results for the 3 different media are given in figures 8, 9 and 10.

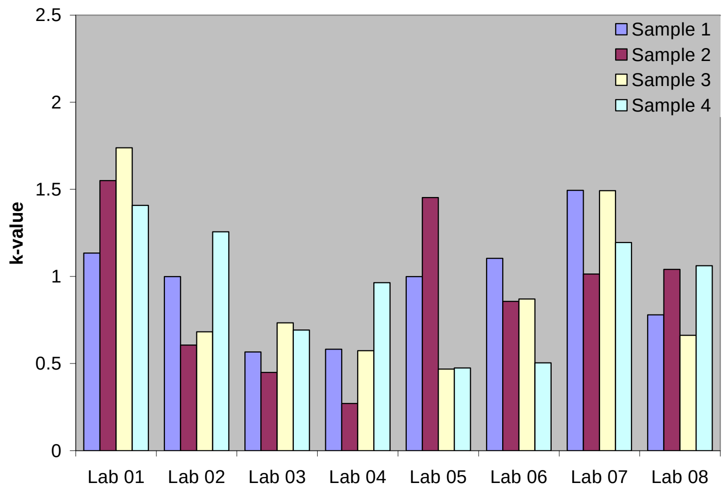

Figure 8. The k-values obtained by participating laboratories carrying out germination tests **BP** on the 4 *Pisum* seed lots used in this comparative test

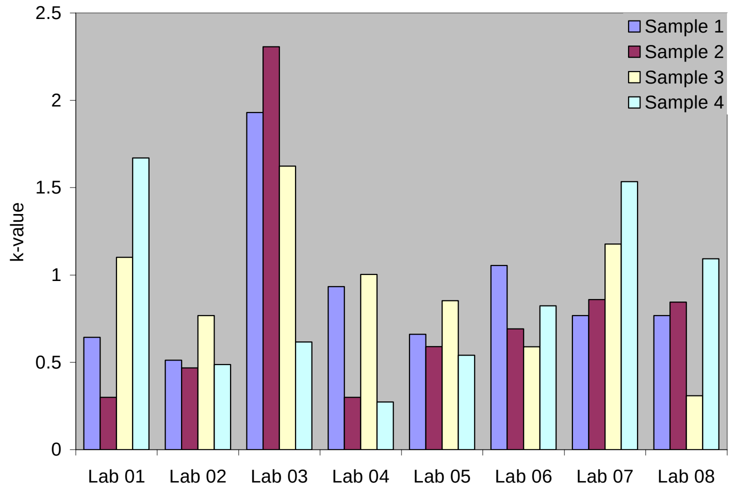

Figure 9. The k-values obtained by participating laboratories carrying out germination tests using **S** on the 4 *Pisum* seed lots used in this comparative test

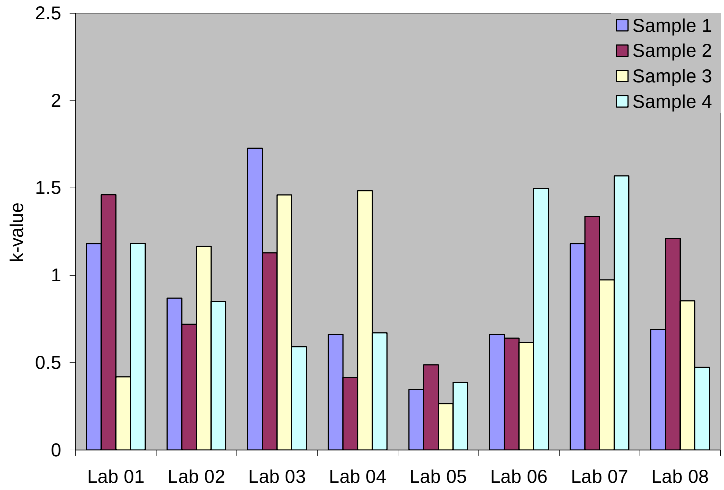

Figure 10. The k-values obtained by participating laboratories carrying out germination tests using **TCPS** on the 4 *Pisum* seed lots used in this comparative test

k-values give a measure of variability between replicates and for this comparative test a k-valve of 1.56 is significant at 5% while a value of 1.81 is significant at 1%. Significant k-values were obtained by Lab 1 when testing seed lot 3 using BP and seed lot 4 in S (both $p<0.05>0.01$). Lab 3 had significant k-values when testing seed lots 1, 2 and 3 in S ($p<0.01$ for seed lots 1 and 2 and $p<0.05>0.01$ for seed lot 3) and seed lot 1 in TCPS ($p<0.05>0.01$). Lab 7 had a significant k-value when testing seed lot 4 in TCPS $p<0.05>0.01$).

Overall repeatability and reproducibility results for the 3 media and the 4 seed lots are given in Table 17.

Table 17. Overall Repeatability and Reproducibility values for germination tests conducted on 4 Pisum seed lots using 3 different germination mediums

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
TCPS	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

The magnitude of repeatability and reproducibility values are related to the size of the attribute being measured and in general seed lots with higher germinations will have lower repeatability and reproducibility values. To allow meaningful comparisons of differences in repeatability and reproducibility values obtained from the different media plots of the square roots of the values were made (figures 11 and 12). From these plots it is clear that the performance of TCPS is as good if not better than BP or S.

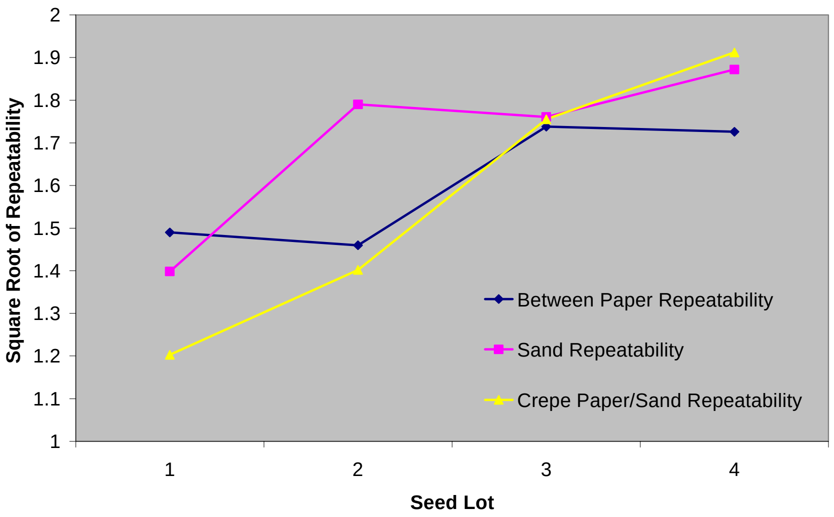

Figure 11. Repeatability measures obtained from the 3 germination media used in this comparative test

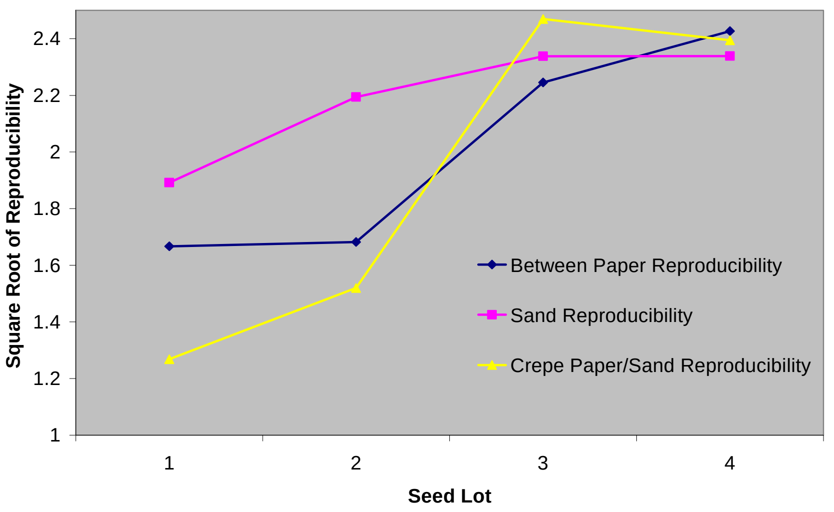

Figure 12. Reproducibility measures obtained from the 3 germination media used in this comparative test

Results of variance analysis

The analysis of variance and mean separation obtained shows that neither BP nor S were significantly different from the TCPS medium (Table 1). However S gave significantly lower results than BP (Table 18).

Table 18. Mean germination percentages for media types averaged across eight laboratories and four seed lots

Treatment	Germination %	Significance code
BP	91.8	B
S	91.1	A
TCPS	91.4	AB
LSD (P =0.05)	0.68	---

Significant differences were detected between laboratories and seed lots (figures13 and 14).

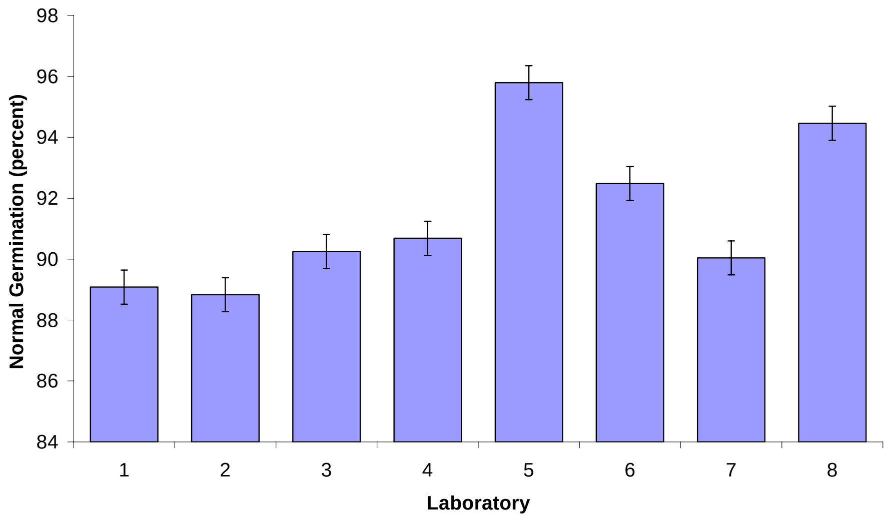

Figure 13. Mean Germinations[2] obtained from the eight participating Laboratories

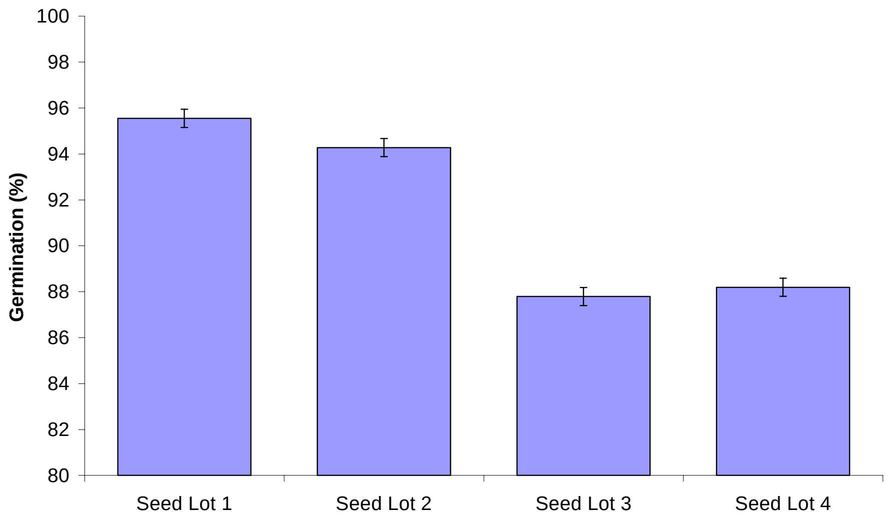

Figure 14. Mean Germinations[3] obtained from the four seed lots

ANOVA analysis also indicated that the interaction terms for laboratory by media, laboratory by lot, media by lot, and laboratory by lot by media were all significant. The interaction for laboratory by media is explained, in part, by laboratories obtaining significantly different germination percentages in different germination media (Figures 15, 16 and 17 and Table 19).

