



International Seed Testing Association

Secretariat, Zürichstrasse 50, CH-8303 Bassersdorf, Switzerland

Phone: +41 44 838 60 00 Fax: +41 44 838 60 01

Email: ista.office@ista.ch - <http://www.seedtest.org>

Document OM11-06

Method Validation Reports on proposed changes to the International Rules for Seed Testing 2012

Contents

ISTA validation study for germination test of <i>Solanum nigrum</i>	2
Alternative embryo extraction procedure to seed health method 7-013: Detection of <i>Ustilago nuda</i> on <i>Hordeum vulgare</i>	11
The osmotic method for detection of <i>Pyrenophora teres</i> (<i>Drechslera teres</i>) and <i>P. graminea</i> (<i>D. graminea</i>) on <i>Hordeum vulgare</i>	17
Evaluation of early counts of radicle emergence during germination as a repeatable and reproducible vigour test for maize	30

ISTA validation study for germination test of *Solanum nigrum*

Mazor, L., Dekalo-Keren M., Abu-Aklin W., Berger V.

Volcani Center, ARO, Bet-Dagan, 50250, ISRAEL

Summary

The objective of this validation test is to introduce the species *Solanum nigrum* into Chapter 5 of the ISTA Rules. The genus *Solanum* is already represented in the ISTA Rules by the species *S. melongena* and *S. tuberosum*.

The experiment was carried out by five ISTA-accredited laboratories using three seed lots. The *S. nigrum* seeds were germinated on a top-of-paper medium, using the alternating temperature regime 20<=>30 °C. Light was supplied for 8 hours during the high-temperature phase, and germination counts were made at 7 and 14 days, and at 21 days when required.

The validation test showed no significant differences between the results obtained by the five laboratories, and that repeatability and reproducibility requirements were therefore met.

The results of this validation study support the introduction of the TP 20<=>30 °C germination method for *Solanum nigrum*.

Introduction

Solanum nigrum (Black Nightshade) is a common plant utilized as a vegetable and fruit source in Africa, India, Indonesia etc. The plant is cultivated as a food crop both for its fruit and its leaves and has medicinal usage. The unripe fruit may contain high concentration of toxins. In many other countries it is considered as a common garden weed.

Solanum nigrum seeds remain viable for years, depending on the storage conditions and moisture content.

Preliminary experiments conducted in our lab confirmed the findings of laboratories in Kenya, Zambia, Sudan and Botswana that the optimum germination temperature for *Solanum nigrum* is 20<=>30 °C when light is provided during the 30 °C phase.

In the peer validation test, there was a significant lot x lab interaction. Jean-Louis Laffont recommended the performance of additional tests by additional labs to assess the method better. A multi-laboratory validation study was therefore carried out, in order to develop an ISTA germination method for *Solanum nigrum*.

Material and methods

Seed material

Seven lots of untreated *Solanum nigrum* seeds were obtained from Mary Chipili (Zambia) and Joseph Ahenda (Kenya). Three lots were selected (4, 5 and 6) and sent to the participating laboratories.

Participating laboratories

Samples were sent to five ISTA-accredited laboratories in France (FRDL0200), Norway (NODL0100), Scotland (GBDL0400), the Netherlands (NLDL0300) and Israel (ILDL0100).

Germination methods

Three seed lots were tested on top-of-paper (TP) medium, using the alternating temperature regime 20<=>30 °C.

For each lot, a total of 400 seeds were tested in replicates of 50–100 seeds. Light was supplied for 8 hours during the high-temperature phase, and germination counts were made at 7 (first counts) and 14 days (final

counts). Since the seed lots were produced more than 3 years ago, the seedlings developed more slowly, and final counts were sometimes done after 21 days.

Table 1. Germination testing conditions on TP at 20<=>30 °C

Lab No.	Germination apparatus	Type of box/ plate (size)	Type of substrate (size)	Type of water (amount)	No. of seeds per replicate
1		Germination box (12 × 18 × 5.5 cm)	Paper, 4 layers (12 × 18 cm)	Deionized water (25 mL per replicate; 0.9 mL per gram paper)	50
2	Jacobsen tank	Bell jars (Ø 8 cm)	Filter paper, 2 layers (Ø 7.5 cm)	Tap water (3.1 mL per replicate; 2.6 mL per gram paper)	50
3	Incubator	Germination box (12.5 × 17.5 × 5.5 cm)	Pleated paper, 1 layer (11 × 17.5 × 2) + envelope strip (11 × 75)	Tap water (1.2 mL per gram PP + 1.1 mL/g = 2.3 mL per gram paper)	100
4	Incubator	Germination tray (45 × 54 cm)	Filter paper (Ø 10 cm) on large filter paper (45 × 45 cm)	Deionized water 2.4 mL/g + 1–1.8 mL/g	100
5	Room germinator	Petri dish (Ø 14 cm)	Anchor brown paper, 2 layers (Ø 14 cm)	Tap water (12.3 mL per replicate; 3.6 mL per gram paper)	50

The evaluation of the seedlings was done in accordance with the ISTA seedling evaluation criteria for *Solanum melongena*. Seedling evaluation group: A.2.1.1.1.

Statistical analysis

The germination results were checked to ensure that the sum of the percentages equalled 100%.

Possible outliers were assessed using side-by-side boxplots and using tolerance Table 5B (between replicates). The performance of the method was assessed through the estimation of repeatability and reproducibility parameters in the context of binomial data.

The statistical analysis of the preliminary experiments was done by Waffa Abu-Aklin of the Israeli Seed Testing Laboratory.

The statistical analysis of the validation study was performed by Jean-Louis Laffont- Chairman of the ISTA Statistical Committee.

Preliminary experiments

In December 2008, in order to assess the optimal germination temperature, the Israeli laboratory pregerminated two lots at 20, 25 and 20<=>30 °C, with 8 hours' light. Since the results with these lots were similar, only the result of lot No. 4 is given.



Figure 1. Germination of *Solanum nigrum* at 20, 25 and 20<=>30 °C after 7 days.

Appendices 1 and 2 show germination at 7 and 14 days, respectively. Germination was enhanced, accelerated and increased at the alternating temperature of 20<=>30 °C. Constant temperatures (20 and 25 °C) were found to inhibit and decrease germination.

This preliminary experiment confirmed the findings of the laboratories in Kenya, Zambia, Sudan and Botswana.

Statistical analysis of the results

1. Data exploration with side-by-side boxplots

Grouping factor: lot

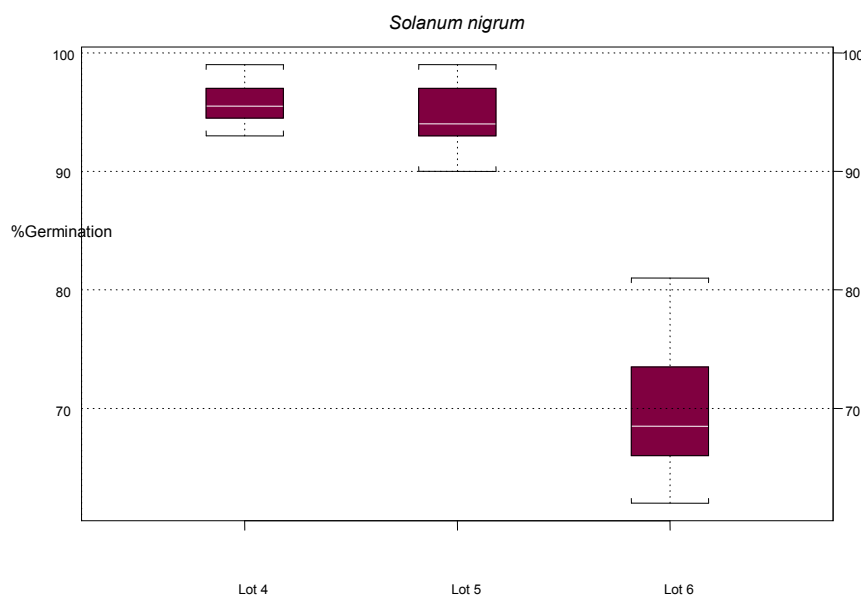


Figure 2. Germination (percentage of normal seedlings) obtained by the participating labs from the 3 seed lots. The germinations of Lots 4 and 5 was similar, while the germination of Lot 6 was much lower and more dispersed (which is a known feature of binomial data).

Grouping factor: lab

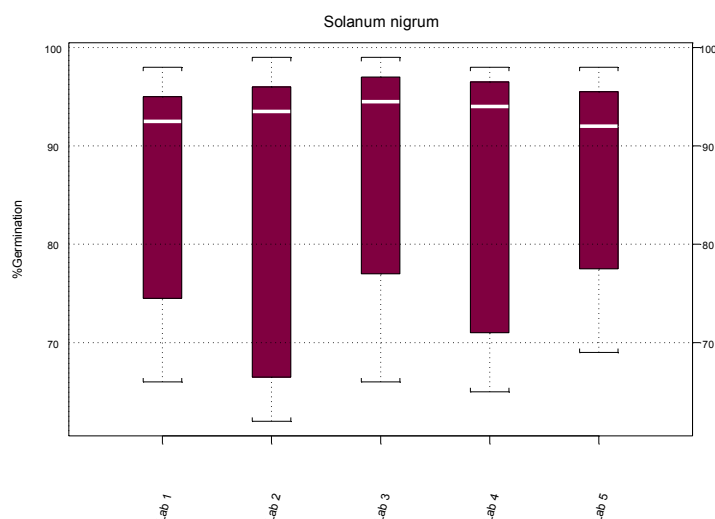


Figure 3. The germination results (normal seedling percentage) obtained by the participating labs were similar.

Grouping factor: lot × lab

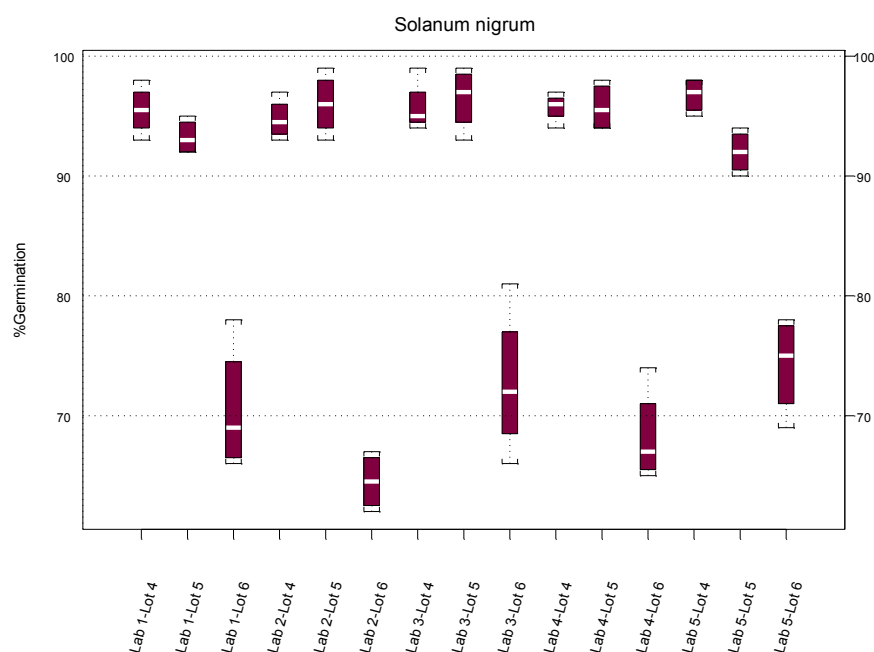


Figure 4: A strong lot effect was shown between lot 4 or 5 and 6. No lot × lab interaction was exhibited in these side-by-side boxplots.

2. Data checking

The data were checked according to the ISTA Rules by calculating the tolerances for germination test replicates.

Table 2: Germination results and tolerances

	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5
Lot 4					
Tolerance range (max.)	8	9	8	8	7
Observed range	5	4	5	3	3
Mean	95.5	94.75	95.75	95.75	96.75
Lot 5					
Tolerance range (max.)	10	8	8	8	11
Observed range	3	6	6	4	4
Mean	93.25	96	96.5	95.75	92
Lot 6					
Tolerance range (max.)	18	19	17	18	17
Observed range	12	5	15	9	9
Mean	70.5	64.5	72.75	68.25	74.25

All the results were within tolerance (Table 2).

3. Repeatability

Let:

- I be the total number of lots;
- J be the total number of labs;
- K be the number of replicates of m seeds for a given lot in a given lab;
- p_{ijk} be the percentage of germinated seeds for lot i , lab j and replicate k .

The standard deviation of repeatability is computed as:

$$S_r = \sqrt{\bar{p} - \frac{100 - \dots}{m}}$$

where:

- \bar{p} is the overall average percentage of germinated seeds;
- f_r^2 is an estimate of the dispersion parameter:

$$(1) \quad f_r^2 = \frac{1}{IJ} \sum_{i,j} \text{var}_{obs_{ij}}$$

where:

$$\text{var}_{obs_{ij}} = \frac{1}{K} \sum$$

and

$$\text{var}_{bin_{ij}} = \frac{\bar{p} - 100 - \dots}{m}$$

with \bar{p}_{ij} being the average percentage of germinated seeds in lot i and lab j .

If $f_r^2 > 1$, one speaks of overdispersion, because the data have larger variance than expected, if a binomial distribution is assumed.

For this dataset, $I = 3$, $J = 5$, $K = 4$ and $m = 100$.

Results

$$\bar{p}_{...} = 86.82$$

$$S_r = 3.37$$

$$f_r = 0.99$$

There is no evidence for overdispersion.