[2] Error bars indicate LSD at p=0.05 (1.12 percent)

[3] Error bars indicate LSD at p=0.05 (0.79 percent)

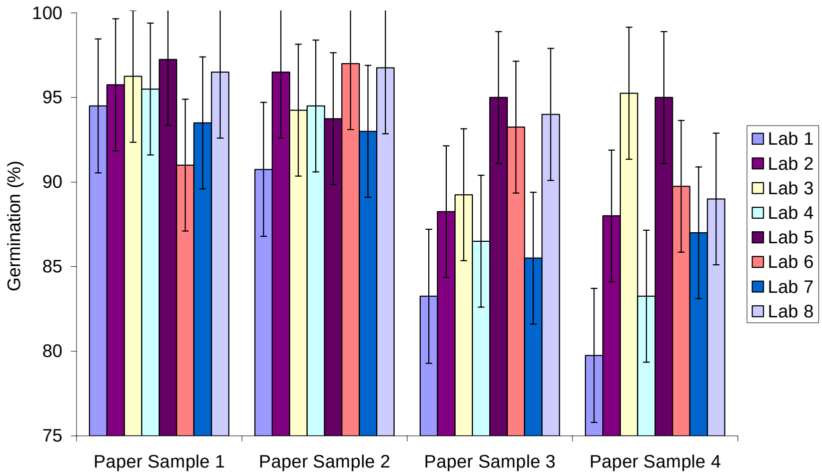

Figure 15 Mean Germinations[4] obtained using BP media

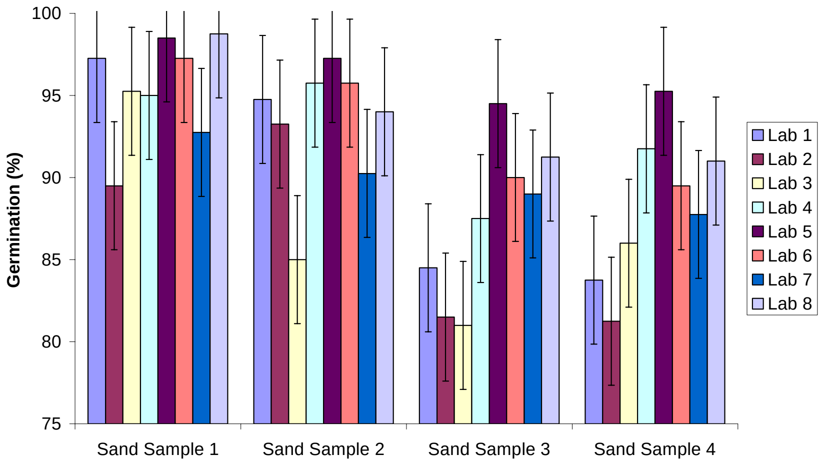

Figure 16 Mean Germinations[5] obtained using S media

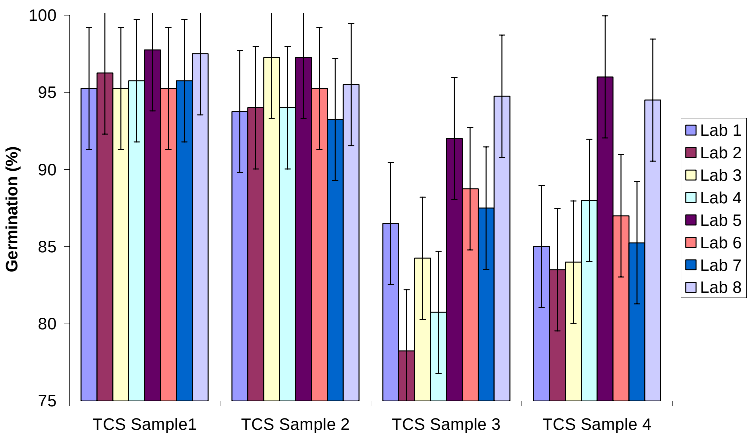

Figure 17. Mean Germinations[6] obtained using TCPS media

[4] Error bars indicate LSD at p=0.05 (3.89 percent)

[5] Error bars indicate LSD at p=0.05 (3.89 percent)

Table 19. Mean germination percentage for three medium types by laboratory and averaged across four seed lots.

Lab	**BP**		**S**		**TCPS**	
	Germination %		**Germination %**		**Germination %**	
1	87.1	A	90.1	B	90.1	AB
2	92.1	C	86.4	A	88.0	A
3	93.8	CDE	86.8	A	90.2	AB
4	89.9	B	92.5	C	89.6	AB
5	95.2	E	96.4	D	95.8	C
6	92.8	CD	93.1	C	91.6	B
7	89.8	B	89.9	B	90.4	B
8	94.1	DE	93.8	C	95.6	C
LSD (P =0.05)	1.87		1.95		2.24	

Discussion and conclusion

Results obtained in this validation study support inclusion of top of crepe cellulose paper (Versa-pak ®) covered with S (TCPS) as a medium option in the ISTA rules for Pisum *sativum*.

The results of ISO approved z-score analysis tests for laboratory proficiency tests reveal that using the TCP method laboratories performed as well if not better than when using BP and S methods for the germination testing of pea seed (Table 20).

[6] Error bars indicate LSD at p=0.05 (3.89 percent)

Table 20. Summary of the results of z-score analysis

	Germination Media		
z-score analysis	BP	S	TCPS
A-Individual z-scores (32 in total for each germination attribute)	**Normal Seedlings** 31 Satisfactory 1 Questionable **Abnormal Seedlings** 32 Satisfactory **Dead Seed** 30 Satisfactory 2 Questionable	**Normal Seedlings** 31 Satisfactory 1 Questionable **Abnormal Seedlings** 30 Satisfactory 2 Questionable **Dead Seed** 32 Satisfactory	**Normal Seedlings** 32 Satisfactory **Abnormal Seedlings** 32 Satisfactory **Dead Seed** 31 Satisfactory 1 Questionable
B-Distribution of z-scores **(**Ratings for the eight laboratories**)**	**Normal Seedlings** 7 Laboratories “Good” 1 “Satisfactory” **Abnormal Seedlings** 8 Laboratories “Good” **Dead Seed** 7 Laboratories “Good” 1 “Satisfactory”	**Normal Seedlings** 7 Laboratories “Good” 1 “Satisfactory” **Abnormal Seedlings** 6 Laboratories “Good” 2 “Satisfactory **Dead Seed** 8 Laboratories “Good”	**Normal Seedlings** 8 Laboratories “Good” **Abnormal Seedlings** 8 Laboratories “Good” **Dead Seed** 8 Laboratories “Good”
C- Re-scaled sum of z-scores **(**Ratings for the eight laboratories**)**	**Normal Seedlings** 7 Laboratories “Good” 1 “Satisfactory” **Abnormal Seedlings** 7 Laboratories “Good” 1 “Questionable” **Dead Seed** 7 Laboratories “Good” 1 “Satisfactory”	**Normal Seedlings** 5 Laboratories “Good” 3 “Satisfactory” **Abnormal Seedlings** 5 Laboratories “Good” 3 “Satisfactory **Dead Seed** 7 Laboratories “Good” 1 “Satisfactory”	**Normal Seedlings** 6 Laboratories “Good” 2 “Satisfactory” **Abnormal Seedlings** 5 Laboratories “Good” 3 “Satisfactory **Dead Seed** 7 Laboratories “Good” 1 “Satisfactory”
D-Squared sum of z-scores **(**Ratings for the eight laboratories**)**	**Normal Seedlings** 8 Laboratories “Satisfactory” **Abnormal Seedlings** 7 Laboratories “Satisfactory” 1 “Questionable” **Dead Seed** 8 Laboratories “Satisfactory”	**Normal Seedlings** 8 Laboratories “Satisfactory” **Abnormal Seedlings** 8 Laboratories “Satisfactory” **Dead Seed** 8 Laboratories “Satisfactory”	**Normal Seedlings** 8 Laboratories “Satisfactory” **Abnormal Seedlings** 8 Laboratories “Satisfactory” **Dead Seed** 7 Laboratories “Satisfactory” 1 “Questionable”

In terms of analysis of repeatability and reproducibility, based on ISO 5725-2 none of the participating laboratories obtained significant h values when testing in TCPS, compared to 2 laboratories each for BP and S methods, and the number of significant k-values was less in TCPS (2) than S (4) but more than in BP (1). Overall repeatability and reproducibility levels for TCPS are as good if not better than those for BP and sand (figures 11 and 12).

It should be noted that it is difficult to compare reproducibility and repeatability values with previous trials; very few ISTA trials have been analysed according to ISO 5725-2. Moreover, measurements of reproducibility and repeatability depend on the scale and unit of measurement. So if these elements are not the same in another study, it is not possible to simply compare the values obtained in the two different studies. With this in mind, the values of reproducibility and repeatability are of a similar magnitude or lower (indicating better reproducibility and repeatability) than those reported in the study of the effect of growing media and temperature on sunflower germination (Ducournau *et al*, 2007) (Table 21)

Table 21: Mean values of repeatability and reproducibility obtained in a comparative testing study on the effect of temperature and germination media on the germination of Sunflower seed and in a comparative test on the use of different germination media for pea seed germination.

Media and Temperature Regime	**Repeatability**	**Reproducibility**
Sunflower BP 20°C	2,97	8,21
Sunflower BP 25°C	4,35	10,88
Sunflower BP 20-30°C	3,76	8,34
Sunflower S 20°C	3,69	8,22
Sunflower S 25°C	3,37	18,74
Sunflower S 20-30°C	3,21	5,01
Sunflower O 20°C	3,53	4,57
Sunflower O 25°C	2,96	3,72
Sunflower O 20-30°C	2,99	4,24
Peas BP	2.59	4.14
Peas S	2.94	4.83
Peas TCPS	2.53	3.94

Variance analysis is a powerful tool in the evaluation of research projects and in this study we see that differences as small as 0.68% are enough to indicate significant differences between the germinations obtained in the different media. Such differences have little practical meaning when the tolerance for the overall mean germination is 10. Care must therefore be taken in the interpretation of ANOVA data.

It is clear that there is no significant difference between overall germinations obtained using TCPS compared to the two approved ISTA germination media (BP and S). Significant interactions are apparently due to the experience of laboratories with the different media (Table 22).

Table 22. Mean Germination results obtained by participating laboratories and the laboratories usual testing media for Pea seed

Laboratory	Normal Testing Media	Mean Germination BP	Mean Germination S	Mean Germination TCP
1	TCPS	87.1	90.1	90.1
2	BP	92.1	86.4	88.0
3	BP	93.8	86.8	90.2
4	S	89.9	92.5	89.6
5	TCPS	95.2	96.4	95.8
6	S	92.8	93.1	91.6
7	BP	89.8	89.9	90.4
8	TCPS	94.1	93.8	95.6

Highest germinations are generally obtained when a laboratory uses its usual germination medium and laboratories find it easier to assess seedlings using their normal germination media. This is particularly the case with the seed lots of lower germination potential (Lots 3 and 4). Some Laboratories had particular difficulties with some of the media, e.g. laboratory 3 with S, and this resulted in significantly lower germination results from such laboratory in both ANOVA and z-score tests. Such laboratory differences lead to significant interaction factors in variance analysis.

The data generated in this validation study supports the inclusion of TCPS as a new media testing option for ISTA laboratories. The TCPS method utilizes a lean manufacturing approach to seed testing through the tray and cart method. The TCPS method has the potential to save time and increase uniformity of results among seed testing laboratories.

The authors propose ISTA consider adopting TCPS as a recommended medium method and recognize TCPS as a germination medium for: *Pisum sativum*. Further work will be carried out to support the inclusion of TCPS as a germination medium for *Glycine max, Phaseolus vulgaris, Zea mays, Gossypium spp. and Helianthus annuus.*

Acknowledgments: The authors would like to thank Seminis for the seed donation, National Packaging Service Corporation for the Versa-Pak ® donation, participants; Sue Alvarez, Ronald Don, Dr. Norbert Leist, Lea Mazor, Dr. Günter Müeller, Kim North, and Victor Vankus. Thanks also go to Ronald Don, Amanda Patin, and Terry Lemme, for comments on design and editorial review.

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MVR-01-003
Viability testing of onion *(Allium cepa)* [Rules Proposal 2008 item C.6.1]

Romeo Herr and Michael Kruse, Universität Hohenheim, Stuttgart, Germany

March 2008

Summary

A validation study about TZ seed viability testing in onion was carried out. Nine laboratories were involved and each tested 400 seeds of four seed lots. The analysis of the results demonstrate that the method used is of sufficient repeatability and reproducibility to be included in the ISTA Rules.

1. Plant material

Four seed samples of onion were ordered from three seed companies in Germany between November 2006 and March 2007. The seeds were stored in the refrigerator at 7 °C.

a.) All E 13: *Allium cepa* cv. Piroska,

Enza Zaden Deutschland GmbH & Co. KG, Dannstadt-Schauernheim,

Lot no. 231350, date of delivery: 08 December 2006

b.) All E 79: *Allium cepa* cv. Piroska,

Enza Zaden Deutschland,

Lot no. 237956, date of delivery: 08 December 2006

c.) All H 94: *Allium cepa* cv. Matina,

Hild Samen GmbH, Marbach,

lot no. a9416, date of delivery: 28 November 2006

d.) All SG: *Allium cepa* cv. Giugnese,

Syngenta Seeds, Kleve,

no lot number available, date of delivery: 19 March 2007

As a short name for each sample a combination of the genus (*Allium*), the first letter of the seed company and two figures of the lot number (if available) is used in this report.

The samples obtained from the seed companies were mechanically divided into subsamples by use of a soil divider. In an in-house study using 1,000 seeds and a comparative study in a second laboratory using 400 seeds the homogeneity of the seed samples was confirmed. About 4 – 5 g of each sample were sent to each of the laboratories in April and May 2007. The seeds were packed as blind samples (lot no. 1 – lot no. 4), the numbering of the four samples was different for each laboratory.

2. Participating laboratories

Nine laboratories from seven countries participated in this validation study.

<u>Argentina</u>

Dow Agro Sciences Argentina, Maria Belén Aranguren, Ruta 8 Knm 362, Venado Tuerto (CP:2600), Provincia de Santa Fe, Argentina

Augusto Martinelli, Rayen Laboratories S.R.L, Bv. Almafuerte 163, 2700 Pergamino, Pcia.Buenos Aires, Argentina

<u>France</u>

GEVES-SNES, Valerie Blouin, 4 rue Georges Morel, F-49071 Beaucouze Cedex,

Germany

LTZ Augustenberg, Stefanie Krämer, Neßlerstr. 23-31, 76227 Karlsruhe

Latvia

National Seed Testing Laboratory, Benita Derilo and Irene Jumburga, Lubanas Street 49, LV-1073 Riga

The Netherlands

NAK, Anny van Pijlen, Randweg 14, NL-8300 BC Emmeloord

United Kingdom

CPVS NIAB, Linda Maile, Huntingdon Road, Cambridge CB 3 0Le

Official Seed Testing Station, Ronald Don and Caroline Cadger, SASA Headquarters, 1 Roddinglaw Road, Edingburgh EH 12 9FJ

USA

USDA/ARS, Annette Miller, National Center for Genetic Resources Preservation, 1111 South Mason St., Fort Collins, CO 80521-4500, U.S.A

In this report the laboratories are anonymously numbered as Lab 1–Lab 9; the sequence of these numbers is not identical to the alphabetical list given here.

3. Procedure for TTC test

The testing method is described in table 1, which is the proposal for inclusion in the ISTA-Rules. Each laboratory tested 4 × 100 seeds from each of the 4 lots.

Table 1: Testing method for *Allium* as proposed for the ISTA Rules Change Proposals 2009.

Species	Pretreatment: type/min. time (h)	Preparation before staining	Staining solution (%)	Optimum staining time (h)	Preparation for evaluation	Permitted non-viable tissue	Remarks
1	2	3	4	5	6	7	8
Allium spp.	W/18	Cut off a thin slice at the linear side of the seed and longitudinally ⅔ into the endosperm near the middle of the seed between radicle and cotyledons	1	18	Cut longitudinally from the flat side through endosperm to expose the embryo	None, including endosperm, except small superficial necrosis on the outer part of the endosperm, not in connection with the embryo cavity	–

4. Results

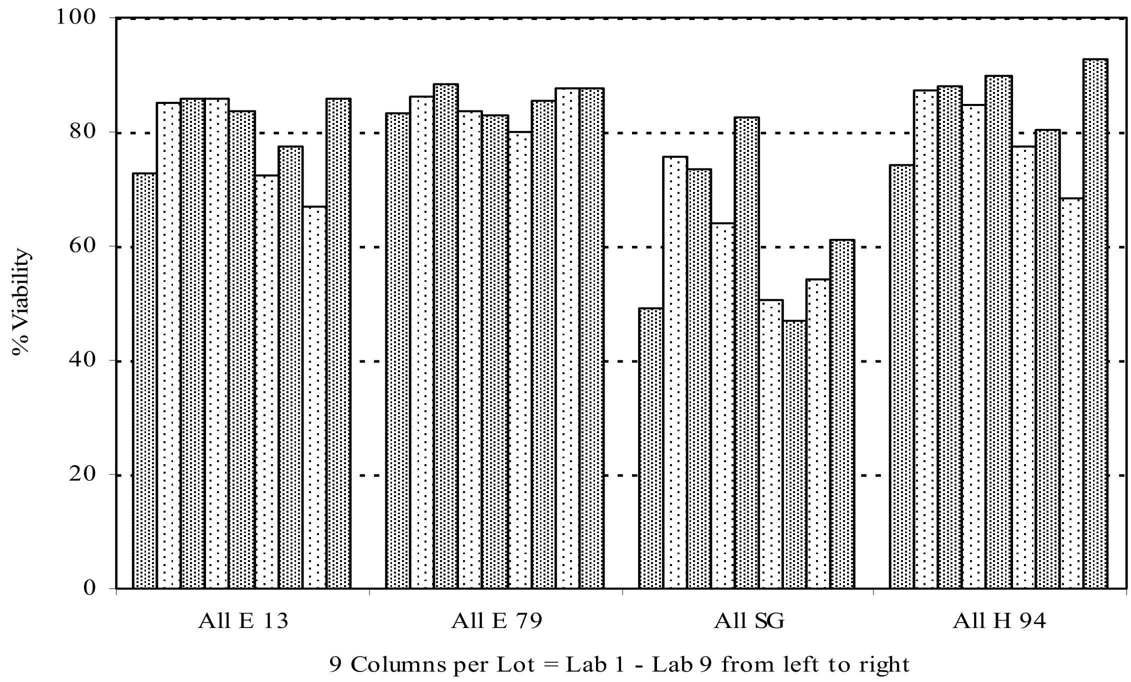
The results of the TTC viability tests were reported between March and July 2007. The results are given in table 2 and shown in figure 1.

Table 2: Seed viability (%) as reported for the four onion seed samples by the 9 participating laboratories (results of the four replicates each containing 100 seeds)

	Lot			
	All E 13	**All E 79**	**All H 94**	**All SG**
Lab 1	77	84	78	56
	67	87	77	48
	80	82	73	45
	67	80	69	48
Lab 2	85	85	91	75
	86	86	84	72
	87	89	84	75
	83	85	90	80
Lab 3	83	88	87	78
	87	86	88	76
	85	91	89	73
	88	89	88	67
Lab 4	86	80	85	67
	83	82	84	56
	91	87	81	62
	84	86	89	71
Lab 5	87	81	89	79
	86	88	88	88
	77	82	91	81
	85	81	91	82
Lab 6	72	79	73	47
	70	80	82	51
	74	78	76	55
	73	83	79	49
Lab 7	75	82	84	48
	78	90	81	52
	79	83	82	50
	78	87	74	38
Lab 8	65	90	69	55
	63	83	65	51
	70	89	68	57
	70	88	72	54
Lab 9	89	87	91	68
	84	89	90	52
	85	86	97	62
	85	88	93	62
mean	**80**	**85**	**83**	**62**

The highest mean viability was 85 ± 4 % for “All E 79”, the lowest mean viability was 62 ± 13 % for “All SG”.

Fig. 1: Viability test results for four onion seed lots as reported by the 9 participating laboratories



5. Statistical Analysis

For statistical analysis the experimental error 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:

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

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

p : % TTC-viability as mean;

q : 100 – p;

n = number of seeds.

Experimental error among the replicates

In table 3 the factors f for experimental error among the 4 replicates within a viability test in each of the 9 laboratories are given. The average factor f for 9 labs and 4 lots is 0.84 and with it below 1.00.

Experimental error among tests in different laboratories

In table 4 the factors f for experimental errors among the 9 laboratories are given. The average factor f for 4 lots is 3.67. The individual f-values for the lots are between 1.54 and 5.29.

Table 3: 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.

	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	mean
All E 13										
mean	73	85	86	86	84	72	78	67	86	
SD obs.	6.75	1.71	2.22	3.56	4.57	1.71	1.73	3.56	2.22	
SD exp.	4.45	3.55	3.50	3.47	3.69	4.48	4.18	4.70	3.50	
f	**1.52**	**0.48**	**0.63**	**1.03**	**1.24**	**0.38**	**0.41**	**0.76**	**0.63**	**0.79**
All E 79										
mean	83	86	89	84	83	80	86	88	88	
SD obs.	2.99	1.89	2.08	3.30	3.37	2.16	3.70	3.11	1.29	
SD exp.	3.73	3.44	3.19	3.69	3.76	4.00	3.52	3.31	3.31	
f	**0.8**	**0.55**	**0.65**	**0.9**	**0.9**	**0.54**	**1.05**	**0.94**	**0.39**	**0.75**
All H 94										
mean	74	87	88	85	90	78	80	69	93	
SD obs.	4.11	3.77	0.82	3.30	1.50	3.87	4.35	2.89	3.10	
SD exp.	4.37	3.34	3.25	3.60	3.03	4.18	3.98	4.65	2.59	
f	**0.94**	**1.13**	**0.25**	**0.92**	**0.49**	**0.93**	**1.09**	**0.62**	**1.19**	**0.84**
All SG										
mean	49	76	74	64	83	51	47	54	61	
SD obs.	4.72	3.32	4.80	6.48	3.87	3.42	6.22	2.50	6.63	
SD exp.	5.00	4.30	4.41	4.80	3.80	5.00	4.99	4.98	4.88	
f	**0.94**	**0.77**	**1.09**	**1.35**	**1.02**	**0.68**	**1.25**	**0.50**	**1.36**	**1.00**
								total average		**0.84**

Table 4: Experimental errors between the laboratories. For each lot the mean, the observed standard deviation, the expected standard deviation (based on the binomial distribution) and the f-values are shown.

Lot	**mean viability (%)**	**observed sd (%)**	**expected sd (%)**	**f-value**
All E 13	80	7.33	2.02	**3.64**
All E 79	85	2.74	1.78	**1.54**
All H 94	83	7.98	1.90	**4.21**
All SG	62	12.84	2.43	**5.29**
			mean	**3.67**

The f-value used for establishing the tolerance tables for seed viability test results the ISTA-Rules is 2.82. Thus, the average f-factor of 3.67 indicates a high but still acceptable experimental error among tests in different laboratories. The individual f-value for seed lot “All SG” (f = 5.29) is particularly high. The reason may be that this seed lot does not represent commercial seed quality as average viability is only 62%. Seed lots of such low quality may cause extraordinary variation because of specific quality problems.

In addition, as a further test, maximum tolerated ranges for the mean viabilities were calculated by the formula $S = f \times SD \times F$, given by Miles (1963). For lot “All SG” the observed range of mean values of the 9 laboratories is from 83 % to 47 % = 36 % (table 3). This range is lower than the calculated maximum range S = 40.4 % (table 5), so the range as a further measure for experimental error is still acceptable.

Table 5: Maximum tolerated ranges S(%) according to Miles (1963)

lot	S (%)	mean	f	SD exp.	F
All E 13	**33.3**	80	2.82	2.02	5.90
All E 79	**29.7**	85	2.82	1.78	5.90
All H 94	**31.2**	83	2.82	1.90	5.90
All SG	**40.4**	92	2.82	2.43	5.90

6. Conclusion

The f-factors in table 3 indicate an acceptable experimental error among the 4 replicates within the tests. The average f-factor of 3.67 indicates a still acceptable experimental error among tests (table 4). Thus, there is no reason to assume that the procedure given in table 1 should not be introduced in the ISTA-Rules.

MVR-01-004
Viability testing of cucumber *(Cucumis sativus)* [Rules Proposal 2008 item C.6.1]

Romeo Herr and Michael Kruse, Universität Hohenheim, Stuttgart, Germany

March 2008

Summary

A validation study about TZ seed viability testing in cucumber was carried out. Six laboratories were involved and each tested 400 seeds of three seed lots. The analysis of the results demonstrate that the method used is of sufficient repeatability and reproducibility to be included in the ISTA Rules.

1. Plant material

Three seed samples of cucumber were ordered from three seed companies in Germany in June 2007. The seeds were stored in the refrigerator at 7 °C.

a.) Cuc E 23: *Cucumis sativus* cv. Vorgebirgstrauben, 20 x 30 g, germination 96 %,

Enza Zaden Deutschland GmbH & Co. KG, Dannstadt-Schauernheim,

lot no. 230851S, date of delivery: 01 June 2007

b.) Cuc H 48: *Cucumis sativus* cv. Hokus, Freiland-Einlegegurke, 2 x 100 g, germination 96 %,

Hild Samen GmbH, Marbach,

lot no. h4820, date of delivery: 04 June 2007

c.) Cuc Q 60: *Cucumis sativus* cv. Delikatess, 15 x 30 g, reduced germination,

Saatzucht Quedlinburg GmbH, Quedlinburg,

lot no. 603940, date of delivery: 11 June 2007

As a short name for each lot a combination of the genus (*Cucumis*), the first letter of the seed company and two figures of the lot number is used in this report.

The samples obtained from the seed companies were mechanically divided into subsamples by use of a soil divider. In an in-house study using 1,000 seeds and a comparative study in a second laboratory using 400 seeds the homogeneity of the seed samples was confirmed. About 10 – 13 g (Cuc H 48) and 12 – 17 g (Cuc E 23, Cuc Q 60) were sent to each of the laboratories in June 2007. The seeds were packed as blind samples (lot no. 1 – lot no. 3), the numbering of the three samples was different for each laboratory.

2. Participating laboratories

Six laboratories from six countries participated in this validation study.

<u>France</u>

GEVES-SNES, Valerie Blouin, 4 rue Georges Morel, F-49071 Beaucouze Cedex,

<u>Germany</u>

LTZ Augustenberg, Stefanie Krämer, Neßlerstr. 23-31, 76227 Karlsruhe

<u>Latvia</u>

National Seed Testing Laboratory, Benita Derilo and Irene Jumburga, Lubanas Street 49, LV-1073 Riga

<u>The Netherlands</u>

NAK, Anny van Pijlen, Randweg 14, NL-8300 BC Emmeloord

United Kingdom

Official Seed Testing Station, Ronald Don and Caroline Cadger, SASA Headquarters, 1 Roddinglaw Road, Edingburgh EH 12 9FJ

USA

USDA/ARS, Annette Miller, National Center for Genetic Resources Preservation, 1111 South Mason St., Fort Collins, CO 80521-4500, U.S.A

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

3. Procedure for TTC test

The testing method is described in table 1, which is the proposal for inclusion in the ISTA Rules. Each laboratory tested 4 × 100 seeds from each of the 3 lots.

Table 1: Testing method for *Cucumis* as proposed for the ISTA Rules Change Proposals 2009.