Note: consider the following generalized linear model (GLM):

$$y_{ijk} \sim \text{Binomial}(m_{ijk}, \pi_{ijk})$$

$$\text{logit } \pi_{ijk} = \left(\begin{array}{c} \mu \\ \alpha_i \\ \beta_j \\ (\alpha\beta)_{ij} \end{array} \right) + \epsilon_{ijk}$$

where:

- $i = 1, 2, \dots, I$ $j = 1, 2, \dots, J$ $k = 1, 2, \dots, K$
- y_{ijk} is the number of germinated seeds out of m_{ijk} in lot i , lab j and replicate k
- μ is the general effect
- α is the fixed effect of lot i
- β is the fixed effect of lab j
- $(\alpha\beta)_{ij}$ is the fixed interaction effect between lot i and lab j .

The ϕ^2 factor, characterizing overdispersion, can be estimated by dividing the squared sum of the Pearson residuals after fitting the model by the residuals degrees of freedom [here, $IJ(K-1)$]. For this particular GLM, the algebraic expression of this estimate is:

$$(2) \quad f_r^2 = \frac{1}{IJ(K-1)} \sum_i \sum_j \sum_k \frac{(y_{ijk} - \pi_{ijk})^2}{\pi_{ijk}(1-\pi_{ijk})}$$

$$\text{where } \pi_{ijk} = \sum_k \pi_{ijk}$$

When $m_{ijk} = m = 100$, expression (2) simplifies to expression (1).

4. Reproducibility

Consider the following linear mixed-effects model (LMM):

$$p_{ij} = \mu + \alpha_i + \beta_j + \epsilon_{ij}$$

where:

- $i = 1, 2, \dots, I$ $j = 1, 2, \dots, J$;
- p_{ij} is the percentage of germinated seeds out of n in lot i and lab j ;
- μ is the general mean;
- α_i is the fixed effect of lot i ;

- b_j is the random effect of lab j . The b_j are iid $N(0, \sigma_{b_j}^2)$;
- e_{ij} are the residuals. The e_{ij} are iid $N(0, \sigma^2)$.

In a context of an LMM, the reproducibility standard deviation is then defined to be:

$$S_R = \sqrt{\sigma_{b_j}^2 + \sigma^2} \quad \text{where } \sigma_{b_j}^2 \text{ and } \sigma^2 \text{ are the variance component estimates.}$$

When data are perfectly balanced (no missing lot \times lab combination), we have:

$$S_R = \sqrt{\frac{1}{I} \sum_i \sum_j \frac{(p_{ij} - \bar{p}_{i..})^2}{p_{ij}}} \quad \text{where } \bar{p}_{i..} = \frac{\sum_j p_{ij}}{J}$$

Assuming a binomial distribution, the variance of p_{ij} is:

$$\text{Var } p_{ij} = \frac{n \cdot p_{ij} (1 - p_{ij})}{n}$$

We then compute the following quantity to characterize overdispersion when lab and lot by lab variations are considered:

$$f_R^2 = \frac{S_R^2}{p_{i..} (1 - p_{i..})} \quad \text{where } \bar{p}_{i..} = \frac{\sum_j p_{ij}}{J}$$

The square root of f_R^2 is then compared to the f value defined by Miles (1963) in equation AG4, and which is used to develop ISTA tolerance tables for comparing germination results from different labs.

Results

For this dataset, $n = 400$.

$$\bar{p}_{i..} : 86.82$$

$$S_R : 2.53$$

$$f_R : 1.49$$

$$f : 1.66$$

The f_R value is lower than the f values, showing no higher variation among laboratories than the one established by Miles for the development of tolerance tables.

Conclusions

The statistical evaluation of this study shows that repeatability and reproducibility requirements are met.

The results obtained in this validation test indicate that TP can be proposed as a suitable substrate, and 20 \leq 30 °C as an optimum temperature regime, for the germination of *Solanum nigrum*.

Acknowledgements

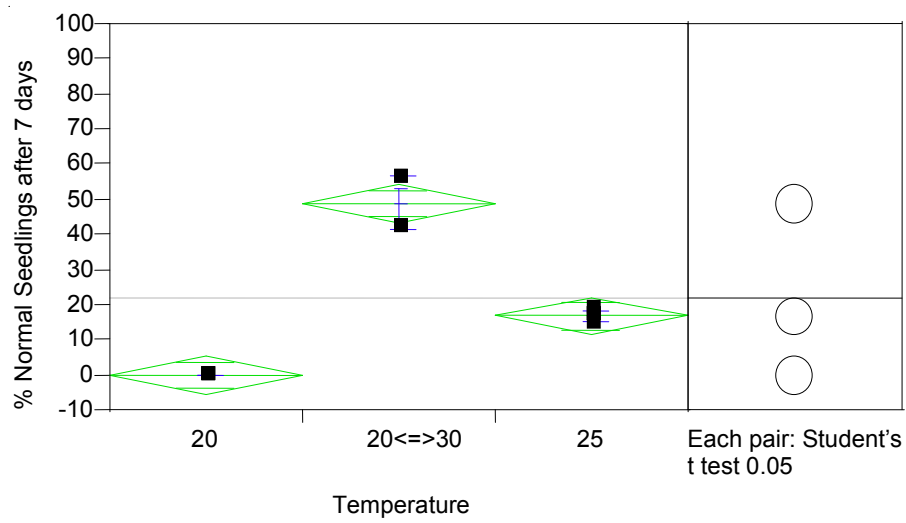
Sincere thanks to Joseph Ahenda (Kenya), who started and promoted the study, and to Mary Chipili (Zambia). Special thanks are due to Jean-Louis Laffont for the statistical analysis, and to Ronald Don and John Hampton for their support.

We are grateful to Sylvie Ducournau, Håkon Tangerås, Gillian McLaren, Gerarda de Boer-Raatgever and Karen Hill for performing the tests, and to Grethe Tarp and Joël Léchappé for their assessment of the test plan and their review of the validation report.

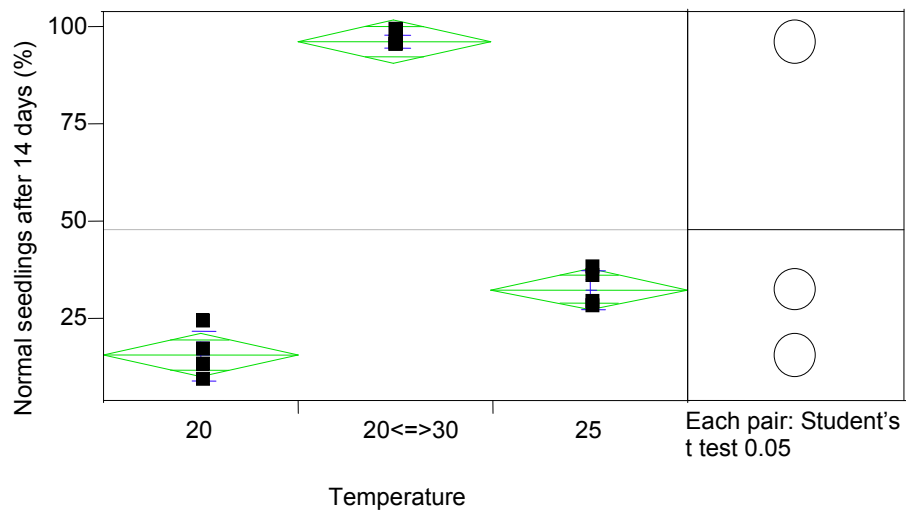
References

- Ahenda J. (2007). Report on Tropical Species Method Validation. Seed Testing International. ISTA News Bulletin No.133: 33-34.
- Miles, S. R. (1963). Handbook of Tolerances and of Measures of Precision for Seed Testing, Proceedings of the International Seed Testing Association. Vol. 28, No. 3 525-686.
- ISTA Rules (2011). Chapter 5: the Germination Test. ISBN-13 978-3-906549-53-8. Published by the International Seed Testing Association. Bassersdorf, Switzerland.
- ISTA Handbook on Seedling Evaluation (2009). Section 16: Seedling Type E- Seedling Group A-2-1-1-1. Editor: Ronnie Don. 3rd Edition. ISBN-13 978-3-906549-39-2, Published by the International Seed Testing Association, Bassersdorf, Switzerland.

Appendix 1: Normal seedlings (%) at 20, 25 and 20<=>30 °C after 7 days



Appendix 2: Normal seedlings (%) at 20, 25 and 20<=>30 °C after 14 days



Alternative embryo extraction procedure to seed health method 7-013: Detection of *Ustilago nuda* on *Hordeum vulgare*

Karin Sperlingsson

Swedish Board of Agriculture Box 83 SE 268 22 Svalöv Sweden

karin.sperlingsson@jordbruksverket.se

October 2010

Summary

For forty years, the Nordic laboratories have used a modification of ISTA seed health method 7-013. This Nordic method differs from 7-013 in the embryo extraction technique and the procedure used to clear embryos for examination for the *Ustilago* mycelium. A validation study comparing the two methods was carried out. Three seed lots with infection levels between 1% and 4% were tested by three laboratories using both the current 7-013 and the Nordic method. The validation study showed that the two methods produce equivalent results. Statistical analysis showed that the only significant differences were among samples. There were no differences between methods, laboratories or any interactions. The Nordic method offers an alternative method for laboratories that do not have access to plentiful warm water, nor a fume hood. The alternative embryo-clearing process adds a day to the duration of the test, so may not be suitable where a quicker turnaround is required. It does, however, offer an alternative clearing procedure which could be used in combination with the existing method to provide flexibility of resources within laboratories during busy periods. The data supports the inclusion of the Nordic method in the ISTA Rules as an equivalent method to the existing method 7-013.

Introduction

ISTA method 7-013 describes the current international method for the detection of *Ustilago nuda* in barley (*Hordeum vulgare*) seed. Since the early seventies, the Nordic countries have used a method described by Joelson, 1968. This method differs from 7-013 in the embryo extraction technique and the procedure used to clear embryos for examination for the *Ustilago* mycelium.

Joelson's method includes an additional dehulling step prior to embryo extraction. Following dehulling, the separation of the embryos from the endosperm is similar to 7-013 but with salt added to the sodium hydroxide solution, to help separate the lighter embryos, which float to the top of the solution from the endosperm. The Nordic laboratories adopted this method in preference to 7-013, as they considered it to be a more efficient process for producing good-quality embryos with a high extraction rate, and it did not require an expensive flotation device described in 7-013 (Ref.).

Early comparative tests conducted by the ISTA Plant Disease Committee (PDC) revealed difficulties with broken embryos encountered by some participants when using the Joelson method. Possible reasons included too concentrated a solution of sulfuric acid for dehulling, too long a soaking period, too high a temperature during the process, and mechanical damage (Anon., 1972). It was argued that the method provided consistent results when applied by experienced analysts, and in 1976 a comparative test showed that, as participating laboratories gained experience, there was no difference between results from the Joelson method and 7-013 (Anon., 1976).

Since the extraction method was first developed, various laboratories in Scandinavia have modified the procedure to overcome some of the earlier problems. For example, the temperature and concentration of the sodium hydroxide and sulfuric acid were altered, making the method more suitable for inexperienced laboratories to use successfully. The Nordic laboratories have carried out comparative tests for many years, with good agreement between the results. The ISTA Seed Health Committee's *Ustilago nuda* Working Group agreed that the Nordic method could be included as an alternative embryo extraction procedure. However, the SHC required that the various laboratories agree to procedures where small differences existed between methods before moving to a peer-validated comparative test. The Nordic laboratories agreed on one description and a peer-validated comparative test was agreed upon. Although the comparative test is a

separate exercise to a proficiency test, on this occasion the SHC agreed to the use of additional samples from ISTA PT round 08-SH for the comparative test.

The aim of the peer-validated comparative test was to determine whether the two methods are equivalent.

Materials and methods

Seed lots

Three seed lots with infection levels of 4.3%, 1.6% and 1.2% were evaluated. These were the same seed lots used in PT round 08-SH, and were obtained from the test organizer. The organizer provided the Swedish laboratory with additional coded samples for distribution to the laboratories in Norway and Finland, with sample codes known only to the organizer. The participating laboratories each received nine samples of 120 g (three per lot) in addition to their PT samples. Thus, the three laboratories in Norway, Finland and Sweden each examined 18 samples, nine following method 7-013 (as part of the proficiency test) and nine following the Nordic method.

The laboratories were:

- Kimen S varelaboratoriet AS,  s, Norway (contact: Barbro E Isaksen, Dagny Stave-Larsen)
- Finnish Food Safety, Loimaa, Finland (contact: Hanna Ranta)
- Swedish Board of Agriculture, Seed Division, Sval v, Sweden (contact: Karin Sperlingsson)

ISTA procedure

Samples were tested according to ISTA Method 7-013. From each sample, 1000 embryos were examined, and the number of infected embryos found per sample was recorded.

Nordic extraction procedure

Dehulling

Each sample was placed in a 600 mL beaker, and sulfuric acid of 25–37% (by weight) (Joelson, no date) was added until the seeds were covered. The seeds were then incubated at 75 °C for 50 minutes or until the seeds turned a medium-brown colour. The sulfuric acid was drained and water added to approximately two thirds of the volume of the beaker. Loosened hulls were removed by stirring the seeds with a glass rod and then being carefully poured away with the water. If not all hulls were removed, new water was added, and either a rod or an electric hand mixer was used to stir for approximately 3 minutes to loosen the remaining hulls. The procedure was repeated until no loose hulls were left.

Embryo removal

The kernels (seeds without hulls) were placed in a container with approximately 1 L of 10–15% sodium hydroxide solution with 130–175 g salt added per litre (NaOH-NaCl). The kernels were then incubated overnight at 22 ± 3 °C. After incubation, the mixture was stirred gently to release the loosened embryos from the dissolved kernels. The released embryos floated to the top of the container and were poured into a new beaker (600 mL). More NaOH-NaCl solution was added to the mixture and left for 5–20 minutes and stirred as before. This process was repeated until no further embryos were released. The required time depends on the variety and how often the mixture is stirred.

To ensure that no embryos remained, the dissolved kernels were placed on top of a sieve with an approximately 2.4 mm mesh (coarse enough to let the embryos pass but retain the remains of the kernels), and washed to allow collection of the remaining embryos in a fine sieve (1 mm mesh) below.

Any large quantity of chaff among the embryos was removed by filling the beaker with water and pouring off the floating chaff. All embryos were then washed in running water for approximately 10 s to rinse off the NaOH-NaCl solution.

Clearing the embryos

The water was drained and the embryos were collected in a fine sieve before being placed in a clean beaker and covered with lactic acid. The beaker was covered with a lid. The embryos were then incubated overnight in an incubator at 75 ± 5 °C.

After incubation, the lactic acid was drained using a fine sieve. Where the embryos were not transparent they were washed in ethanol using a fine sieve or placed in 95% ethanol for a few minutes. The embryos were then covered with a glycerine-ethanol solution (1:3) (Joelson, no date) or with a pure solution of glycerine, ready for examination.

Examination

1000 embryos were examined using a microscope at 16–25× magnification with substage illumination. The number of embryos examined and the number of infected embryos found per sample were recorded.

Statistical analysis

The data was analysed on Genstat Version 8 using a logistic regression (general linear model, binomial data, logistic link).

Results

The total numbers of infected embryos found are summarized in Table 1. Variation was greatest in sample 1.

The accumulated analysis of deviance (Table 2) shows that the sample factor is significant. Method and laboratory are not significant, and neither is the interaction (also Figure 1). The estimated dispersion value 2.27 is greater than that normally expected of binomial data (dispersion = 1). However, this is consistent with the over-dispersion seen in ISTA PT round 08-SH, where variability for *U. nuda* was higher than expected (Cockerell and Roberts, 2010).

Table 1. Total number of infected embryos found by all laboratories per sample and method

Seed lot	Method 7-013	Nordic method
1	487	391
2	171	179
3	100	120
Grand total	758	690

Table 2. Accumulated analysis of deviance

Change	d.f.	Deviance	Mean deviance	Deviance ratio	Approx. F pr.
+ Sample	2	490.307	245.153	107.98	<0.001
+ Lab	2	7.394	3.697	1.63	0.210
+ Method	1	2.835	2.835	1.25	0.271
+ Sample.Lab	4	12.314	3.079	1.36	0.268
+ Sample.Method	2	10.607	5.304	2.34	0.111
+ Lab.Method	2	2.090	1.045	0.46	0.635
+ Sample.Lab.Method	4	10.804	2.701	1.19	0.332
Residual	36	81.730	2.270		
Total	53	618.081	11.662		

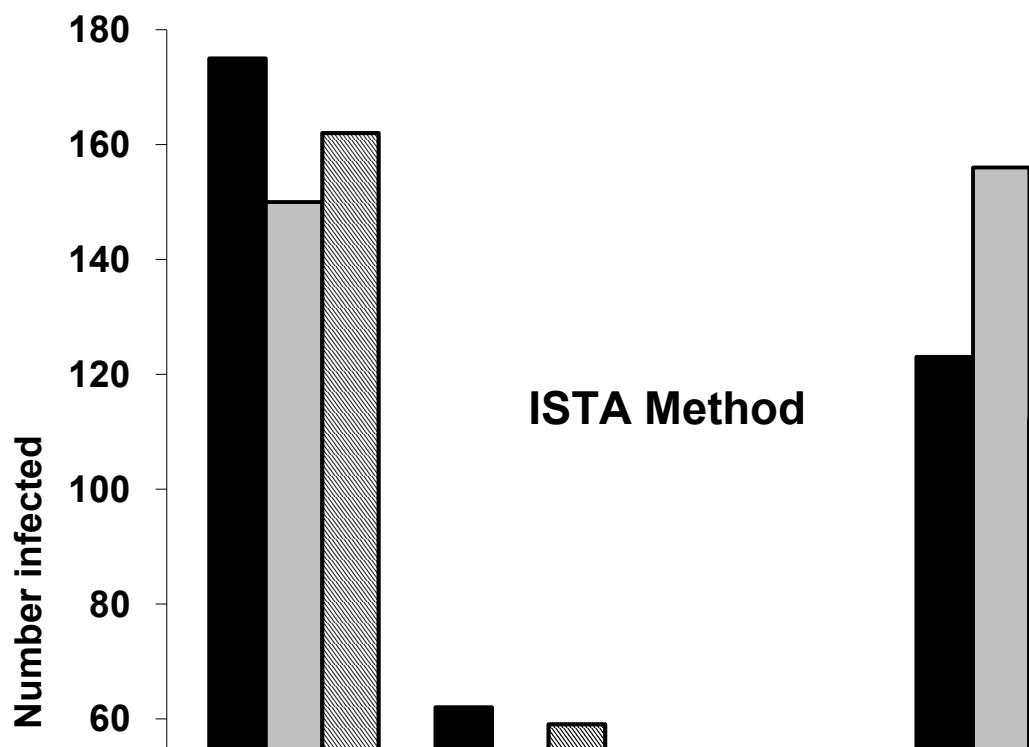


Figure 1. Total number of infected embryos found per sample, laboratory and method.

Discussion

The results confirm earlier comparative tests showing that the ISTA method and the Nordic method do not differ in the number of infected embryos found. Although the analysis shows an over-dispersion, this is consistent with the over-dispersion seen in the ISTA proficiency test, where the variability for *U. nuda* was higher than expected in the seed lots (Cockerell and Roberts, 2011).

The Nordic method differs from the current method in two main areas. Firstly, the extraction of the embryos involves an additional step to de-hull the seed, followed by separation of the embryos from the endosperm in a solution of sodium hydroxide and salt. The loosened embryos float to the top of the solution and are collected. Secondly, in order to clear the tissues, the embryos are not boiled in a lactic acid solution, but are incubated overnight in lactic acid at 75 °C.

Extraction and cleaning of embryo samples

The Nordic method provides an alternative method for extracting and cleaning the embryo samples by introducing an additional step to remove the hulls prior to soaking in a solution of sodium hydroxide. Compared to method 7-013, the potential advantage of this additional step before the soak is that it does not require warm water and reduces the amount of water needed to aid separation of the embryos. In method 7-013, warm water is an important factor for good extraction. Past data (Rennie, 1984) states that extraction rates can differ from 40 to 80%, depending on the sample, the temperature during soaking and the temperature of the water used to wash the sample. The Nordic method can be useful for laboratories where large amounts of warm water are not readily available.

The amount of time spent cleaning the embryo sample may be reduced, since most of the hulls are removed at an early stage, reducing the problem of hulls among the extracted embryos. The time spent on cleaning embryo samples will depend on the seed sample and the experience of the analyst; this applies to both methods equally.

Both methods use specific gravity to separate the embryos from the chaff and endosperm. In method 7-013, the extracted embryos are added to a mixture of water and glycerol; the embryos then float to the top and

are collected. With the Nordic method, the kernels are incubated in a solution of sodium hydroxide and salt. The free embryos float to the top and are collected.

The size of the embryos that are retrieved can be affected by the salt concentration. When this is low, it is a little more difficult to release the loosened embryos, but the embryos are larger. Higher concentrations make release of the embryos easier, but the embryos shrink somewhat. The salt concentration does not affect the result of the analysis.

With regard to the concentrations of sulfuric acid, sodium hydroxide and salt, during the test both low and high concentrations were used by various laboratories. There was no effect on the result.

Neither is the result affected by the interval between adding further sodium hydroxide and stirring the embryos to loosen them during extraction. Some laboratories do other work in between and leave the embryos during that time.

The Nordic method can achieve 98% embryo recovery (Joelson, 1968). For both methods, embryo release can be affected by the cultivar and the field conditions prior to harvest.

With the Nordic method, less than 1% of the embryos are collected in the sieve. The remainder float to the surface and are poured from the container, rather than being collected in a series of sieves. The Nordic laboratories consider that the risk of embryos being damaged is smaller using this method.

Clearing embryos

In method 7-013, the embryos are cleared by boiling the embryos in a solution of lactic acid and glycerol in a fume hood. In the Nordic method, a fume hood is not required, since embryos are cleared using concentrated lactic acid and overnight incubation in an oven at 75 °C. For laboratories that only need to test few samples, and do not want to go to the expense of purchasing a fume hood, the Nordic Method offers a suitable alternative.

The disadvantage of this method is that it extends the duration of the test, which may not suit all customers.

The examination of the embryos requires substage illumination and a magnification of 16–25×. Embryos can be examined in either glycerol or a mixture of glycerol and ethanol (3:1).

Conclusion and recommendation

The Nordic method provides equivalent results to the present ISTA method 7-013. The method of extraction allows laboratories to test for *U. nuda* in barley when a fume hood is not available. It is also an alternative where warm/hot water is not freely available.

It provides for an additional method for clearing the embryos which could also be used with the current ISTA method, when results are not required within 24 hours of receipt of the sample. This would free up staff resources.

It is recommended that an equivalent method for detection of *Ustilago nuda* in *Hordeum vulgare* is introduced to the Annexe to Chapter 7 as Method 7-013b.

Acknowledgements

The author would like to thank Valerie Cockerell, SASA, Scotland for providing extra samples that made it possible to conduct the comparative test. She also helped with the statistical analyses, and gave valuable advice on how to write this validation report.

I also wish to thank the staff at Kimen S  varelaboratoriet AS, Norway, the Finnish Food Safety Authority Evira, and the staff at the Swedish Board of Agriculture, Seed Division, Sval  v, Sweden.

References

Anon (1972). Report on the Twelfth International Workshop on Seed Pathology, sponsored by the International Seed Testing Association, 31 August–5 September 1970, Solna, Sweden, ISTA-PDC, Copenhagen 1972.

Anon (1976). Report on the Fifteenth International Workshop on Seed Pathology, sponsored by the International Seed Testing Association, 8–14 September 1975, Paris, ISTA-PDC, Paris 1976.

Anon (2001). 7-013: Detection of *Ustilago nuda* on *Hordeum vulgare* (Barley).

Cockerell, V. and Roberts Report of ISTA proficiency round 08-SH Method 7-013 Detection of *Ustilago nuda* on *Hordeum vulgare* (Submitted to Seed Testing International February 2011)

Joelson, G. (1968). Laboratoriemetod för bestämning av naket sot hos korn. Årsredogörelse 1967–1968, Lokala Frökontrollen i Skara.

Joelson, G. (no date). Analys av naket sot enligt metod utarbetad i Skara av G.Joelson (copy available from ISTA Secretariat).

Rennie, W. J. (1984). Working Sheet No 25 (2 Ed) Barley Loose smut. ISTA Handbook on Seed Health Testing. International Seed Testing Association, Zurich, Switzerland.

The osmotic method for detection of *Pyrenophora teres* (*Drechslera teres*) and *P. graminea* (*D. graminea*) on *Hordeum vulgare*

K. Sperlingsson¹ and G. Brodal²

¹ Swedish Board of Agriculture, Box 83, SE-268 22 Svalöv, Sweden

² Bioforsk: Norwegian Institute for Agricultural and Environmental Research, Plant Health and Plant Protection Division, Høgskoleveien 7, N-1432 Ås, Norway

Summary

The osmotic method has been used for many years in Norway and Sweden as a routine method for detection of *Pyrenophora teres* (anamorph *Drechslera teres*) and *P. graminea* (anamorph *D. graminea*) on barley. The method is based on the ability of *Pyrenophora* spp. to produce red pigments. However, it cannot distinguish between *P. teres* and *P. graminea*, because they produce the same pigment. A validation study was carried out with the aim to provide the necessary documentation for including the method in the International Rules for Seed Testing (ISTA Rules). Seven laboratories participated, and each tested 3 × 300 seeds from three barley seed lots. Analyses of the results demonstrate that the method provides sufficient repeatability, and that there are no particular problems with this test at the laboratory level. Furthermore, in previous studies with the osmotic method, organized by a Nordic working group, it was shown that the osmotic method easily gives reproducible results for *Pyrenophora teres*/*P. graminea* in barley when used by experienced laboratories. Moreover, the osmotic method is well suited for routine analyses, because it is quick and easy to carry out. The study showed that, if used correctly and with proper equipment, the osmotic method for detection of *Pyrenophora teres*/*P. graminea* is easy to perform, and shows good conformity between laboratories.

Introduction

There is no method for detection of *Pyrenophora teres* Drechsler (anamorph: *Drechslera teres* (Sacc.) Shoem.) or *P. graminea* Ito & Kurib., in Ito (anamorph: *D. graminea* (Rabenh. ex Schlecht.) Shoem.) in barley seed in the ISTA Rules (ISTA, 2010). The most common methods used in seed health testing laboratories for detection of these pathogens are agar plate methods, such as potato dextrose agar (PDA) or malt agar (MA), and the freezing blotter method published by ISTA (1964a) in S. 3 No. 6 (barley leaf stripe), revised as Working Sheet No. 6 (2nd ed.) in 1984 (Rennie and Tomlin, 1984), and Working Sheet S. 3. No. 7 (barley net blotch) (ISTA, 1964b). However, these methods are both time-consuming and laborious. A cheap and less laborious method, the osmotic method, was developed by Joelson in Sweden for rapid detection of *Pyrenophora* spp. in cereal seeds (Svensson, 1981; Joelson, 1983). With this method, seeds are incubated on filter paper moistened by a sugar solution. The osmotic pressure from the sugar inhibits the germination of the kernels. The osmotic method is based on the ability of *Pyrenophora* spp. to produce brick-red pigments (anthraquinones) by incubation of seeds under certain conditions (Braverman, 1960; Kietreiber, 1977; Knudsen, 1982). However, the method cannot distinguish between *P. teres* and *P. graminea*, because they produce the same pigment – catenarin (Engström *et al.*, 1993). The pigments turn from brick-red to violet when NaOH is added. Some saprophytes produce pigments, but not of the same colour, and will not react in the same way when NaOH is added. The osmotic method has been used in routine seed health testing of barley in Sweden and Norway for many years (Brodal, 1993).