Species	Pretreatment: type/min. time (h)	Preparation before staining	Staining solution (%)	Optimum staining time (h)	Preparation for evaluation	Permitted non-viable tissue	Remarks
1	2	3	4	5	6	7	8
Cucumis spp.	W/18	Cut off transversally a small part of the seed at distal end. Cut lateral longitudinally through the seed coat. Remove seed coat and the thin inner skin	1	6	Observe embryo	⅓ radicle, measured from the radicle tip, ½ of the distal end of cotyledons	–

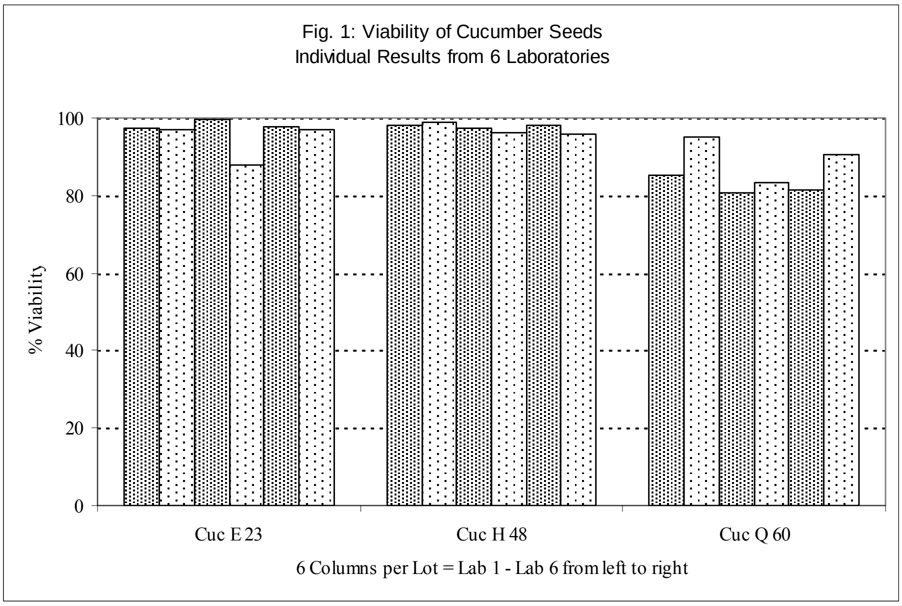
4. Results

The results of the viability tests were reported between August and October 2007. The results are given in table 2 and shown in figure 1.

Table 2: Seed viability as reported for the three cucumber seed lots by the 6 participating laboratories (results of the four replicates of 100 seeds)

Laboratory	Lot **Cuc E 23**	**Cuc H 48**	**Cuc Q 60**
Lab 1	97	99	85
	98	99	88
	97	97	86
	97	97	82
Lab 2	98	100	96
	96	97	97
	96	99	95
	98	99	92
Lab 3	99	99	78
	100	97	82
	99	97	79
	100	97	84
Lab 4	86	97	81
	89	96	83
	88	95	86
	88	97	84
Lab 5	99	97	86
	99	99	84
	95	99	83
	98	98	73
Lab 6	94	97	90
	99	95	91
	99	95	90
	96	96	91
mean	**96**	**97**	**86**

Two lots of cucumber seeds had a viability of 96 ± 4 % and 97 ± 1 %, one lot had a lower viability of 86 ± 6 %.



5. Statistical analysis

For statistical analysis the experimental error 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:

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

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

p : % TTC-viability as mean;

q : 100 – p;

n = number of seeds.

Experimental error among the replicates in one laboratory

In table 3 the factors f for experimental error among the 4 replicates within a viability tests in each of the 6 laboratories are given. The average factor f for 6 labs and 3 lots is 0.77, which is below 1.00.

Table 3: 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.

Cuc E 23	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	mean all labs
mean	97	97	100	88	98	97	
SD obs.	0.50	1.15	0.58	1.26	1.89	2.45	
SD exp.	1.64	1.71	0.71	3.28	1.48	1.71	
f	**0.31**	**0.68**	**0.82**	**0.38**	**1.28**	**1.44**	**0.82**
Cuc H 48	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	
mean	98	99	98	96	98	96	
SD obs.	1.15	1.26	1.00	0.96	0.96	0.96	
SD exp.	1.40	1.11	1.56	1.90	1.31	2.02	
f	**0.82**	**1.13**	**0.64**	**0.5**	**0.73**	**0.47**	**0.72**
Cuc Q 60	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	
mean	85	95	81	84	82	91	
SD obs.	2.50	2.16	2.75	2.08	5.80	0.58	
SD exp.	3.55	2.18	3.94	3.71	3.88	2.93	
f	**0.71**	**0.99**	**0.70**	**0.56**	**1.49**	**0.20**	**0.77**
						total average	**0.77**

Experimental error among tests in different laboratories

In table 4 the factors f for experimental errors among the 6 laboratories are given. The average factor f for 3 lots is 3.00, individual f-values are between 1.49 and 4.28.

Table 4: Experimental errors between the laboratories. For each lot the mean, the observed standard deviation, the expected standard deviation (based on the binomial distribution) and the f-values are shown

Lot	mean viability (%)	observed sd	expected sd	f-value
Cuc E 23	96	4.17	0.97	4.28
Cuc H 48	97	1.18	0.79	1.49
Cuc Q 60	86	5.58	1.73	3.23
			mean	3.00

The average factor f = 3.00 (table 4) indicates a high but still acceptable experimental error among the 6 laboratories for each lot. To estimate whether the variation is acceptable, the maximum tolerated range S for test results was calculated by the formula S = f × SD × F, given by Miles (1963). The calculation is shown in table 5. The range of observed mean values of 6 laboratories for lot "Cuc E 23" (experimental error f = 4.28) is between 100 % and 88 % = 12 % (table 3). This range is lower than S = 15.5 % (tab. 5), so even this experimental error (f = 4.28) can still be accepted.

Table 5: Maximum tolerated ranges S(%) according to Miles (1963)

lot	S (%)	p	f	SD exp.	F
Cuc E 23	**15.5**	96	2.82	0.97	5.90
Cuc H 48	**13.5**	97	2.82	0.79	5.90
Cuc Q 60	**27.5**	86	2.82	1.73	5.90

6. Conclusion

The f-factors in table 3 and 4 indicate an acceptable experimental error within and among the tests in the participating laboratories. There is no reason to assume that the procedure given in table 1 should not be introduced in the ISTA-Rules.

MVR-01-005
Viability testing of lettuce *(Lactuca sativa)* [Rules Proposal 2008 item C.6.1]

Romeo Herr and Michael Kruse, Universität Hohenheim, Stuttgart, Germany

March 2008

Summary

A validation study about TZ seed viability testing in lettuce was carried out. Eight laboratories were involved and each tested 400 seeds of four seed lots. The analysis of the results demonstrate that the method used is of sufficient repeatability and reproducibility to be included in the ISTA Rules.

1. Plant material

Four seed samples of lettuce were ordered from two seed companies in Germany in April 2007. The samples were stored in the refrigerator at 7 °C.

a.) Lac E 48: *Lactuca sativa* cv. Red Salad Bowl,

Enza Zaden Deutschland GmbH & Co. KG, Dannstadt-Schauernheim,

lot no. 234831

date of delivery: 27 April 2007

b.) Lac E 88: *Lactuca sativa* cv. Maikönig,

Enza Zaden Deutschland,

lot no. 238864

date of delivery: 27 April 2007

c.) Lac H 20: *Lactuca sativa* cv. Rebato,

Hild Samen GmbH, Marbach,

lot no. U 2040

date of delivery: 17 April 2007

d.) Lac H 49: *Lactuca sativa* cv. Oliver,

Hild Samen GmbH, Marbach,

lot no. U 4980

date of delivery: 17 April 2007

As a short name for each sample a combination of the genus (*Lactuca*), the first letter of the seed company and two figures of the lot number is used in this report.

The samples obtained from the seed companies were mechanically divided into subsamples by use of a soil divider. In an in-house study using 1,000 seeds and a comparative study in a second laboratory using 400 seeds, the homogeneity of the seed samples was confirmed. About 1 – 2 g of each sample were sent to each of the participating laboratories in June 2007. The seeds were packed as blind samples (lot no. 1 – lot no. 4), the numbering of the four samples was different for each laboratory.

2. Participating laboratories

Eight laboratories from seven countries participated in this validation study.

Argentina

Augusto Martinelli, Rayen Laboratories S.R.L, Bv. Almafuerte 163, 2700 Pergamino, Pcia.Buenos Aires, Argentina

France

GEVES-SNES, Valerie Blouin, 4 rue Georges Morel, F-49071 Beaucouze Cedex,

Germany

LTZ Augustenberg, Stefanie Krämer, Neßlerstr. 23-31, 76227 Karlsruhe

Latvia

National Seed Testing Laboratory, Benita Derilo and Irene Jumburga, Lubanas Street 49, LV-1073 Riga

The Netherlands

NAK, Anny van Pijlen, Randweg 14, NL-8300 BC Emmeloord

United Kingdom

CPVS NIAB, Linda Maile, Huntingdon Road, Cambridge CB 3 0Le

Official Seed Testing Station, Ronald Don and Caroline Cadger, SASA Headquarters, 1 Roddinglaw Road, Edinburgh EH 12 9FJ

USA

USDA/ARS, Annette Miller, National Center for Genetic Resources Preservation, 1111 South Mason St., Fort Collins, CO 80521-4500, U.S.A

In this report the laboratories are anonymously numbered as Lab 1 – Lab 8, the sequence of these numbers is not identical to the alphabetical list given here.

3. Procedure for viability testing

The testing method is described in table 1, which is the proposal for inclusion in the ISTA-Rules. Each laboratory tested 4 × 100 seeds from each of the 4 lots.

Table 1: Testing method for *Lactuca* as proposed for the ISTA Rules Change Proposals 2009.

Species	Pretreatment: type/min. time (h)	Preparation before staining	Staining solution (%)	Optimum staining time (h)	Preparation for evaluation	Permitted non-viable tissue	Remarks
1	2	3	4	5	6	7	8
Lactuca spp.	Prepare the dry seed, cut longitudinally ¼ through the distal end of the fruit (achene) and W/18	Expose the embryo by gently pressing the seed coat	1	3	Observe embryo	⅓ radicle, measured from the radicle tip; ½ of the distal end of the cotyledons,if superficial; ⅓ at distal end, if pervading	–

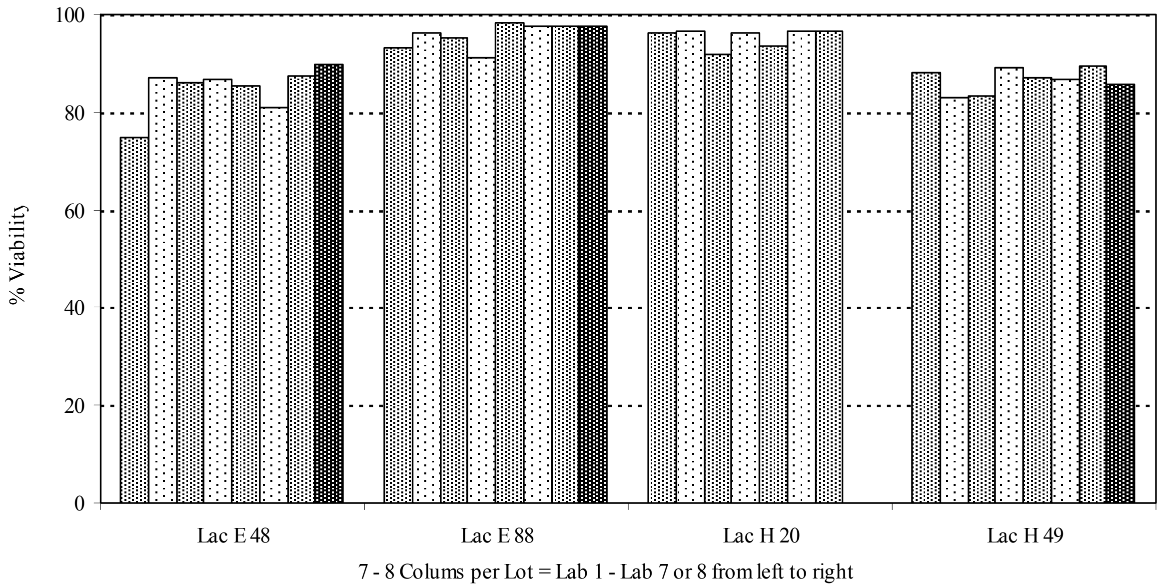
4. Results

The results of the viability tests were reported between July and September 2007. One laboratory (Lab 8) sent results only for three of four lots. The results are given in table 2 and shown in figure 1.

Table 2: Seed viability as reported for the four lettuce seed samples by the 8 participating laboratories (results of the replicates each containing 100 seeds)

Laboratory	**Lot**			
	Lac E 48	**Lac E 88**	**Lac H 20**	**Lac H 49**
Lab 1	65	93	95	89
	81	95	96	89
	82	90	98	87
	71	95	96	88
Lab 2	86	95	96	80
	92	95	97	85
	83	98	98	81
	87	97	96	86
Lab 3	85	97	93	82
	87	96	95	85
	85	95	95	84
	87	93	85	83
Lab 4	91	91	96	92
	84	92	97	90
	87	90	96	89
	85	91	96	86
Lab 5	85	99	91	81
	78	100	90	91
	90	95	97	88
	89	99	96	88
Lab 6	82	100	94	83
	79	96	99	87
	83	97	97	87
	80	98	96	90
Lab 7	90	100	97	88
	90	97	98	91
	86	97	94	91
	84	96	98	88
Lab 8	94	97	-	86
	84	99	-	85
	93	97	-	87
	88	98	-	85
mean	**85**	**96**	**95**	**87**

Fig 1: Viability test results for four lettuce seed lots as reported by the participating laboratories



The highest mean viability was 96 ± 3 % for “Lac E 88”, the lowest mean viability was 85 ± 6 % for “Lac E 48”.

5. Statistical analysis

For statistical analysis an experimental error 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:

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

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

p : % TTC-viability as mean;

q : 100 – p;

n = number of seeds.

<u>Experimental error among the replicates</u>

In table 3 the f-factors for experimental error among the 4 replicates within a viability test in the 8 laboratories are given. The average factor for 8 labs and 4 lots is f = 0.91, which is below 1.00.

<u>Experimental error among tests in different laboratories</u>

In table 4 the factors f for experimental errors among the 8 laboratories are given. The average factor f for 4 lots is 2.12. Individual f-values for each of the 4 lots are between 1.43 – 2.64.

Table 3: 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.