Previous studies of the osmotic method and comparisons with agar plate and freezing blotter methods

In 1990, 1992 and 1993, a Nordic working group on seed pathology organized meetings and comparative tests to harmonize procedures and performance of the osmotic method for detection of *P. graminea* and *P. teres* in barley seed (Brodal *et al.*, 1994). During these years samples from 57 barley seed lots (range of infection frequencies 0–87% infected seeds) were analyzed in six laboratories in three separate testing series. Correlation coefficients between the results obtained with the osmotic method in the laboratories varied between 0.75 and 0.97 (all significant at the 0.001% level). Results from Swedish and Norwegian

laboratories, where the method had been widely used, were best correlated. The same samples were tested with the freezing blotter method. Results from the freezing blotter method correlated well with results from the osmotic method, with correlation coefficients between 0.83 and 0.92 (Brodal, 1995).

Another comparative test of the osmotic method was organized by a sub-working group of the Plant Disease Committee of ISTA in 1994–1995 (Brodal, 1997). Samples from ten barley seed lots (range of infection frequencies 3–90% infected seeds) were tested in 13 laboratories. Four of the participating laboratories had long experience with the osmotic method, whereas other labs were using the method for the first time. The frequencies of *P. teres* and *P. graminea* recorded with the osmotic method were well correlated ($r > 0.90$) between most of the participating laboratories. In addition to the osmotic method, some of the laboratories carried out the freezing blotter and/or the agar plate (PDA) method on the same set of seed samples. Since a high level of agreement was found between the osmotic blotter results from the four experienced laboratories, the average results obtained in these laboratories were used for comparisons with the other methods. The results from the osmotic method were highly correlated to the results from the freezing blotter method and the agar plate method. However, approximately 50% higher infection frequencies were recorded with the osmotic than with the freezing blotter method, and approximately 20% higher frequencies were recorded with the osmotic method than with the agar plate (PDA) method. This indicates a higher sensitivity of the osmotic method than that of the other methods. The reason might be that with the osmotic method, small pigment spots can be observed from slightly infected seeds, but the low inoculum amount might not be enough to produce any conidia in the freezing blotter method or mycelium on agar, or that slight or superficial inocula might have been removed by pretreatment with NaOCl.

In these studies, the time needed to carry out the various methods was not measured. However, the osmotic method was considered to be less time consuming than the other methods. The main reason was the quick examination based on pigment spots.

From the Nordic and the ISTA-PDC comparative studies, it was concluded that the osmotic method easily gives reproducible results for *Pyrenophora teres* and *P. graminea* in barley when used by experienced laboratories. Moreover, the osmotic method is well suited for routine analyses, because it is quick and easy to carry out with no need for a microscope, preparation of agar media or sterile facilities.

In Sweden and Norway, thousands of barley samples have been tested annually with the osmotic method, to assess the need for seed treatment against *Pyrenophora teres* and *P. graminea*. In order to obtain ISTA accreditation for such tests, it is necessary that this method is included in the ISTA Rules. A final comparative test was organized in 2007 with the aim to provide the necessary documentation (validation) of the method. Seven seed health testing laboratories from Estonia, Finland, Norway and Sweden participated in this test. The repeatability and reproducibility of the osmotic method were evaluated. Results from this validation study are presented below.

Materials and methods

Seed samples

Based on the infection levels determined by two laboratories of the Swedish Board of Agriculture (JV) in Svalöv and Landskrona, three naturally infected lots of barley seed (infection frequencies 6–25% infected seeds) were selected for the validation test. Each seed lot was sampled and divided into three subsamples of 300 seeds. Subsamples were coded randomly and sent to participating laboratories as a blind comparative test.

The osmotic method

The method was, with slight modifications, carried out as described by Joelson (1983). Before plating, seed samples were pretreated by heat treatment in an oven at 90 °C for 2 hours in thin layers in open paper trays or dishes, to reduce the occurrence of saprophytes (Fig. 1). Seeds were placed on filter paper (Munktell quality 1731, 400 g/m²) moistened by a sugar solution (0.5 M sucrose), made from 170 g sugar per litre of water. The sugar (sucrose) was ordinary table sugar, as used for human consumption. The paper was soaked quickly in the sugar solution and surplus solution drained off. The osmotic pressure from the sugar solution inhibits the germination of the kernels. Development of roots and coleoptiles results in poor contact between seed and filter paper, so to keep the seeds in the correct position and to provide good contact between the seeds and the paper, the seeds were plated in wells or indentations in the paper, which were

impressed into the paper after it was soaked in the sugar solution. Samples were incubated in transparent plastic dishes for 7 days with alternating photoperiods of 16 hours at 26 ± 2 °C in strong white light (5000–6000 lux) and 8 hours at 22 ± 2 °C in darkness. These incubation conditions inhibit the development of conidia and mycelium, and favour the development of brick-red pigment spots on the paper. These turn violet after a weak solution of NaOH is added.



Figure 1. Seeds prepared for pretreatment at 90 °C for two hours.

Two of the participating laboratories did not use the osmotic method routinely, and therefore had no equipment for forming wells in the paper. As it was essential for all laboratories to use filter paper with wells, these two laboratories were sent prepared filter papers together with the seed samples. The filter papers were first dipped in water, the wells were formed and the papers were dried. It only remained for these laboratories to dip the filter papers in the sugar solution. These laboratories were also sent transparent plastic incubation dishes.

For examination, the seeds were removed, and a solution of 1% NaOH was poured onto the paper (Figs. 2 and 3). The colour of the pigment spots then immediately changed to violet, which made the spots easier to discern and count. The sizes of the spots usually varied from 1–2 mm up to 1–2 cm in diameter (Fig. 4). Very faint spots were not recorded (Fig. 5). The wells in the paper made it easy to see exactly where the seeds had been placed.

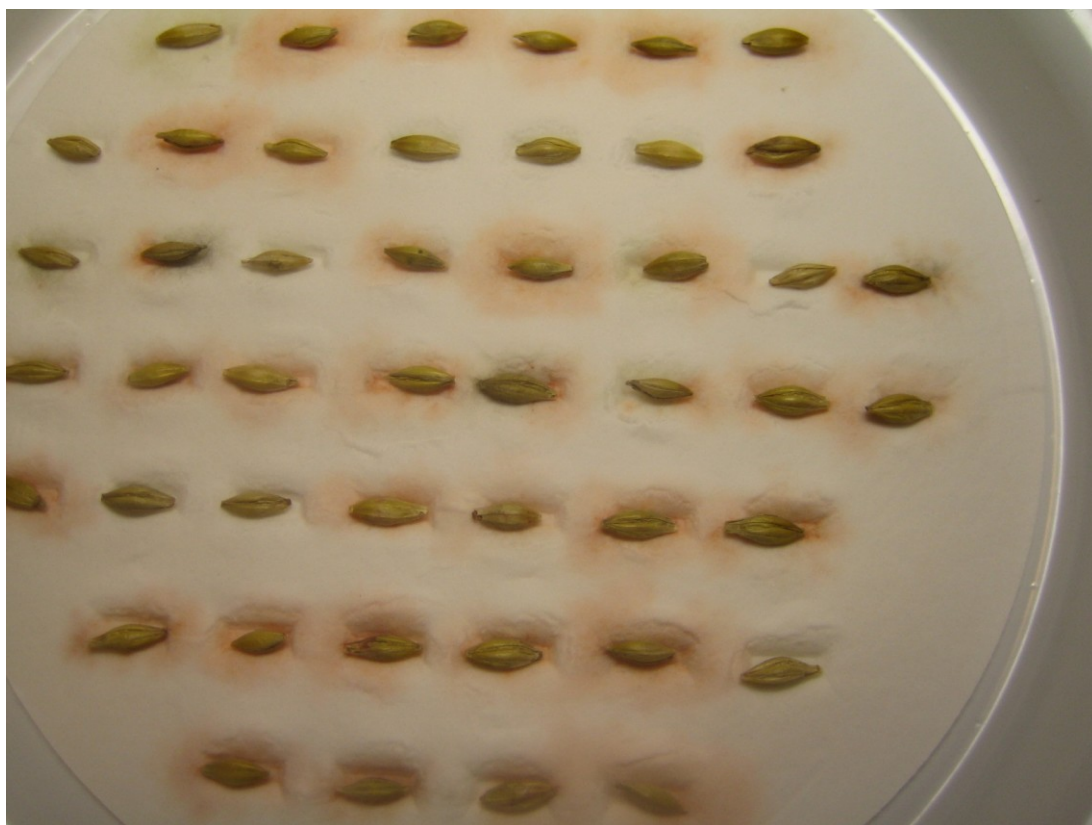


Figure 2. A dish after seed incubation.

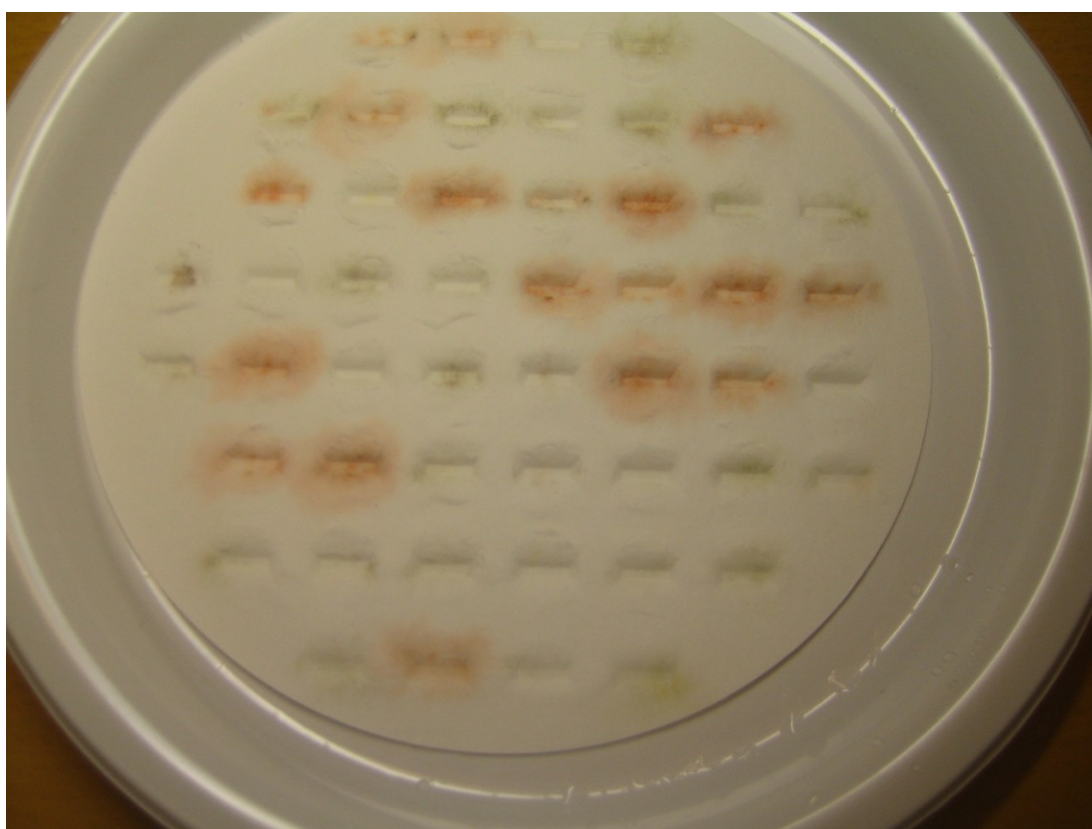


Figure 3. A dish after removal of incubated seeds, before adding the NaOH solution.

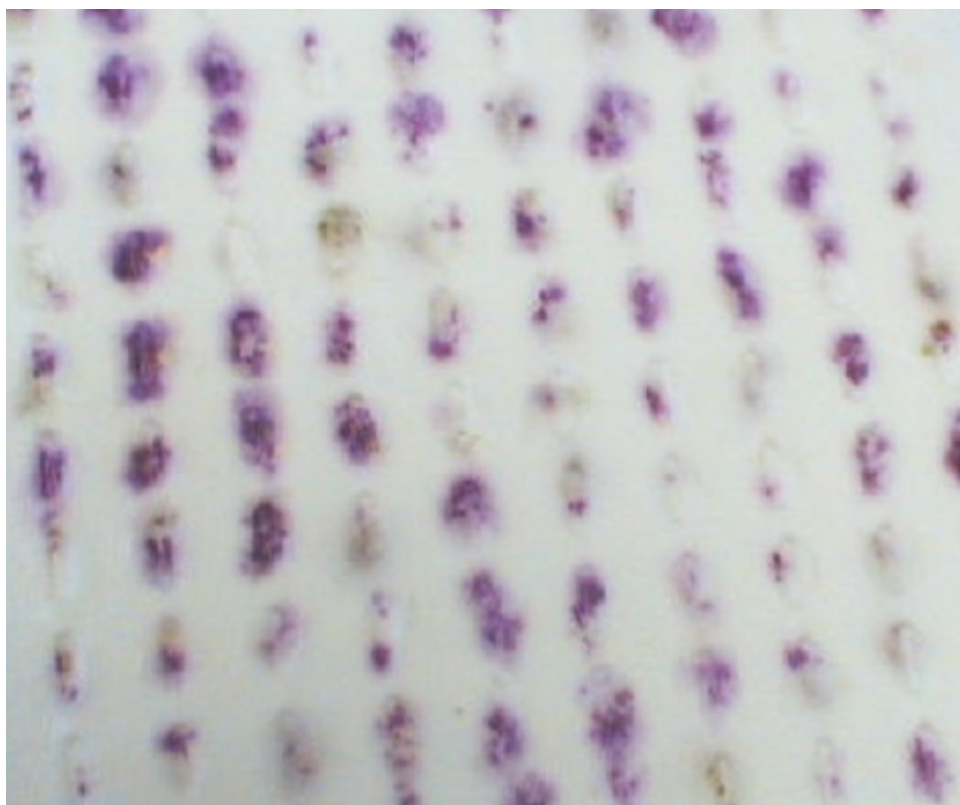


Figure 4. Blotter after addition of the NaOH solution. There are visible differences in spot size.

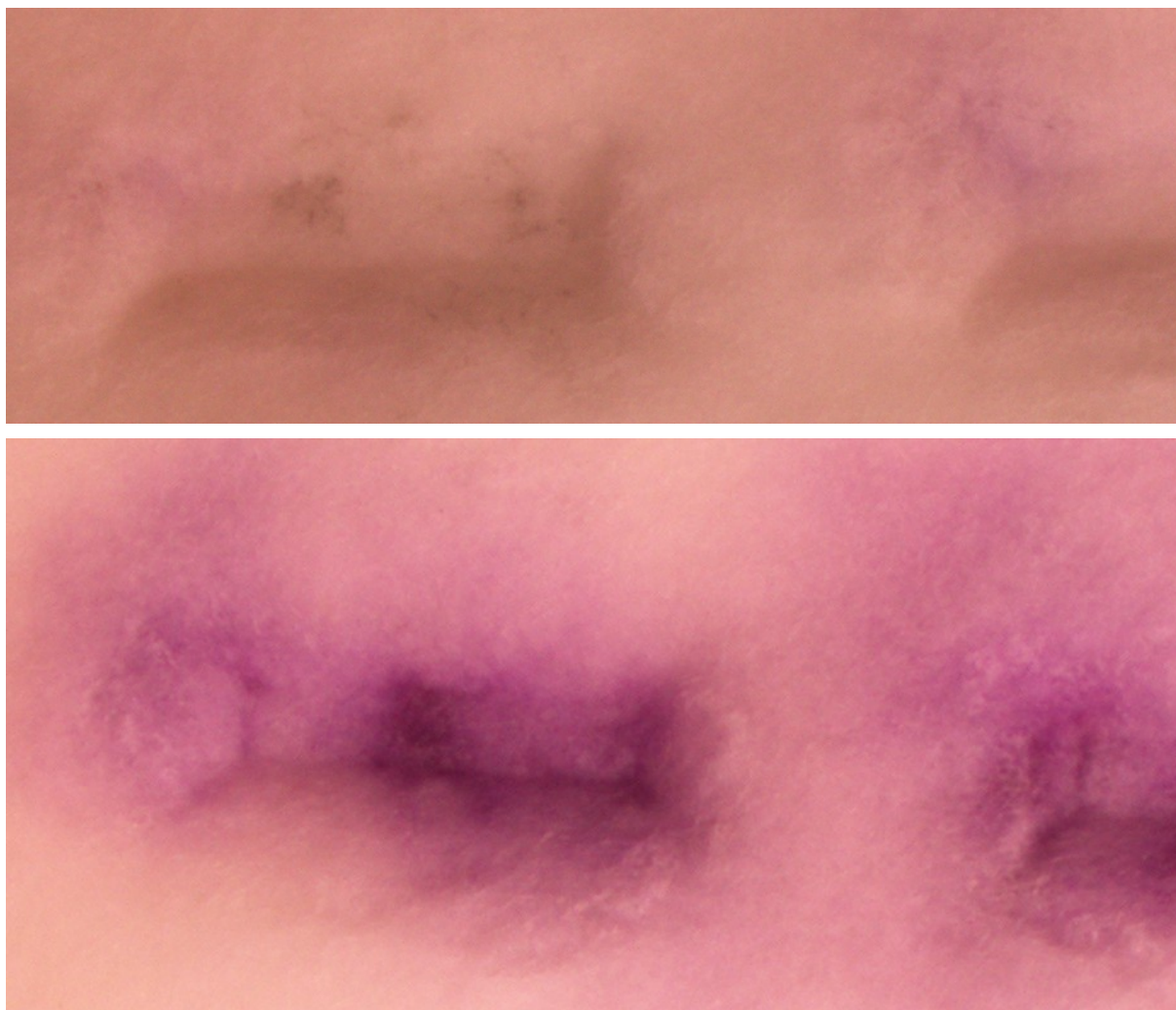


Figure 5. Enlargement of spots visible on the blotters after addition of the NaOH solution. Above: faint spots, not to be recorded; below: normal spots, to be recorded.

Statistical analysis

Statistical analysis was conducted by Jean-Louis Laffont of the ISTA Statistics Committee. Generalized Linear Mixed Models (GLMM) were used to assess the effects of the different factors and to estimate repeatability and reproducibility variances. The details of the analyses performed are included in the Appendix.

Results and discussion

The results of the test are shown in Figures 6–8.

All the participating laboratories had some experience in using the osmotic method, and five of the seven labs used the method frequently. Some of the laboratories participating in earlier ring tests had little experience in using the method.

There is a tendency for some laboratories to have higher or lower results than the majority, but in the statistical analysis, no crossover interaction between laboratory and by was detected, meaning that the lot ranking is consistent across laboratories.

Lab 5 tested under unusual conditions: at the time of the test, it merged with another laboratory, and was closed down. Therefore, in order to participate in the test, a temporary installation was arranged. This could be the reason for their low result.

Lab 4 did not use the osmotic method routinely.

Lab 3 tested under ultra violet (UV) light, as described by Joelson (unpublished) and Nylund (1990). When UV light is applied, it must have a wavelength of 366 or 254 nm, and the plates must be analysed before being sprayed with the NaOH solution. Since the majority of laboratories do not use UV light, and since it must be used only with proper safety precautions, this will not be recommended in the ISTA method.

Statistical analysis found a strong lot effect, a strong laboratory effect and a significant lot x laboratory interaction effect (Appendix). This interaction effect has limited consequences on the ability of the laboratories in ranking the lots consistently, since no crossover interaction effect was detected. The standard deviations of repeatability, computed for three levels of infection, are close to the binomial standard deviation, thus showing limited over-dispersion within laboratories. The standard-deviations of reproducibility were computed for information only, since there was no reference to which they could be compared.

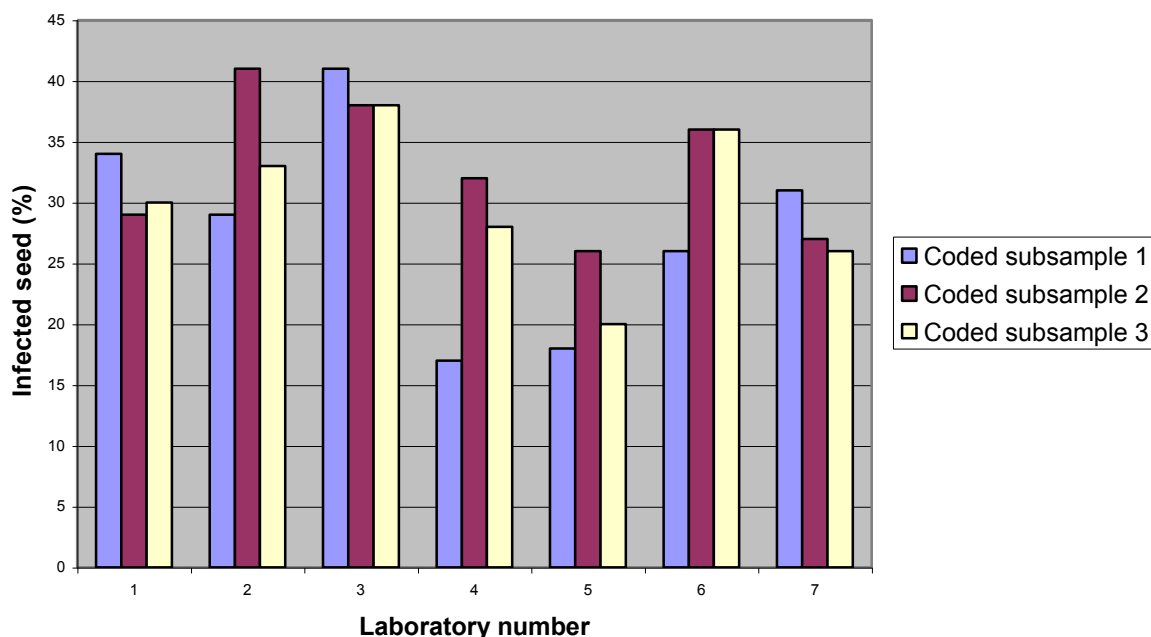


Figure 6. Incidence of *Pyrenophora* spp. in seed lot No. 1 as detected by the osmotic method.

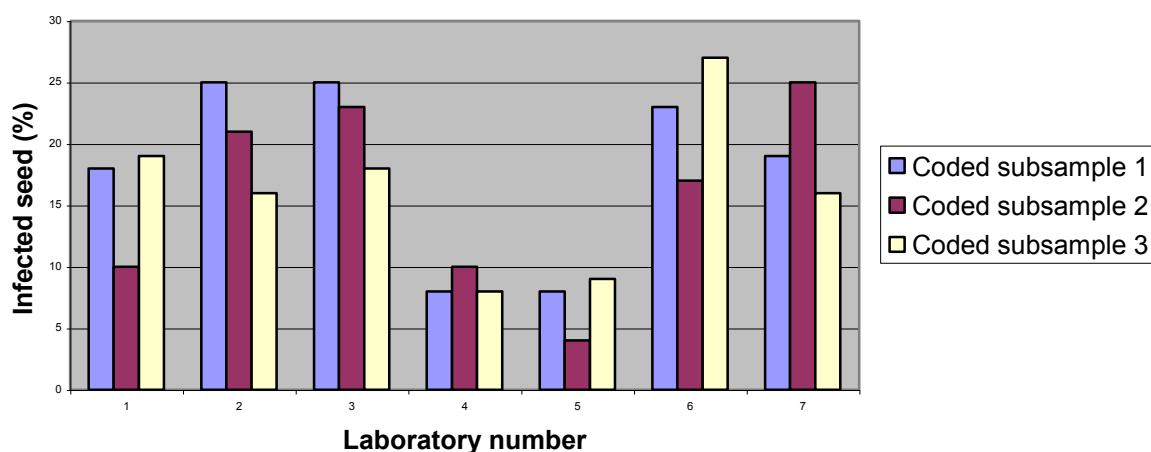


Figure 7. Incidence of *Pyrenophora* spp. in seed lot No. 2 as detected by the osmotic method.

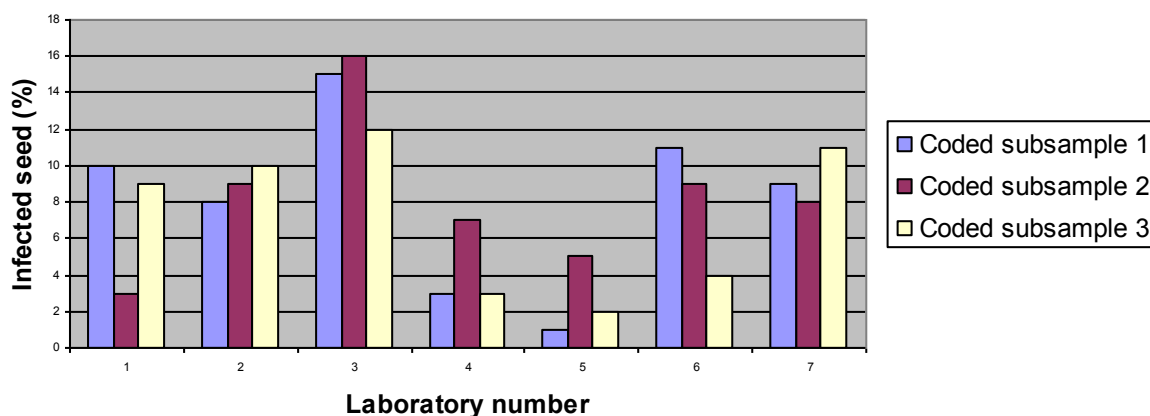


Figure 8. Incidence of *Pyrenophora* spp. in seed lot No. 3 as detected by the osmotic method.

The use of proper equipment, especially the light source, is critical (Joelson, unpublished). The white light must be at least 4000 lux. The amount of light is most important during the first three to four days of incubation (Joelson, unpublished).

It is essential that filter papers with wells are used. There are some advantages with this procedure compared with placing the seeds on flat paper. First, there is better contact between the moistened filter paper and the seeds; second, the seeds remain in the correct position, and it is easy to ascertain their exact position after incubation (Brodal, 1997; Nylund, 1990).

Water quality is not as important, since the sugar solution is highly concentrated (Joelson, unpublished). There was no advantage in using a buffer solution (Joelson, unpublished).

During incubation, the illumination causes temperature fluctuations; therefore, the incubation room or cabinet must be equipped with a temperature controlling system. During the dark period, the temperature should be 22 °C, and during the light period 25–27 °C (Joelson, unpublished).

The size of the pigment spots on the paper can vary, ranging from a diameter of 10–20 mm to a small spot under the seed (Fig. 4). This variation is probably due to the amount of inoculum on the individual seed.

The main advantage of the osmotic method is that it requires a low input of manpower compared with other methods. The method is less laborious than the freezing blotter and agar plate methods, and is well suited to routine testing.

The osmotic method cannot distinguish between *Pyrenophora teres* and *P. graminea*.

Only pure seed should be used for analysis. Seed not graded before being sent to laboratory must be graded to the same quality as certified seed.

Detection of *Pyrenophora* spp. after seed treatment

The method has not been validated for treated seed. It must only be used for untreated seed.

Conclusions and recommendations

The study showed that when applied correctly and with proper equipment, the osmotic method for detection of *Pyrenophora teres* or *P. graminea* is easy to perform, and shows good conformity between laboratories. It is therefore recommended to include the osmotic method in ISTA Rules as a routine test for *Pyrenophora teres* and *P. graminea*, when it is not necessary to distinguish the two species.