	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	mean
Lac E 48									
mean	75	87	86	87	86	81	88	90	
SD obs.	8.18	3.74	1.15	3.10	5.45	1.83	3.00	4.65	
SD exp.	4.34	3.36	3.47	3.39	3.52	3.92	3.31	3.03	
f	**1.88**	**1.11**	**0.33**	**0.91**	**1.55**	**0.47**	**0.91**	**1.53**	**1.09**
Lac E 88									
mean	93	96	95	91	98	98	98	98	
SD obs.	2.36	1.50	1.71	0.82	2.22	1.71	1.73	0.96	
SD exp.	2.51	1.90	2.13	2.86	1.31	1.48	1.56	1.48	
f	**0.94**	**0.79**	**0.80**	**0.29**	**1.69**	**1.15**	**1.11**	**0.65**	**0.93**
Lac H 20									
mean	96	97	92	96	94	97	97		
SD obs.	1.26	0.96	4.76	0.50	3.51	2.08	1.89	no	
SD exp.	1.90	1.77	2.71	1.90	2.47	1.84	1.77	data	
f	**0.66**	**0.54**	**1.75**	**0.26**	**1.42**	**1.13**	**1.07**		**0.98**
Lac H 49									
mean	88	83	84	89	87	87	90	86	
SD obs.	0.96	2.94	1.29	2.50	4.24	2.87	1.73	0.96	
SD exp.	3.22	3.76	3.71	3.10	3.36	3.39	3.07	3.50	
f	**0.30**	**0.78**	**0.35**	**0.81**	**1.26**	**0.85**	**0.57**	**0.27**	**0.65**
							total average		**0.91**

Table 4: Experimental errors between the laboratories. For each lot the mean, the observed standard deviation, the expected standard deviation (based on the binomial distribution) and the f-values are shown.

Lot	mean viability (%)	observed sd (%)	expected sd (%)	f-value
Lac E 48	85	4.75	1.80	**2.64**
Lac E 88	96	2.58	0.99	**2.59**
Lac H 20	95	1.89	1.04	**1.81**
Lac H 49	87	2.44	1.70	**1.43**
			mean	**2.12**

6. Conclusion

The f-factors in table 3 and table 4 indicate an acceptable experimental error among the 4 replicates in the laboratories and among the 8 laboratories for one lot. There is no reason to assume that the procedure given in table 1 should not be introduced in the ISTA-Rules.

MVR-01-006
Viability testing of tomato *(Lycopersicon esculentum)* [Rules Proposal 2008 item C.6.1]

Romeo Herr and Michael Kruse, Universität Hohenheim, Stuttgart, Germany

March 2008

Summary

A validation study about TZ seed viability testing in tomato was carried out. Nine laboratories were involved and each tested 400 seeds of four seed lots. The analysis of the results demonstrate that the method used is of sufficient repeatability and reproducibility to be included in the ISTA Rules.

1. Plant material

Four seed samples of tomato were ordered from three seed companies in Germany in November and December 2006. The seeds were stored in the refrigerator at 7 °C.

a.) Lyc E 22: *Lycopersicon esculentum* cv. Tigerella,

Enza Zaden Deutschland GmbH & Co. KG, Dannstadt-Schauernheim,

lot no. 222498, date of delivery: 08 December 2006

b.) Lyc E 23: *Lycopersicon esculentum* cv. Hellfrucht,

Enza Zaden Deutschland,

lot no. 235522, date of delivery: 08 December 2006

c.) Lyc H 84: *Lycopersicon esculentum* cv. Matina,

Hild Samen GmbH, Marbach,

lot no. a84790, date of delivery: 28 November 2006

d.) Lyc Q 68: *Lycopersicon esculentum* cv. Balkonzauber,

Saatzucht Quedlinburg GmbH, Quedlinburg,

lot no. 680360, date of delivery: 27 November 2006

As a short name for each sample a combination of the genus (*Lycopersicon*), the first letter of the seed company and two figures of the lot number is used in this report.

The samples obtained from the seed companies were mechanically divided into subsamples by use of a soil divider. In an in-house study using 1,000 seeds and a comparative study in a second laboratory using 400 seeds the homogeneity of the seed samples was confirmed. About 5 g of each sample were sent to each of the laboratories between February and April 2007. The seeds were packed as blind samples (lot no. 1 – lot no. 4), the numbering of the four samples was different for each laboratory.

2. Participating laboratories

Nine laboratories from seven countries participated in this validation study:

<u>Argentina</u>

Dow Agro Sciences Argentina, Maria Belén Aranguren, Ruta 8 Knm 362, Venado Tuerto (CP:2600), Provincia de Santa Fe, Argentina

Augusto Martinelli, Rayen Laboratories S.R.L, Bv. Almafuerte 163, 2700 Pergamino, Pcia.Buenos Aires, Argentina

<u>France</u>

GEVES-SNES, Valerie Blouin, 4 rue Georges Morel, F-49071 Beaucouze Cedex,

Germany

LTZ Augustenberg, Stefanie Krämer, Neßlerstr. 23-31, 76227 Karlsruhe

Latvia

National Seed Testing Laboratory, Benita Derilo and Irene Jumburga, Lubanas Street 49, LV-1073 Riga

The Netherlands

NAK, Anny van Pijlen, Randweg 14, NL-8300 BC Emmeloord

United Kingdom

CPVS NIAB, Linda Maile, Huntingdon Road, Cambridge CB 3 0Le

Official Seed Testing Station, Ronald Don and Caroline Cadger, SASA Headquarters, 1 Roddinglaw Road, Edingburgh EH 12 9FJ

USA

USDA/ARS, Annette Miller, National Center for Genetic Resources Preservation, 1111 South Mason St., Fort Collins, CO 80521-4500, U.S.A

In this report the laboratories are anonymously numbered as Lab 1 – Lab 9, the sequence of these numbers is not identical to the alphabetical list given here.

3. Procedure for viability testing

The testing method is described in table 1, which is the proposal for inclusion in the ISTA-Rules. Each laboratory tested 4 × 100 seeds from each of the 4 lots.

Table 1: Testing method for *Lycopersicon* as proposed for the ISTA Rules Change Proposals 2008.

Species	Pretreatment: type/min. time (h)	Preparation before staining	Staining solution (%)	Optimum staining time (h)	Preparation for evaluation	Permitted non-viable tissue	Remarks
1	2	3	4	5	6	7	8
Lycopersicon esculentum	W/18	Cut between radicle and cotyledons ⅓ into the endosperm	1	18	Cut the seed at the flat side into two halves, observe cut surfaces	None	Sometimes a staining of 42 h gives a clearer and darker staining. The size of the embryo must be more than one half of the normal size

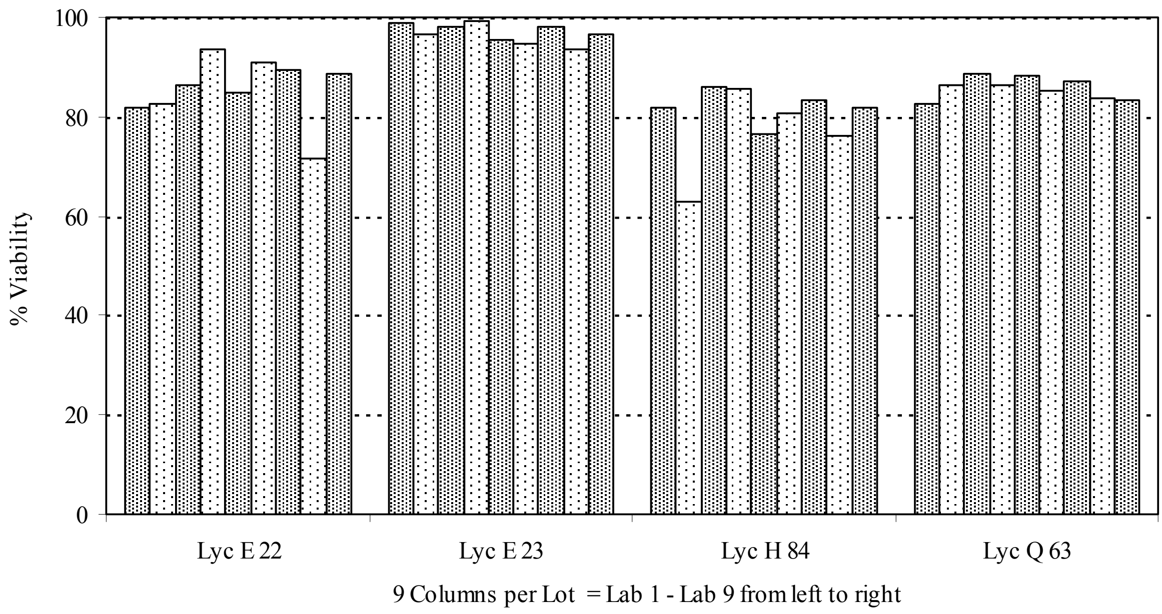
4. Results

The results of the TTC viability tests were reported between March and July 2007. The results are given in table 2 and shown in figure 1.

Table 2: Seed viability (%) as reported for the four tomato seed samples by the 9 participating laboratories (results of the four replicates each containing 100 seeds)

Laboratory	**Lot** **Lyc E 22**	**Lyc E 23**	**Lyc H 84**	**Lyc Q 68**
Lab 1	83	99	84	83
	84	100	83	80
	80	99	78	82
	81	98	82	85
Lab 2	80	95	67	82
	83	98	57	90
	85	97	65	87
	83	97	62	87
Lab 3	86	98	88	91
	85	98	83	88
	84	98	87	86
	90	98	86	89
Lab 4	94	100	89	82
	96	100	87	90
	97	99	83	88
	88	98	83	86
Lab 5	85	97	74	88
	85	97	71	88
	85	96	85	91
	84	92	76	86
Lab 6	90	96	81	86
	91	94	77	85
	90	92	85	87
	93	97	80	83
Lab 7	90	96	79	85
	87	99	84	88
	86	100	86	90
	94	97	84	86
Lab 8	66	95	75	87
	75	93	81	81
	72	94	74	83
	73	93	75	84
Lab 9	89	98	82	80
	87	96	84	87
	91	95	78	83
	88	97	83	84
mean	**86**	**97**	**79**	**86**

Fig. 1: Viability test results for four tomato seed lots as reported by the 9 participating laboratories



The highest mean viability was 97 ± 2 % for "Lyc E 23", the lowest mean viability was 79 ± 8 % for "Lyc H 84".

5. Statistical analysis

For statistical analysis the experimental error 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:

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

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

p : % TTC-viability as mean;

q : 100 – p;

n = number of seeds.

<u>Experimental error among the replicates within the laboratories</u>

In table 3 the factors f for experimental error among the 4 replicates within a viability test in each of the 9 laboratories are given. The average factor f for 9 labs and 4 lots is 0.77 and with it smaller than 1.00.

<u>Experimental error among tests in different laboratories</u>

In table 4 the factors f for experimental errors among the 9 laboratories are given. The average factor f for 4 lots is 2.65. The individual f-values for the 4 lots are between 1.23 and 3.70.

Table 3: 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.

	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	mean
Lyc E 22										
mean	82	83	86	94	85	91	89	72	89	
SD obs.	1.83	2.06	2.63	4.03	0.50	1.41	3.59	3.87	1.71	
SD exp.	3.84	3.78	3.44	2.42	3.60	2.86	3.10	4.51	3.16	
f	**0.48**	**0.55**	**0.76**	**1.67**	**0.14**	**0.49**	**1.16**	**0.86**	**0.54**	**0.74**
Lyc E 23										
mean	99	97	98	99	96	95	98	94	97	
SD obs.	0.82	1.26	0.00	0.96	2.38	2.22	1.83	0.96	1.29	
SD exp.	0.99	1.77	1.40	0.86	2.07	2.23	1.40	2.42	1.84	
f	**0.82**	**0.71**	**0.00**	**1.11**	**1.15**	**0.99**	**1.30**	**0.40**	**0.70**	**0.80**
Lyc H 84										
mean	82	63	86	86	77	81	83	76	82	
SD obs.	2.63	4.35	2.16	3.00	6.03	3.30	2.99	3.20	2.63	
SD exp.	3.86	4.83	3.47	3.52	4.24	3.94	3.73	4.26	3.86	
f	**0.68**	**0.90**	**0.62**	**0.85**	**1.42**	**0.84**	**0.80**	**0.75**	**0.68**	**0.84**
Lyc Q 68										
mean	83	87	89	87	88	85	87	84	84	
SD obs.	2.08	3.32	2.08	3.42	2.06	1.71	2.22	2.50	2.89	
SD exp.	3.80	3.42	3.19	3.42	3.22	3.55	3.34	3.69	3.71	
f	**0.55**	**0.97**	**0.65**	**1.00**	**0.64**	**0.48**	**0.66**	**0.68**	**0.78**	**0.71**
								total average		**0.77**

Table 4: Experimental errors between the laboratories. For each lot the mean, the observed standard deviation, the expected standard deviation (based on the binomial distribution) and the f-values are shown.

Lot	mean viability (%)	observed sd (%)	expected sd (%)	f-value
Lyc E 22	86	6,52	1,75	**3,70**
Lyc E 23	97	1,90	0,88	**2,17**
Lyc H 84	79	7,11	2,02	**3,51**
Lyc Q 68	86	2,15	1,75	**1,23**
			mean	**2,65**

6. Conclusion

The f-factors in table 3 and table 4 indicate an acceptable experimental error among the 4 replicates in the laboratories and among the 9 laboratories. The f-factors are equal or below those f-values used for establishing tolerance tables as given in the ISTA-Rules. Thus variation is acceptable and there is no reason to assume that the procedure given in table 1 should not be introduced in the ISTA-Rules.