Acknowledgements

We would like to thank Jean-Louis Laffont, ISTA Statistics Committee Chair, for his help in the data analysis. We also wish to thank all the participating laboratories: Eurofins (former AnalyCen), Sweden, Frökontrollen Mellansverige AB, Sweden, Kimen Såvarelabortoriet AS, Norway, the Finnish Food Safety Authority Evira (former KTTK Kasvintuotannon tarkastuskeskus siementarkastusosasto), Finland, PMK, Põllumajandusuuringute Keskus, Estonia, Jordbruksverket Utsädesenheten, Sverige. The authors want to thank the participants for their invaluable contribution to this report.

References

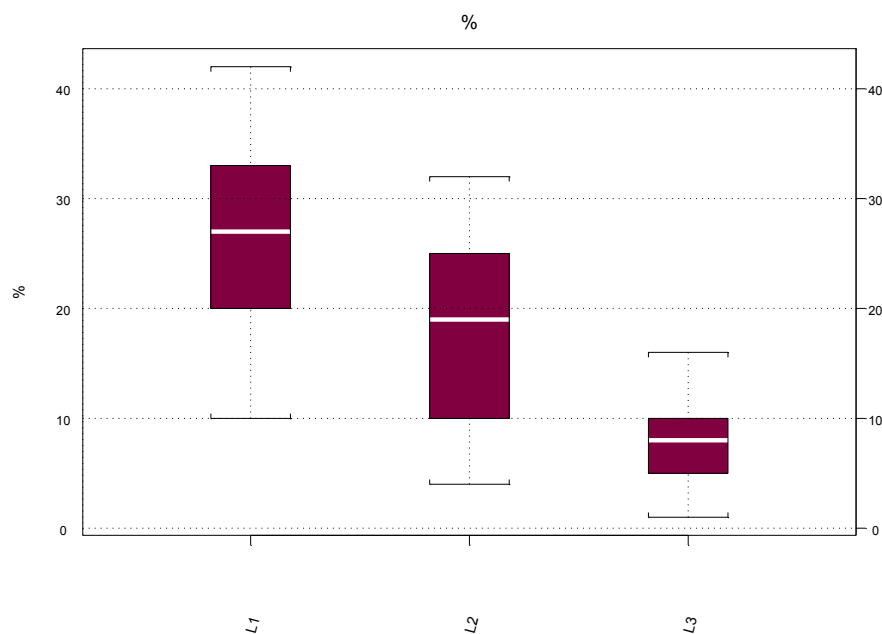
- Braverman, S.W. (1960). The *Helminthosporium gramineum* complex and related species on cereals and forage grasses. *Phytopathology*, **50**, 688-691.
- Brodal, G. (1993). Fungicide treatment of cereals according to need in the Nordic countries. *Crop Protection in Northern Britain*, The Association for Crop Protection in Northern Britain 1993: 7-16.
- Brodal, G., Halkilahti, A.M., Jørgensen, J. and Sperlingsson, K. (1994). *Frøbårne plantesjukdommer og frøpatologiske undersøkelser* [Seedborne diseases and seed pathological investigations]. Nordic Council of Ministers, Copenhagen, TemaNord 1994: 630, 50 pp.
- Brodal, G. (1995). The osmotic method for detection of *Drechslera graminea*/D. *teres* on barley seed compared with the freezing blotter method. In: *Abstracts 24th ISTA Congress Seed Symposium*, Copenhagen, Denmark, June 7–16, 1995, p 24.
- Brodal, G. (1997). Comparative Tests with the Osmotic Blotter Method for Detection of *Drechslera* spp. in Barley Seeds. In *Seed Health Testing – Progress Towards the 21st Century*, (eds J.D. Hutchins and J.C. Reeves), pp. 211-218, CAB International, Wallingford, UK.
- Engström, K., Brishammar S., Svensson C., Bengtsson M., Andersson R. (1993). Anthraquinones from some *Drechslera* species and *Bipolaris sorokiniana*. *Mycological Research*, **97**, 381-384.
- ISTA (1964a). Barley Leaf Stripe - *Drechslera graminea*. Working sheet S. 3 No. 6. *Handbook on Seed Health Testing*. International Seed Testing Association, Bassersdorf, Switzerland.
- ISTA (1964b). Barley Net Blotch – *Drechslera teres*. Working sheet S. 3. No. 7. *Handbook on Seed Health Testing*. International Seed Testing Association, Bassersdorf, Switzerland.
- International Rules for Seed Testing: Annexe to Chapter 7 Seed Health Testing, Seed Health Testing Methods (2010).
- Joelson, G. (1983). The osmotic method – a method for rapid determination of seed-borne fungi. Preprint No. 104, 20th ISTA Congress, Ottawa, June 17–25, 1983. International Seed Testing Association, Zürich, 9 pp.
- Joelson, G. Unpublished. *Osmometoden snabbmetod för att bestämma angrepp av parasitsvampar*.
- Kieteriber, M. (1977). Makroskopische Saatgutbefallserkennung von *Drechslera graminea*, dem Erreger der Streifenkrankheit der Gerste. *Jahrbuch 1976 der Bundesanstalt für Pflanzenbau und Samenprüfung in Wien*, 115-121.
- Knudsen, J.C.N. (1982). Production of pigments by *Pyrenophora graminea* and *Pyrenophora teres* with special reference to the use of this characteristic in seed disease testing. *Seed Science and Technology*, **10**, 357-263.
- Nylund, R.M. (1990). *Osmometoden som diagnostiskt hjälpmedel vid artbestämning av parasitsvampar* [osmotic method as diagnostical tool for determination of species of parasite fungi] Examensarbete inom Lantbruksbiologi med miljö- och naturvård. Lantmästarlinjen, Sveriges Lantbruksuniversitet, Alnarp 24 pp.
- Rennie, W.J. and Tomlin, M.M. (1984). Barley Leaf Stripe - *Pyrenophora graminea*. Working sheet No. 6 (2. ed.) *ISTA Handbook on Seed Health Testing*. Section 2 Working Sheets. International Seed Testing Association, Bassersdorf, Switzerland.
- Svensson, C. (1981). Ny metod för betämning av utsädesburna sjukdomar [New method for detection of seed-borne diseases]. *Växtskyddsrapport, Jordbruk 15*, Sveriges Lantbruksuniversitet, 115-118.

Appendix

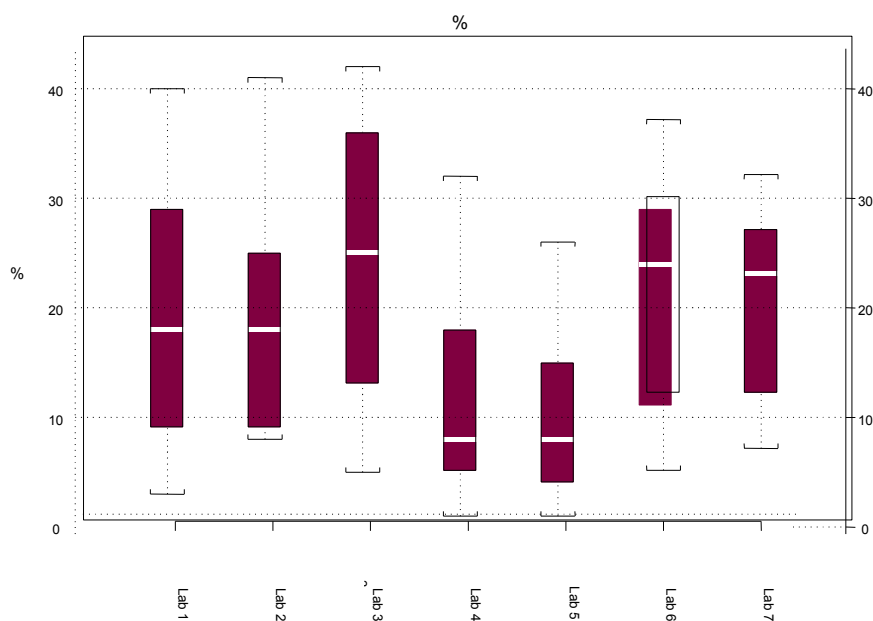
Statistical analysis of osmotic method validation data

1. Data exploration with side-by-side boxplots

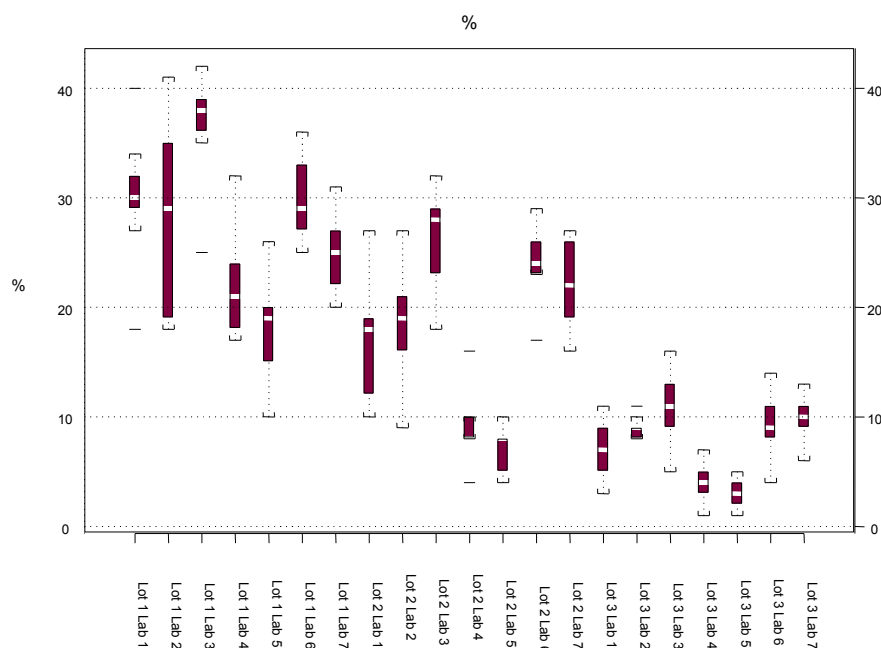
Grouping factor: lot



Grouping factor: Lab



Grouping factor: lab x lot



A strong lot effect and a strong lab effect are exhibited in these side-by-side boxplots. Interactions between lot and lab seem to be limited.

As expected (binomial data), heteroscedasticity as well as non-symmetry is exhibited in these side-by-side boxplots.

2. Modeling 1: assessing the significance of the effects

Model: generalized linear mixed model

$Counts \sim \text{Binomial}(100, \pi_{ijkl})$

$$\text{logit } \pi_{ijkl} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + c_{k(ij)}$$

where:

$i = 1, 2, 3$ $j = 1, 2, 3, 4, 5, 6, 7$ $k = 1, 2, 3$ $l = 1, 2, 3$;

μ is the general effect;

α_i is the fixed effect of lot i ;

β_j is the fixed effect of lab j ;

$(\alpha\beta)_{ij}$ is the interaction effect between lot i and lab j ;

$c_{k(ij)}$ is the random effect of sample k from lot i in lab j . The $c_{k(ij)}$ are iid $N(0, \sigma^2_{c_{k(ij)}})$.

This model was fitted using the SAS GLIMMIX procedure.

Results:

Fit statistics:

-2 Res Log pseudo-likelihood 131.67

Generalized chi-square 166.28

Generalized chi-square / DF 0.99

The generalized chi-square statistic is very close to one, indicating no lack of fit of the above model.

Variance component

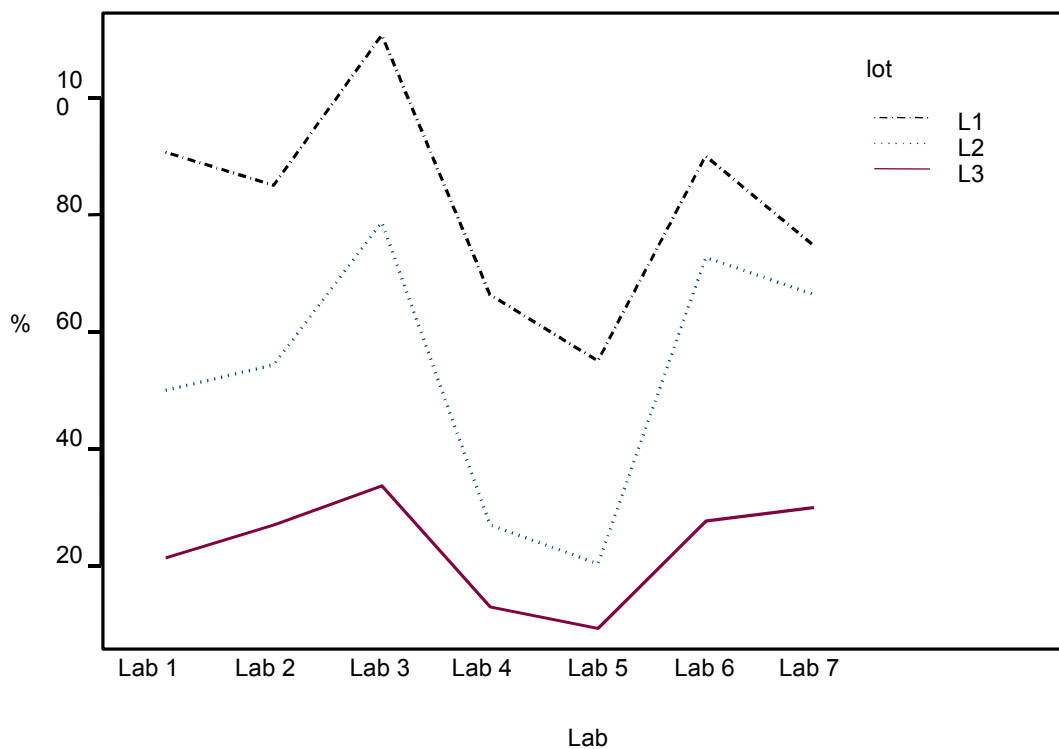
Sample (lot x lab)	0.02116
---------------------------	----------------

Type III tests of fixed effects:

	Num DF	Den DF	F value	Pr > F
Lot	2	42	223.84	<0.0001
Lab	6	42	29.83	<0.0001
Lot x lab	12	42	2.53	0.0130

The lot and lab main effects are highly significant, which is not surprising.