MVR-01-007
Modified method for the detection of *Aphelenchoides besseyi* Christie in *Oryza sativa* L. seeds [Rules Proposal 2008 item C.7.1]

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Summary

A method extracting dehulled seed was compared to the EPPO method (PM 3/38(1)) for the detection of *Aphelenchoides besseyi* in rice seed. The new method was evaluated in a peer validation study with three laboratories. The study was organized by the Seed Health Committee of the International Seed Testing Association. Two naturally infested seed lots and one non-infested seed lot were tested in five sub-samples of 100 seeds each, giving a total of 15 sub-samples per laboratory. For each sub-sample the numbers of ‘suspect’ (*A. besseyi*) and ‘other’ (saprophytes) nematodes were recorded. The identity of suspect nematodes was checked under a microscope at a higher magnification (1,000 x).

Although both methods detected the presence of *A. besseyi* in all infected samples, the overall results indicate a significantly higher number of nematodes extracted from dehulled seed after one day compared to the EPPO method after five days. For testing rice seed on a routine basis for the presence of *A. besseyi* the method extracting dehulled seed is recommended.

Introduction

Aphelenchoides besseyi Christie

White tip disease of rice (*Oryza sativa* L.) caused by *Aphelenchoides besseyi* Christie is widely distributed in all rice growing areas (Fortuner and Williams, 1975). Different countries have imposed quarantine regulations to prevent the entry of nematode infested seed (Gergon and Mew, 1991). Estimated grain yield losses range from 10 to 50% (Yoshii and Yamamoto, 1950; Atkins and Todd, 1959; Hung, 1959).

The nematode infects rice florets before the panicle emerges from the flag leaf sheath and multiplies rapidly in the florescence up to the second week of heading, corresponding to the ‘soft dough’ stage of grain development. At this time, juveniles of the nematode cease to emerge from eggs but those already hatched continue to develop to adults. As soon as the infected grain begins to mature, the nematodes become quiescent due to dehydration (Huang and Huang, 1972). It is this anhydrobiosis stage that enables the nematode to be transmitted by seed. The addition of water reactivates the nematode. In an infected seed, the nematode can remain viable and infective for up to 23 months (Todd and Atkins, 1958). As rice seed is rarely stored for more than a year, the anhydrobiotic capability of the nematode is more than sufficient for seed transmission.

Extraction methods

The method recommended by EPPO (PM 3/38(1)) is intended to detect the presence of *A. besseyi* in seed lots for quarantine purposes. It is a simple technique requiring only basic equipment (EPPO, 1998) and is based on the principle that nematodes will leave the tissue of rice seed if the seed is soaked in water for several days. Earlier workers (Yoshii and Yamamoto, 1950; Goto and Fukatsu, 1956; Todd and Atkins, 1958 & 1959; Fukano, 1962; Prasad and Varaprasad, 1992) manually peeled the husks from infested grains in a small quantity of water, and counted the nematodes that migrated out of the infested seed. It was shown that maximum reactivation of the quiescent nematodes could be obtained by immersion in water for 24 hours at 25 +/- 3 °C (Huang and Huang, 1974; Gergon and Mew, 1991). The technique, however, is laborious and hence impractical for routine testing. The use of a small hand milling equipment, very easy to clean, proved to be suitable for dehulling (Giudici *et al.*, 2003).

Aim of the test

In this validation study the method using dehulled seed for extraction was compared with the EPPO method (PM 3/38(1)) for the detection of *A. besseyi* in rice. The method was evaluated by three seed testing laboratories from the UK (Scotland), Italy and the Netherlands, all with practical experience in detecting this pathogen, in order to determine the most efficient and effective method.

Material and methods

Seed samples

From each of three seed lots, a non-infested, a medium and a heavily infested seed lot, five replicates of 100 seeds of rice were tested for both methods. With 5-fold replication, infection can be guaranteed to be less than 1% with 99% confidence if no A. besseyi was found (Huang, 1983; Gergon and Mew, 1991; Prot and Gergon, 1994, EPPO, 1998).

Sample preparation

For each seed lot 15 sub-samples of 100 seeds were prepared according to the International Rules for Seed Testing (Anonymous, 2001). Each sub-sample was coded randomly. There was no relationship between successive codes and seed lots. Five sub-samples from each lot were sent to the three participating laboratories in the UK, Italy and the Netherlands.

Extraction methods

EPPO method

Soak small samples of rice (e.g. 100 seeds) on a small nylon sieve, in shallow dishes of water, for up to 4-5 days at 25 +/- 2 °C. The sieve is a nylon piece of material with a pore size of 0.25 mm glued to a section of cylindrical tubing. Each day, the sieve carrying the seeds was removed from the dish while the water from the dish was collected for microscopic examination; the fresh water was added to the dish, and the sieve was replaced.

Dehulled seed method

100 seeds are dehulled using a Husker TR-120 (by Kett) with 1 mm of distance between the rolls, and the kernels and hulls are transferred to a nylon sieve, with meshes of 0.25 mm, placed in a beaker of Ø 45 mm filled with 20 ml of water. The apparatus was left undisturbed for 24 h at 25 +/- 2 °C. The sieve was removed from the beaker and gently squeezed. The water was used for microscopic examination.

Examination and confirmation

The water sample was allowed to stand for at least 20 minutes to allow any nematodes to settle to the bottom of the container. Both juveniles and adults of *A. besseyi* are counted in a De Grisse counting dish under a microscope (50x). Because many harmless microbivorous nematodes commonly occur in seed, it is necessary to confirm the identification, at higher magnification (1,000x), of any nematodes suspected of being *A. besseyi*.

Data analysis

General introduction

For each combination of laboratory, method and seed lot the numbers of suspect (*A. besseyi*) and 'other' (saprophytes) nematodes were recorded.

The numbers of suspect nematodes were analyzed using generalized linear modeling fitted with GenStat (Payne *et al.*, 2003).

Poisson model

The generalized linear model for the count data was specified considering a Poisson distribution and a log-link function. The effects of laboratories, methods and seed lots were tested against the mean deviance of laboratory x method x seed lot term in the model. The predictions (based on the model) and corresponding standard error were calculated. The standard errors are based on the dispersion factor that was set to the mean deviance of the laboratory x method x seed lot.

Repeatability and reproducibility

The repeatability and reproducibility variances were computed using the approach described in Laffont (2007) for a nominal count of 50. However, as these variances are usually increasing with the mean in the context pf Poisson data, we will not consider them to assess the performance of the methods.

Results

All sub-samples of the non-infested seed lot were found negative by all participants for both *A. besseyi* and saprophytes and also for both methods.

Table 1 contains the statistical effects for the different factors tested in this peer validation test. The analysis resulted in a significant seed lot effect which is expected by testing a heavy, medium infested lot and a non-infested lot. In addition, a significant method effect is recorded which means that more nematodes, *A. besseyi* and saprophytes, were extracted from the dehulled seeds. No laboratory effect was registered and none of the interactions (laboratory x method; laboratory x seed lot; method x seed lot) were significant, meaning that the results gathered in each laboratory for each lot and method were comparable.

Figure 1 presents the numbers of *A. besseyi* found by three laboratories in the heavily infested seed lot using both methods. All laboratories extracted a significant higher amount of suspect nematodes testing dehulled seed (average 109) compared to the EPPO method (average 23).

The numbers of suspect nematodes found in the medium infested seed lot is presented in Figure 2. The results for the EPPO method is comparable for all laboratories (average 10) while differences occur using the dehulled seed method. Laboratory 2 found less suspect nematodes (average 16) compared to laboratory 1 (average 45) and laboratory 3 (average 42).

Figure 3 and 4 present the numbers of saprophytes recorded in the heavily and medium infested seed lots, respectively. On average laboratory 3 found more saprophytes (2.4 (EPPO) and 0.8 (dehulled)) than laboratory 1 (0.07 (EPPO) and 0.7 (dehulled)) and laboratory 2 (0.1 (EPPO) and 0.3 (dehulled)).

The reproducibility variance (between laboratory variability plus within laboratory variability) and the repeatability variance (within laboratory variability) for both methods based on the count data are presented in Table 2 for information only as they will not be used for the evaluation.

Discussion

All laboratories detected *A. besseyi* in the two infested rice seed lots, heavily and medium infested. And as expected, none of the laboratories detected *A. besseyi* in the non-infested seed lot.

Dehulling of rice seed resulted in significantly more nematodes detected in the grain even after only a day of extraction, making it important in quantitative analysis of *A. besseyi*. The results of the dehulled seed were in agreement with research performed earlier (Gergon and Mew, 1991; Giudici *et al.*, 2003). The amount of target nematodes, *A. besseyi* in this case, increased significantly using dehulled seed. In addition, the extraction period shortens from 5 days for the EPPO method to 1 day by using dehulled seed which can be an advantage for growers who need their results quickly.

Laboratory 2 found less nematodes in the medium infested seed lot compared to the other participants. The number of nematodes extracted was comparable to the results gathered with the EPPO method for this lot. The differences were not significantly different from the other laboratories.

For this test the process of dehulling was not tested but the seed were dehulled by the test organizer. Using standardized samples seemed to be an advantage for the comparison of the two methods.

The small hand mill used for this dehulling is very easy to clean and suited its purpose well. The mill is provided by different companies and is inexpensive. It should be noted that cleaning the mill between each sample is extremely important to prevent cross contamination.

Conclusion

All laboratories were able to detect *A. besseyi* in the contaminated seed lots. The highest number of target nematodes was recorded using the dehulled seed method. Based on this, and the shorter extraction period, it is therefore recommended to use the dehulled seed method for testing of *Aphelenchoides besseyi* in rice seed.

Acknowledgements

The input of the participating laboratories and the ISTA Seed Health Committee members is greatly acknowledged. Special thanks are due to Nora Pelazza who prepared the seed samples and sent them to the participants.

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Tables and figures

Table 1: Determination of statistical significant differences for lot-, laboratory- and method-effects and their interaction for the presence of *Aphelenchoides besseyi*.

Factor	Df	Aphelenchoides besseyi
Laboratory effect	2/72	2.4
Method effect	1/72	159.7*
Seed lot effect	2/72	209.9*
Laboratory.method effect	2/72	2.6
Laboratory.seed lot effect	4/72	07
Method.seed lot effect	2/72	0.9
Laboratory.method.seed lot effect	4/72	1.2

* statistical significant differences compared to the F-value

Table 2: Reproducibility variance and repeatability variance for the detection of *Aphelenchoides besseyi* in rice seeds For a nominal count of 50.

Pathogen	Reproducibility	Repeatability
EPPO	239.7	221.3
Dehulled	807.5	753.3

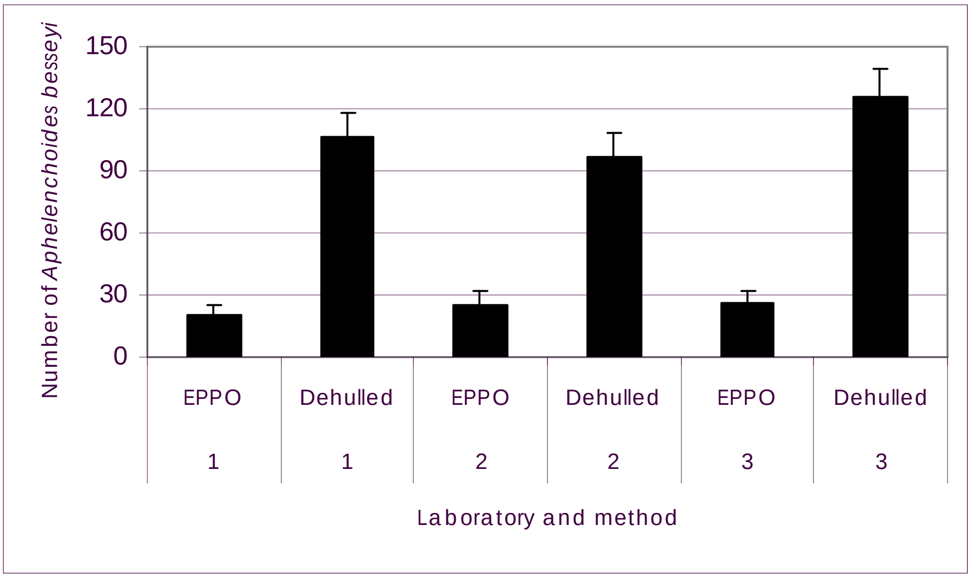

Figure 1: Number of *Aphelenchoides besseyi* found by all participants in the heavily infested lot of rice seeds using the EPPO and dehulled seed method.

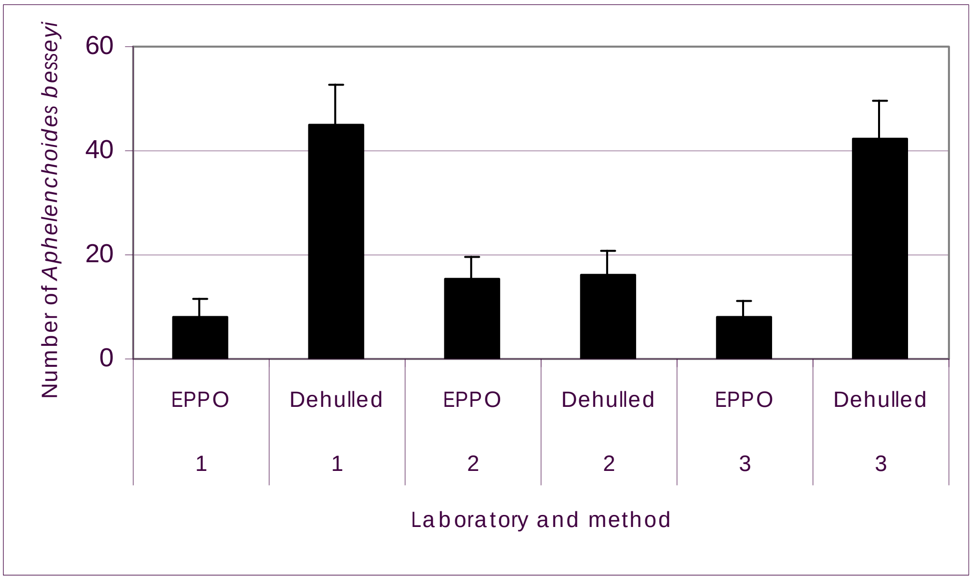

Figure 2: Number of *Aphelenchoides besseyi* found by all participants in the medium infested lot of rice seeds using the EPPO and dehulled seed method.

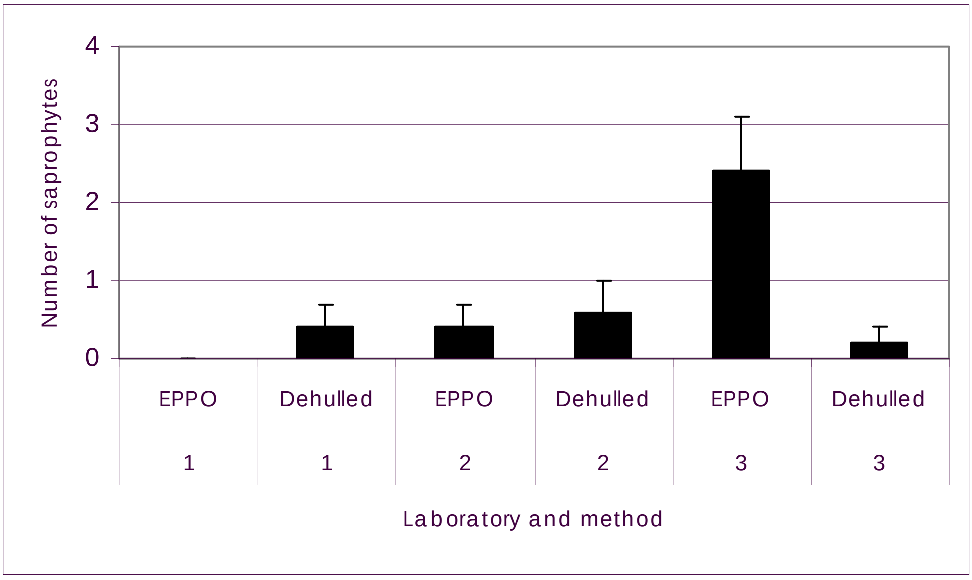

Figure 3: Number of saprophytes found by all participants in the heavily infested lot of rice seeds using the EPPO and dehulled seed method.

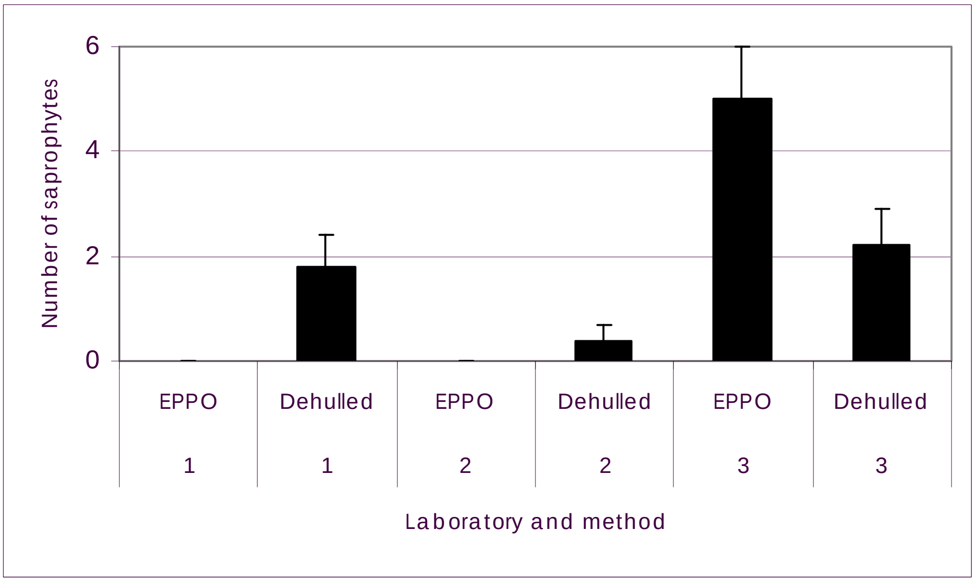

Figure 4: Number of saprophytes found by all participants in the medium infested lot of rice seeds using the EPPO and dehulled seed method.

MVR-01-008
New method for testing bitter seeds in lupin samples (*Lupinus* spp.) [Rules Proposal 2008 item C.8.1]

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Participating laboratories

In this international Comparative Test 14 laboratories (12 ISTA and 2 non ISTA) from four different countries (Austria, France, Germany, Latvia) have participated.

Introduction

In *Lupinus* spp. presence or absence of alkaloid is a diagnostic feature. At present the ISTA method (chapter 8, ISTA Rules) is used to distinguish seeds with low alkaloid content, called „sweet lupins“ (*Lupinus luteus*. *L. angustifolius* and *L. albus*) from seeds with high alkaloid content called „bitter lupins“.

As a guideline it is recommended to analyse 200 single seeds. Very precise estimates of varietal purity require larger samples. For reports and issue of ISTA International Seed Analysis Certificates, analysis of 400 single seeds is required.

In Comparative tests carried out in the past it became evident that seed testing labs are using different kinds of methods for testing bitter lupins (ISTA method, in-housemethods) and furthermore by applying the ISTA method they have problems in evaluating the results accurately.

Therefore a NEW, much more better method for testing bitter lupins was developed, evaluated and validated.

Material and methods

For the Comparative Test four non bitter lupin samples (sweet lupins, basic seed) from two different seed lots (two different varieties) were used. These four samples had to be analysed with the ISTA method as well as the NEW method.

The ISTA method and the NEW method are described as follows:

ISTA method (chapter 8.8.2 *Fabaceae* (*Leguminosae*) *and* Lolium *spp*.

1) soak the seeds in water for 24 h,
2) cut a thin slice from each seed and place the slice on a glass plate over a white surface,
3) add 1-2 drops of Lugol‘s solution to each slice,
4) a distinct brown–red precipitate indicates presence of alkaloid,
5) evaluation is done on a white surface.

In step 3) the ISTA method doesn’t give any advice with respect to the concentration of the Lugol’s solution.

NEW method

1) soak the single seeds in water for 2 h or longer (2.5 – 5.0 ml each seed),
2) scarify or pierce each seed with an appropriate tool (scalpel, needle, plier, …),
3) soak the seeds for further 5 – 24 h in water,
4) add 1–3 drops of 1 % Lugol’s solution to each seed,
5) a distinct brown–red precipitate indicates presence of alkaloid,

6) evaluation can be done either on a white surface or a luminescent screen.

The time targets of step 1) and 3) can be arranged somewhat flexible depending on the number of hard seeds in the sample. The number of added drops of the Lugol's solution depends on the equipment (pipette) used.

Design of the Proficiency Test

sample	1	2	3	4
added bitter seeds	6	10	8	14
method	ISTA method	NEW method	ISTA method	NEW method

Number of samples	4 samples
Number of seeds analysed	200 seeds per sample (2 x 100)
added bitter seeds	different number of bitter seeds
Validation (samples and added bitter seeds)	10 times (neither bitter seeds in the samples nor non bitter seeds in the added bitter seeds were detected)
Lugol's solution	1 % (10.0 g iodine + 20.0 g potassium iodide, made up to 1000 ml)

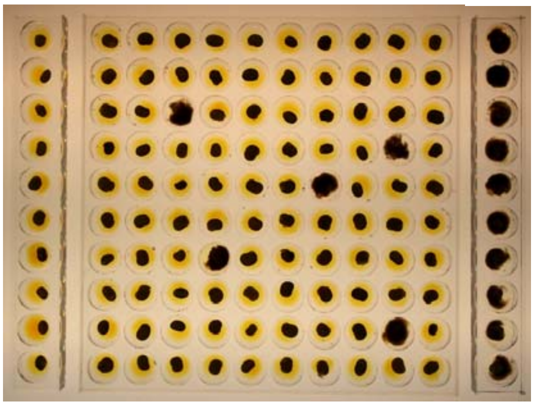
To avoid misunderstandings and errors in the Comparative Test, a 1 % Lugol's solution was dispatched with the samples. In Comparative Tests performed in the past it became evident, that a concentration of 1 % is the most suitable.

Equipment and evaluation

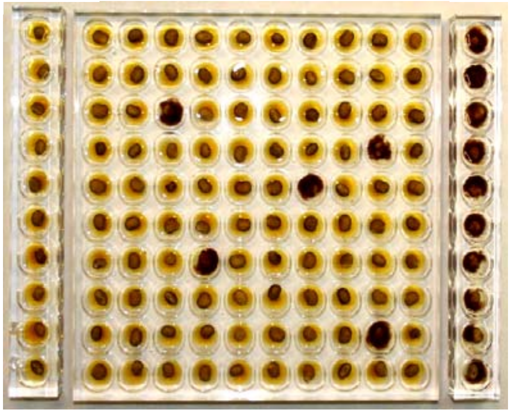
For the NEW method acrylic glass plates with 100 slots for each replicate (23 cm x 23 cm x 2 cm) and small acrylic glass plates (23 cm x 3 cm x 2 cm) each with 10 slots for the reference material (picture 1a and 1b) were manufactured. These acrylic glass plates are a kind of big microtiter plates with a volume of about 5 ml each slot. During soaking the plates should be covered with an appropriate equipment (thin acrylic glass plate, plastic film, glass plate, …) to avoid evaporation.

Evaluation can be done both on a white surface (picture 1a) and on a luminescent screen (picture 1b). An example with 5 bitter seeds added and detected is shown.

(1) (2)



(1) (2)



Picture 1a: Evaluation on a white surface

Picture 1b: Evaluation on a luminescent screen

(1): reference seeds with low alkaloid content (sweet lupins)

(2): reference seeds with high alkaloid contents (bitter lupins)

Data analysis

For data analysis and presentation mean deviation and boxplots were used.

Results and discussion

To the samples analysed with the ISTA method 6 and 8 bitter seeds have been added. To the equal samples analysed with the NEW method 10 and 14 bitter seeds have been added. Sample1 and sample 2 were from the same lot. This did apply to sample 3 and sample 4 too.

The results of the actually added bitter seeds to the samples are shown in figure 1, 2 and 3. As you can see in each case the mean deviation of the NEW method is much better than the mean deviation of the ISTA method (figure 1 and figure 2).

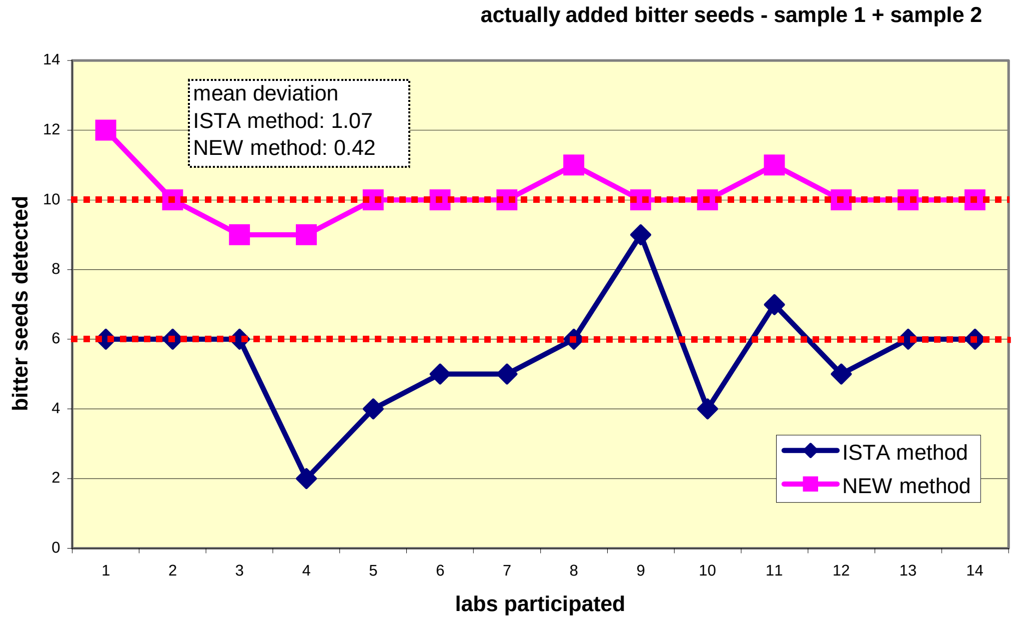


Figure 1: bitter seeds detected in sample 1 (ISTA method) and sample 2 (NEW method) by adding 6 bitter seeds to sample 1 and 10 bitter seeds to sample 2.