The lot x lab interaction effect is also significant. However, looking at the interaction plot for this interaction term, we don't see any crossover between the effects:



3. Modeling 2: assessing repeatability/reproducibility

Data analysed: sum of the counts over the three replicates

Model: generalized linear mixed model

Counts \sim binomial(300, π_{ijk})

$$\text{logit } \pi_{ijk} = \left(\begin{array}{c} \mu + \alpha_i + b_j + (\alpha b)_{ij} \end{array} \right)$$

where:

$i = 1, 2, 3$ $j = 1, 2, 3, 4, 5, 6, 7$ $k = 1, 2, 3$;

μ is the general effect;

α_i is the fixed effect of lot i ;

b_j is the fixed effect of lab j . The b_j are iid $N(0, \sigma_{b_j})$;

$(\alpha b)_{ij}$ is the interaction effect between lot i and lab j . The b_j are iid $N(0, \sigma_{\alpha b_{ij}})$.

Repeatability and reproducibility computations:

The repeatability and the reproducibility variances are computed for three nominal probabilities π_i ("gold" standards), 0.1, 0.2 and 0.3 using the following formulas:

$$\text{Repeatability variance: } S_r^2 = \frac{4 \pi}{300}$$

$$\text{Reproducibility variance: } S_R^2 = \frac{4 \pi}{300} + \frac{4 \pi}{300} - \frac{4 \pi}{300} + \frac{4 \pi}{300}$$

Results:

		Repeatability	Reproducibility	Binomial
Gold standard		std-dev	std-dev	std-dev
10%	0.019824	0.046575	0.017321	
20%	0.026432	0.079451	0.023094	
30%	0.030282	0.102896	0.026458	

Standard deviations of repeatability are very close to binomial standard deviations: there is no particular problem with this test at the laboratory level.

Standard deviations of reproducibility are added here for information only, as there is no reference available to make a comparison.

Evaluation of early counts of radicle emergence during germination as a repeatable and reproducible vigour test for maize

S Matthews¹, M-H Wagner², A Ratzenboeck³, M Khajeh-Hosseini⁴, E Casarini⁵, R El-Khadem⁶, M El Yakhlifi² and A A Powell¹

¹ School of Biological Sciences, 23, St. Machar Drive, University of Aberdeen, Aberdeen AB24 3UU, UK
(e-mail: agr791@abdn.ac.uk)

² SNES, GEVES, Angers, France

³ Department of Seed Testing, Austrian Agency for Health and Food Safety, Vienna, Austria

⁴ Department of Crop Science, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran

⁵ LaRAS, Bologna, Italy

⁶ Pioneer Hi-Bred, Parndorf, Austria

Summary

In an international comparison of six commercial seed lots of maize, single counts of radicle emergence (RE; defined as production of a 2 mm radicle) after 6 d at 13 °C (5 laboratories) and 66 h at 20 °C (6 laboratories) consistently ranked the lots in the same order in each of two runs in each laboratory. The two fastest germinating lots gave significantly higher RE counts than the lowest two lots in all but one run in one laboratory over the two temperatures. Absolute RE values showed some variability between laboratories, and although there was an interaction between labs and lots, this did not affect the ranking and differentiation of the lots. The repeatability and reproducibility of the data was comparable with that for previous vigour tests, except for one lot with the lowest vigour, for which the reproducibility between laboratories was higher. The RE counts in Aberdeen at 13 °C and 20 °C for nine lots (including the six for the comparative test) were significantly related to field emergence (%) determined in Iran, slower germinating lots (low RE) giving lower emergence. We argue that early counts of radicle emergence during germination provide a faster, more clearly discerning vigour test than the cold test, and that the method described in this document provides a repeatable and reproducible method.

Introduction

The cold test for maize is the most used of all vigour tests (TeKrony, 2001). There is much evidence, most recently from Italy using commercial seed lots, that counts of normal seedlings after the cold test is highly significantly related to field emergence in cold soils (Lovato and Balboni, 1997; Noli *et al.*, 2008). However, the test is difficult to standardise (Nijenstein and Kruse, 2000).

An alternative vigour test for maize has been suggested (Matthews and Khajeh-Hosseini, 2006, 2007) in which radicle emergence at 13 °C and 20 °C is measured. In the work on rate of germination, two measurements were used: mean germination time (MGT) based on frequent, often daily counts, and single early counts of radicle emergence.

MGT has given consistent rankings of lots in experimental runs in three laboratories that were indicative of field performance (Khajeh-Hosseini *et al.*, 2009). In later work on six commercially available seed lots, comparisons were made in two runs at 13 °C in four laboratories of both MGT and single early counts of radicle emergence (Matthews *et al.*, 2010). Both measurements were consistent in their ranking of the lots (Matthews *et al.*, 2010). A 6 d count of radicle emergence at 13 °C gave the same ranking in two experimental runs in each of the four laboratories, and was significantly related to the cold test results (normal germination) in all experimental runs.

In this document we present evidence in support of early counts of radicle emergence as a vigour test from a further comparative test at 13 °C and 20 °C on six seed lots of maize in six laboratories.

Materials and methods

Selection of seed lots

Initial assessments were made in late 2007 from stocks of F₁ hybrids held in store at the Pioneer Hi-Bred laboratory in Parndorf, Austria. Nine lots were selected for further tests in Aberdeen and Mashhad, Iran (Table 1). All lots had standard germinations ranging from 90 to 99%, cold test germinations (normal seedlings) from 76 to 96% and similar moisture contents (Table 1).

Further tests on these nine lots of the timing of radicle emergence (RE) at 13 and 20 °C in Aberdeen and on field emergence in Iran were conducted in early 2008 (Appendix I, Table A). On the basis of these tests and the initial assessments, six lots were selected having at least 90% germination in the standard germination test, and which exhibited a range of performance in field and radicle emergence (Table 1; Table A, Appendix I).

Participating laboratories

Five laboratories conducted comparisons of radicle emergence at 13 °C. These were: University of Aberdeen, Department of Crop Science; Ferdowsi University of Mashhad, Iran; LaRAS, University of Bologna, Italy; SNES, GEVES, Angers, France; Pioneer Hi-Bred Services GmbH, Parndorf, Austria.

Comparisons of the radicle emergence at 20 °C included a further laboratory, namely the Department of Seed Testing, Austrian Agency for Health and Food Safety GmbH, Vienna.

The six selected seed lots, highlighted in blue in Table 1 and numbered from 1 to 6, were sent out to all participating laboratories by the Pioneer Hi-Bred laboratory in Austria in April 2008. Recipients were asked to store the seed in a moisture-proof container at a low temperature prior to use.

Standard germination and cold tests

Standard germination tests were conducted on 400 seeds at 25 °C according to the ISTA Rules (ISTA, 2008) and the cold test was that routinely in use at the Parndorf laboratory of Pioneer Hi-Bred. This test placed 4 replicates of 100 seeds on top of a water-saturated Barden clay/sand mixture held in trays, with the embryo placed down to increase anaerobic stress. Seeds were held in the dark for 4 d at 10 °C, then transferred to 25 °C in the dark for 3 d, after which normal seedlings (ISTA 2009) were counted (Matthews *et al.*, 2010).

Radicle emergence test

Two experimental runs were carried out at each of two temperatures using 4 replicates of 25 seeds in each, i.e.:

At 20 °C for each lot:

4 x 25 seeds run 1 = 100 seeds

4 x 25 seeds run 2 = 100 seeds

At 13 °C for each lot:

4 x 25 seeds run 1 = 100 seeds

4 x 25 seeds run 2 = 100 seeds

Substrate

A rolled towel method was used for germination, accepting that small differences in protocol and/or materials would occur between laboratories.

The maize seeds were placed on the papers with the embryo radicle pointing to the bottom of the paper. Two rows of seeds were suggested to assist counting, one of 12 and one of 13 seeds. Towels were held in moisture-proof bags or containers to prevent them drying out.

Experimental design

A randomised block design of 4 blocks of 6 lots was suggested as the experimental design; otherwise it should be completely randomised.

Temperature

Temperature was stressed as the most important potential variable between laboratories and runs, and within runs. Monitoring of temperature was suggested to be desirable, and daily rotation of blocks was advised. Participant laboratories were also advised to keep blocks close together in the incubation room (e.g. on the same shelf).

Counting germination

The criterion for germination was radicle emergence (RE), which for this test is defined as the production of a 2 mm radicle. If the radicle was not obvious, or if the observer had to examine the seed closely to see the radicle, it was not counted as germinated. A clear and obvious radicle was considered a quicker and more uniform method of germination assessment. A length of 2 mm, judged by eye, was the target for germination. A guide marked on a strip of graph paper was suggested as a useful aid to determine whether the minimum length of 2 mm had been reached.

Timing of radicle emergence counts

Radicle emergence counts were completed after:

- 66 h at 20 °C: As a guide to achieve these counts at a reasonable time of day, participants were advised to set the test up at 16.00 hours for counts at 10.00 hours.
- 6 days at 13 °C: Set up at a time of day convenient for making the subsequent counts.

Statistical analysis

The effect of the various factors (laboratory, seed lot, test run) were analysed by analysis of variance, and the LSD values calculated for $p < 0.05$.

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:

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

Results

Comparison of runs and laboratories (ANOVA)

13 °C

The ranking of the lots was largely consistent, with only a few instances where the ranking in the experimental runs was different to that of the overall means (Table 2). Lots 3 and 5 had significantly lower RE counts than the top two lots (6 and 1) in both runs of the test in all laboratories.

The ANOVA (appendix II, Table B) showed a highly significant effect of lots and laboratories. There was a significant interaction between lot and laboratory, which can be seen in Table 2, where lab A with the lowest mean overall RE showed the greatest range in RE, while lab D with the highest overall mean had the smallest range.

20 °C

The ranking of the lots was almost perfectly consistent for all runs (Table 3), even though there were differences in the percentages of RE, particularly between laboratories. The RE of seed lot 5 was consistently lower than that for the other lots, and the REs of the top two lots (6 and 1) were significantly higher than the bottom two, with only one exception (run 2, lab D).

The ANOVA (appendix II, Table C) showed a highly significant effect of lot and laboratory. There was also a highly significant interaction between lot and laboratory. This can be seen in Table 3, where differences in the RE between lots were smaller in laboratories D and E than in the other four laboratories. Nevertheless, the ranking of the lots in these two laboratories remained the same as in the other experimental runs.

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 overestimations or underestimations compared to the mean of all the results available, whereas the k-values give a measure of the variability of the repeats. Higher values indicate greater under- or overestimations (h-values) or greater variability between replicates (k-values).

13 °C

There was a significant underestimation (h-values) of radicle emergence only in laboratory B for two lots (lots 4 and 6) in both runs (Figure 1a, b).

Significant variability between replicates (k-values) was observed in run 1 (Figure 2a) for single lots in labs A (lot 6) and B (lot 3) and for two lots in lab C (lots 2 and 5). In run 2 (Figure 2b) significant k-values occurred for single lots in labs D (lot 3) and E (lot 2).

20 °C

The radicle emergence of only one lot (lot 4, lab D) was significantly overestimated in run 1 (Figure 3a), while in run 2 the radicle emergence of two lots (lot 6, lab B; lot 1, lab C) were underestimated.

Variability between replicates was notable only in lab C, where three lots showed significant variability in both runs. There was also significant variability between replicates in single lots in labs 1 and 2 in both runs (Figure 4a, b).

Values for repeatability and reproducibility were within similar ranges at both temperatures (Table 4), indicating that neither temperature gave more, or less, consistent results.

Relationships with cold test germination and field emergence

Radicle emergence at both 13 and 20 °C was significantly correlated with final field emergence (%) (Table 5). Radicle emergence at 13 °C was also correlated with germination (normal seedlings) after the cold test. These data originated from those obtained during the preliminary assessment of nine lots before the selection of lots for the comparative test (Appendix I, Table A).

Discussion

Two counts of radicle emergence (RE), 6 d at 13 °C and 66 h at 20 °C, ranked the six lots included in this comparative test in the same order in both runs in all laboratories (Tables 2 and 3). This confirmed the findings in previous work on maize at 13 °C in three laboratories (Khajeh-Hosseini *et al.*, 2009) and four laboratories (Matthews *et al.*, 2010). Furthermore, in 21 out of 22 runs of the test (2 runs x 5 labs at 20 °C plus 2 runs x 6 labs at 13 °C), the two lots (6 and 1) with the highest RE (high vigour) were clearly and significantly distinguished from the two lots (2 and 5) with the lowest RE.

There were, however, highly significant differences between laboratories in absolute percentages of RE, and interactions between laboratories and lots, at both temperatures (Appendix II, Tables A and B). The interactions between labs and lots were seen in the magnitude and significance of some of the differences between the lots in different laboratories. Differences were less, for example, between lots in lab D and greater in lab A, especially at 20 °C. However, these differences were not so great as to change the rank order of the lots.

In terms of statistical significance, the differences in the absolute percentage of RE seen between labs could have arisen as a result of one or both of two reasons: 1) differences in the control of temperature over the whole of the experimental area; (2) differences in the application of the criterion of germination. The fact that lab A had the lowest overall mean and lab D the highest mean at both temperatures suggested that the criterion for germination may have been more important. Thus, lab A may have judged some seeds as not having germinated because they had not reached the 2 mm radicle stage, whereas they were regarded as germinated by lab D. Despite the differences in absolute RE, there were clear significant differences between the top two lots (6 and 1) and the lowest two lots (2 and 5) in all but one run of the test (Tables 2 and 3).