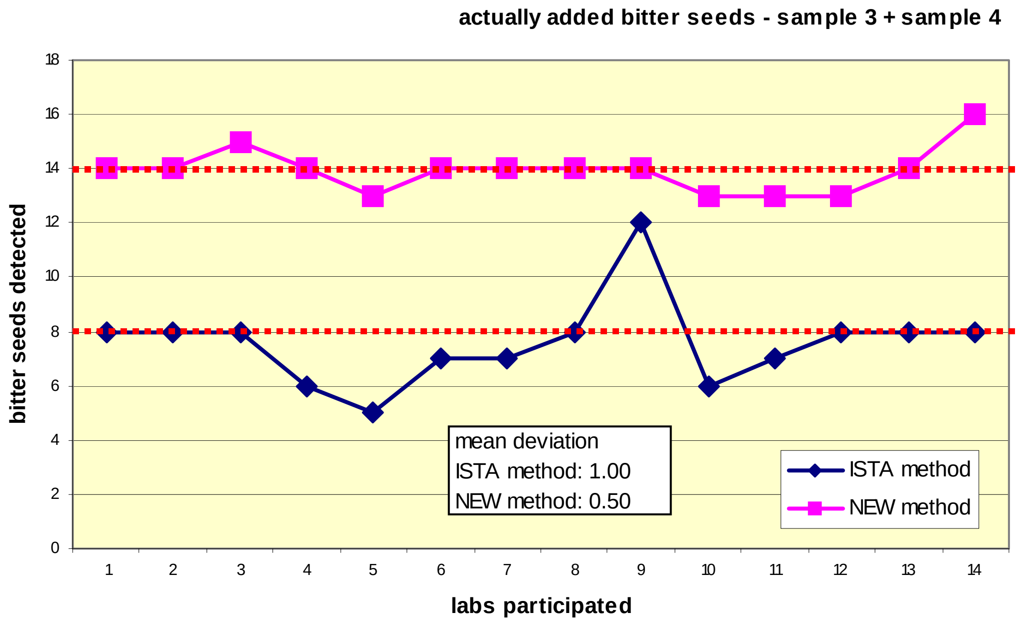


Figure 2: bitter seeds detected in sample 3 (ISTA method) and sample 4 (NEW method) by adding 8 bitter seeds to sample 3 and 14 bitter seeds to sample 4.

The lower the mean deviation, the more the values of a group or a distribution lie together, i.e. the better the method. Consequently the NEW method is better than the ISTA method.

By using the ISTA method more large outside values could be observed. These findings can be seen very clear in figure 3.

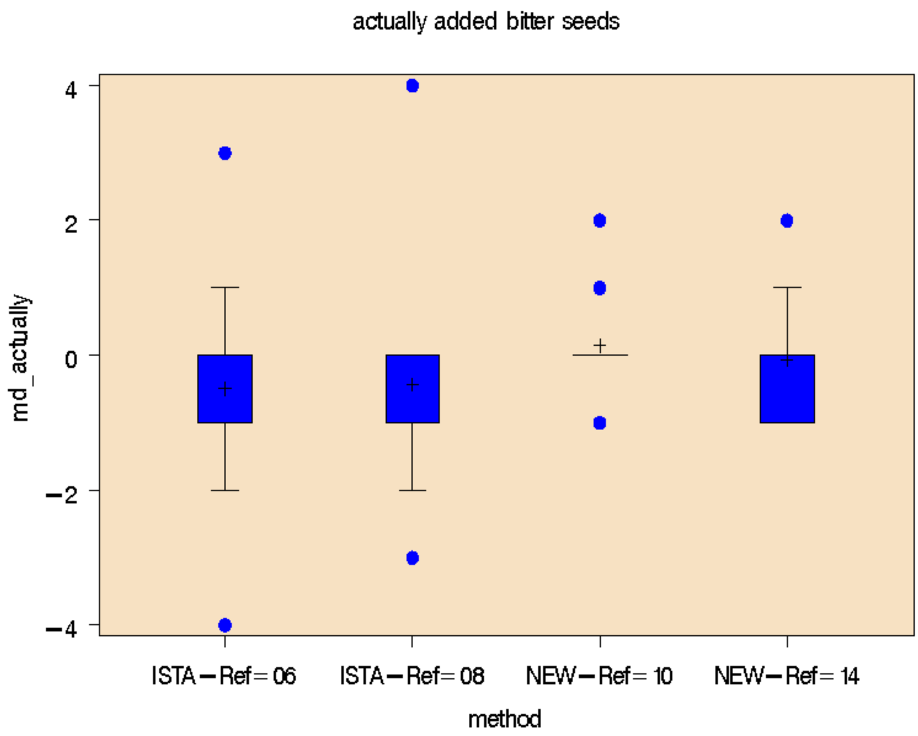


Figure 3: Visualisation of the observed distributions with boxplots (min, max, median, Q 75%, Q 25%).

It is a fact that not the same number of bitter seeds has been added to each sample as mentioned above. In order to compare both methods correctly a conversion of the data set was carried out. The number of actually added bitter seeds to each sample was normalized to 10, i.e. a theoretical adding of 10 bitter seeds to each sample was calculated. With this conversion of the data, all samples and methods can be really compared with each other. The results of the added bitter seeds normalized to 10 are shown in figures 4, 5 and 6. Again the mean deviation by applying the NEW method is much more better (figure 4 and figure 5). Large outside values and the big number of large outside values could only be found by applying the ISTA method. The NEW method is unambiguously the better method. These good results can be seen very clear in figure 6.

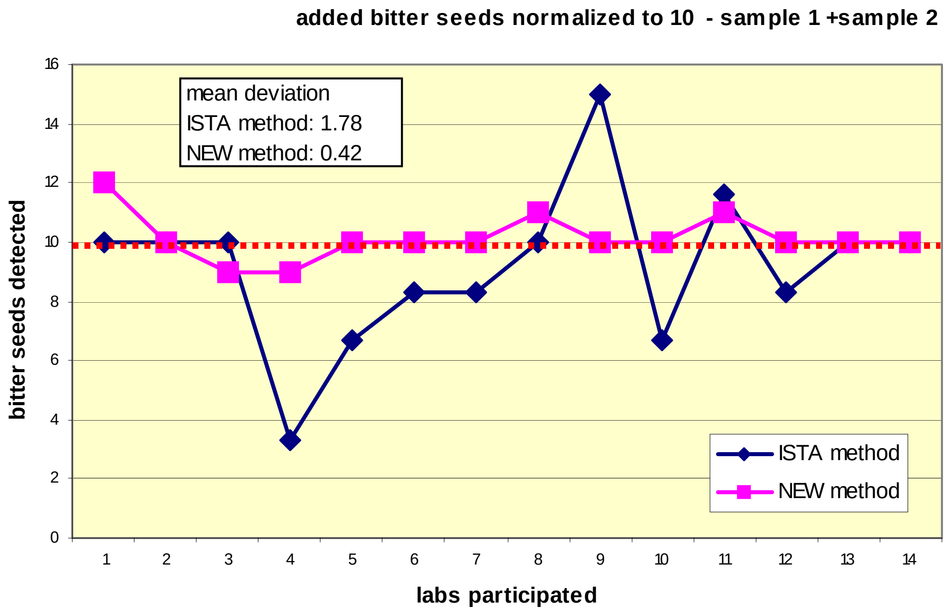


Figure 4: bitter seeds detected in sample 1 (ISTA method) and sample 2 (NEW method) after data conversion (added bitter seeds normalized to 10 to sample 1 and sample 2).

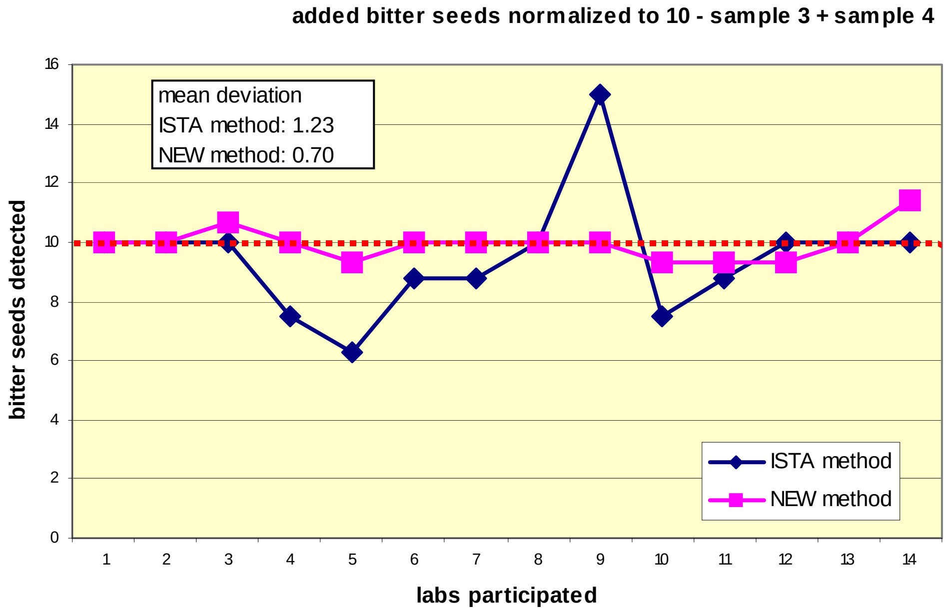


Figure 5: bitter seeds detected in sample 3 (ISTA method) and sample 4 (NEW method) after data conversion (added bitter seeds normalized to 10 to sample 3 and sample 4).

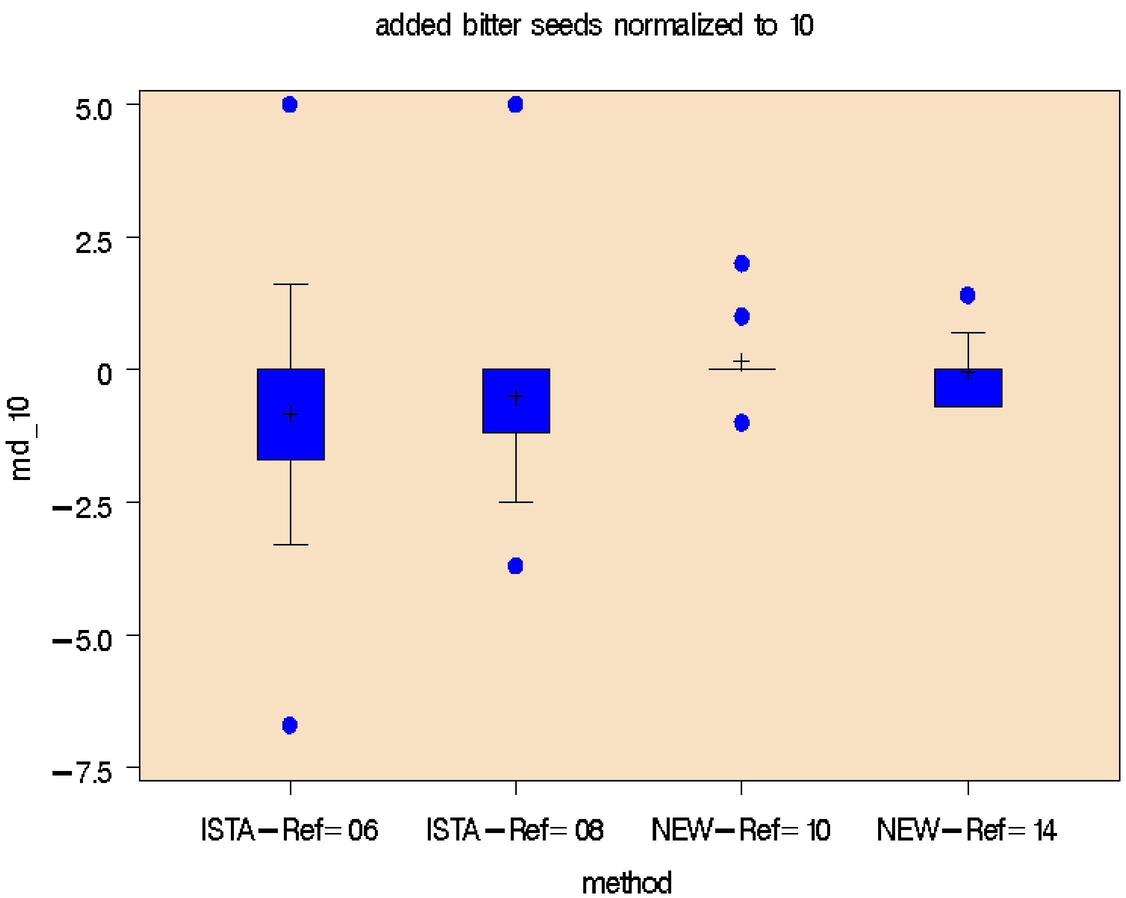


Figure 6: Visualisation of the observed distributions with boxplots (min, max, median, Q 75%, Q 25%).

Conclusions and recommendations

The results show very clearly that compared to the existing ISTA method the NEW method is the best method. It became evident that the labs would prefer the NEW method rather than the ISTA method, because the NEW method is much more sure in evaluation and much easier in handling.

In addition the NEW method has a lot of further advantages. The advantages noted by the laboratories are as follows:

The most important advantage is that the evaluation of the brown-red precipitate is more sure, easier, more reliable, unambiguous and can be done very fastly.

The NEW method is less time-consuming. For example by analysing 2 x 100 seeds the ISTA method lasts nearly doubly as long as the NEW method (depending on the experience of the seed analyst), therefore the NEW method causes much lower costs. Time for soaking is not taken into account.

	sample preparation	scarifying/slicing	evaluation
ISTA method (Σ 115 min)	15 min	60 min	40 min
NEW method (Σ 55 min)	15 min	20 min	20 min

- With respect to the evaluation, cases of doubts appear very rarely. They can be solved easily by adding further drops of a 1 % Lugol's solution.
- The NEW method is much easier to handle, because the seeds mustn't be sliced, only scarifying or piercing is necessary, therefore a lower risk of injury happens.
- The NEW method is much more flexible with respect to the time management.
- By applying the NEW method a considerably higher sample throughput can be achieved.
- Additionally there is a great benefit for the breeders. The tested seeds can be cleaned with water and after drying the seeds can be used for further breeding experiments.

Because of the good results and the large number of advantages of the validated NEW method we propose to replace the ISTA method with the NEW method (or to accept the NEW method as a second method for testing bitter lupins).

In our opinion a replacement is well-founded and recommended.

Acknowledgements

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