Only a few examples of significant under- or overestimation of data were revealed by the h-values (Figures 1 and 3). Examples of significant variability between replicates (k-values) were seen only in single lots (in one lab, run 2; two labs, run 2) at 13 °C, and at 20 °C only lab C showed significant variability between replicates in single lots in both runs (Figs 4a, b). With the exception of the reproducibility values for lot 5 (Table 4), all values for repeatability and reproducibility were comparable with the results previously reported for the controlled deterioration test (Powell, 2009) and a standard germination test for sunflower (Ducournau *et al.*, 2007). The low vigour of lot 5 may explain the greater variability observed, as results for low vigour seed lots are typically more variable than those for high vigour lots.

Comparisons of the data to determine whether replicates, repeat runs of the test in one laboratory and results for the same lots in different labs were in tolerance were made using the ISTA Germination Calculator (www.seedtest.org/upload/cms/user/Germinationtolerancescalculator-V0.3.xlsISTA). In only 14 of the 132 tests completed were the replicates not in tolerance: 2 out of 60 tests at 13 °C and 12 out of 72 tests at 20 °C. The results from the repeat runs in each laboratory were in tolerance, with the exception of lot 5 in labs B and E at 13 °C and lot 3 (lab D) and lot 5 (lab E) at 20 °C. The results from different laboratories were in tolerance, with the exception of lots 2 and 5 in both runs of the test at 13 °C, lot 2 in run 1 at 20 °C and lot 5 in both runs at 20 °C. Where the data from different laboratories were not in tolerance, this was largely due to over- or underestimations of radicle emergence in one or two laboratories. This could be eliminated by training courses to ensure uniformity for routine testing.

The radicle emergence counts at 13 °C and 20 °C made in preliminary work in Aberdeen for nine lots (Appendix I, Table A) were significantly related to final field emergence determined in Iran (Table 5, Figure 5a and b). The RE after 6 d at 13 °C was also significantly related to the cold test result (% normal seedlings) (Table 5), as previously seen by Matthews *et al.* (2010). The cold test in the present work on nine lots was not significantly related to field emergence, but in comparisons of a larger number of commercial lots, for example by Lovato and Balboni (1997) and Noli *et al.* (2008), it was highly significantly related to field emergence.

It has been suggested (Matthews and Khajeh-Hosseini, 2007; Matthews *et al.*, 2010) that the cold test and radicle emergence assessed at any temperature are both assessments of seed age. Physiologically older seeds need to go through a longer period of metabolic repair before radicle emergence, thus leading to a longer lag period from the beginning of imbibition to radicle emergence compared to younger seeds. In the cold test, the early cold period and anaerobic conditions might slow down metabolic repair. As a result, in comparison to the warm germination test, a proportion of the older seed would not repair sufficiently at the lower temperature of the cold test to produce normal seedlings.

We argue that laboratories should consider a test of the radicle emergence as an alternative to the cold test for three reasons. Firstly, a radicle emergence test takes less time than the cold test, as little as 2-3 days at 20 °C, compared to up to 7 to 10 days for the cold test. Secondly, a clearer differentiation between low- and high-vigour lots is often achieved by the radicle emergence test than by the cold test, as seen in Table 3. Finally, as the present work suggests, standardisation of the radicle emergence test is possible.

This comparative test has clearly revealed that the radicle emergence test can consistently identify differences in vigour, and that the test is repeatable and reproducible in the ranking of the lots. At the outset of this work, temperature control over the experimental area in the incubator or controlled temperature room was emphasised as a possible source of variation. This may have been the cause of the differences that were observed between laboratories, along with the interpretation of the criterion for radicle emergence. We would recommend the following for routine application of the method, and to ensure uniformity between labs:

1. The temperature over the experimental area should always be monitored. The overall declared temperature control of many of the controlled temperature rooms or incubators used by participating labs ranged from ± 0.5 °C to ± 2.0 °C. However, data from some laboratories in this current work suggested that in the actual experimental area the temperature control was closer to ± 1.0 °C. As a guide to using this radicle emergence test method, ± 1.0 °C should be the realistic target.
2. Laboratories should consider incorporating an early count of RE (66 h) into a standard germination test carried out at 20 °C in rolled towels. Thus, one test following ISTA Rules would provide a vigour assessment after 2 to 3 days, as well as the standard germination count.
3. A limit should be placed on the number of lots to be tested in any one run, say 15 lots, so that the experimental area covered is not too great and a temperature range is therefore not difficult to avoid.
4. A standard lot of medium vigour (e.g. lot 4 in the comparative test) should be included in all test runs in any one season.

5. Joint training exercises by laboratories in workshops, or collaborative work on the same lots, should be organised to ensure uniformity for routine testing.

Acknowledgements

We thank Pioneer Hi-Bred Services GmbH, Parndorf, Austria for the supply of seed and information. We also thank those who gave technical support to the work. In particular, we acknowledge the contributions of Sandrine Stievenard and Marylène Moron in Angers, Jarad Nezafat and Ali Izadfar in Mashhad, Giovanni Urso and Emma Beltrami in Bologna, and Elisabeth Gorgosilits in Parndorf.

References

- Ducournau, S. Garreau, P. and Léchappé, J. (2008). Effect of temperature and growing media on sunflower germination. *Seed Testing International*, **135**, 34-36.
- Khajeh-Hosseini, M., Lomholt, A. and Matthews, S. (2009). Mean germination time in the laboratory estimates the relative vigour and field performance of commercial seed lots of maize (*Zea mays* L.). *Seed Science and Technology*, **37**, 446-456.
- Lovato, A. and Balboni, M. (1997). Seed vigour in maize (*Zea mays* L.): two-year laboratory and field test compared. *Italian Journal of Agronomy*, **1**, 1-6.
- Matthews, S. and Khajeh-Hosseini, M. (2006). Mean germination time as an indicator of emergence performance in soil of seed lots of maize (*Zea mays*, L). *Seed Science and Technology*, **34**, 361-369.
- Matthews, S. and Khajeh-Hosseini, M. (2007). Length of the lag period of germination and metabolic repair explain vigour differences in seed lots of maize (*Zea mays* L). *Seed Science and Technology*, **35**, 200-212.
- Matthews, S., El-Khadem, R., Casarini, E., Khajeh-Hosseini, M., Nasehzadeh, M. and Wagner, M.-H. (2010). Rate of physiological germination compared with the cold test and accelerated ageing as a repeatable vigour test for maize. *Seed Science and Technology*, **38**, 379-389.
- Nijenstein, J. H. and Kruse, M. (2000). The potential for standardization in cold testing of maize (*Zea mays* L.) *Seed Science and Technology*, **28**, 837-851.
- Noli, E., Casarini, E., Urso, G. and Conti, S. (2008). Suitability of three vigour test procedures to predict field emergence of early sown maize. *Seed Science and Technology*, **36**, 168-176.
- Powell, A.A. (2009). Evaluation of the controlled deterioration test as a repeatable and reproducible vigour test for *Brassica* species. *ISTA Method Validation Reports*, 2009, 92-123.

Table 1: Initial assessments of seed quality of the nine seed lots of F₁ hybrid maize from which six were selected (highlighted lots) for further comparative tests. All tests were conducted by Pioneer Hi-Bred laboratory.

Seed lot code number	Standard germination (%)		Cold test germination		Moisture content (%)
	Normal (%)	Abnormal (%)	Normal (%)	Abnormal (%)	
7	96	3	96	3	13.5
8	96	4	97	3	13.8
1	98	1	90	6	13.9
2	94	5	94	3	13.2
3	92	4	76	10	13.3
9	93	4	88	5	13.9
4	90	7	94	3	12.9
5	96	3	82	10	13.6
6	99	1	96	3	13.5

Table 2: Seed quality evaluation of six seed lots of maize. Radicle emergence (%) assessed after 6 d at 13 °C in two runs in five laboratories; each mean is derived from 4 replicates of 25 seeds. Cold test assessed in the laboratory of Pioneer Hi-Bred; field emergence assessed in Iran. The order of the lots is that of the overall mean, with the highest germinating lot at the top.

Seed lot	Laboratory										Overall mean	Cold test (%)	Field emergence (%)
	A		B		C		D		E				
	1	2	1	2	1	2	1	2	1	2			
6	94a	95a	90a	89a	98a	97a	98a	99a	98a	98a	95a	96	92
	1	1	1	2=	1	1	1	1	1	1	1		
1	90a	94a	86a	89a	89ab	88ab	92a	91b	90a	97a	91a	90	90
	2	2	2	2=	3	3	3=	4	2	2	2		
4	89a	90a	78a	78a	90ab	93a	93a	96ab	91a	92a	89a	94	82
	3	3	4	4	2	2	2	2	3	3	3		
2	52b	59b	81a	89a	66bc	78b	92a	94ab	79a	74b	76b	94	78
	5	4	3	1	4	4	3=	3	4	4=	4		
3	56b	57b	59b	55b	56c	58c	60b	72c	64a	74b	61c	76	84
	4	5	5	5	5	5	5	5	5	4=	5		
5	1c	3c	13c	48b	21d	10d	27c	36d	8b	28c	20d	82	78
	6	6	6	6	6	6	6	6	6	6	6		
Mean	65c		71b		70b		79a		74a				

Any two means without a letter in common are significantly different ($p < 0.05$) as determined by LSD following Analysis of Variance.

Table 3: Radicle emergence (%) of six lots of maize after 66 h at 20 °C in two runs in six laboratories. Each mean is derived from 4 replicates of 25 seeds. The order of the lots is that of the overall mean, with the highest germinating lot at the top.

Seed lot	Laboratory												Overall mean
	A		B		C		D		E		F		
	1	2	1	2	1	2	1	2	1	2	1	2	
6	95a	97a	93a	94a	98a	100a	100a	99a	98a	99a	99a	100a	95a
	1	1	1	1=	1	1	1	1=	1	1	1	1	1
1	92a	96a	90a	94a	90a	94ab	97a	99a	96a	98a	94a	94a	91a
	2	2	2	1=	2	2	2=	1=	2	2	2	2	2
4	90a	94a	88a	90a	89a	88b	97a	94a	93a	92ab	93a	91a	89a
	3	3	3	2	3	3	2=	3	3	3	3	3	3
2	65b	72b	84a	74b	67b	66c	95a	93a	91a	91ab	89a	82b	76b
	4	4	4	3	4	4	4	4	4	4	4	4	4
3	62b	64b	–	–	65b	62c	78b	91a	71b	83b	59b	65c	61c
	5	5	–	–	5	5	5	5	5	5	5	5	5
5	29c	32c	31c	24c	38c	41d	76b	88a	61b	77b	28c	16d	20d
	6	6	6	6	6	6	6	6	6	6	6	6	6
Mean	74c		76c		75c		92a		88b		76c		

Any two means without a letter in common are significantly different ($p < 0.05$) as determined by LSD following analysis of variance.

Table 4: Values for repeatability and reproducibility for six seed lots tested in five (13 °C) or six (20 °C) laboratories for radicle emergence in each of two runs

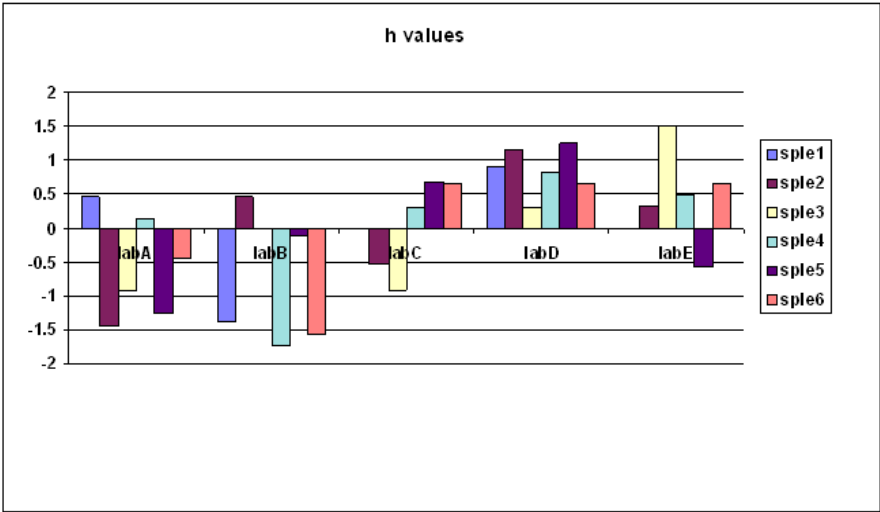
Lot	Repeatability sr2		Reproducibility sR2	
	Run 1	Run 2	Run 1	Run 2
13 °C				
6	4.502	3.578	5.292	5.040
1	7.711	4.033	7.029	5.089
4	5.489	6.733	7.567	9.072
2	10.250	10.558	17.757	16.471
3	9.048	9.587	8.509	12.508
5	13.594	10.721	15.633	20.693
Range	4.502–13.594	3.578–10.721	5.292–17.757	5.040–20.693
20 °C				
6	4.028	1.700	4.374	2.747
1	5.637	16.995	5.727	17.957
4	4.807	6.218	5.379	5.745
2	10.904	8.472	16.330	14.213
3	7.364	11.034	9.309	13.810
5	12.046	7.557	22.552	31.563
Range	4.028–12.046	1.700–11.034	4.374–22.552	2.747–31.563

Table 5: Correlation coefficients between cold test germination, final field emergence (%) and two measures of the rate of germination: radicle emergence (RE) after 66 h at 20 °C and 6 d at 13 °C for nine lots of maize (Table A, Appendix I).

	Cold test germination (%)	Final field emergence (%)
Cold test germination (%)	–	0.415
RE after 66 h at 20 °C (%)	0.605	0.761*
RE after 6 d at 13 °C (%)	0.696*	0.850**

* p<0.05; ** p<0.01

a) 13 °C Run 1



b) 13 °C Run 2

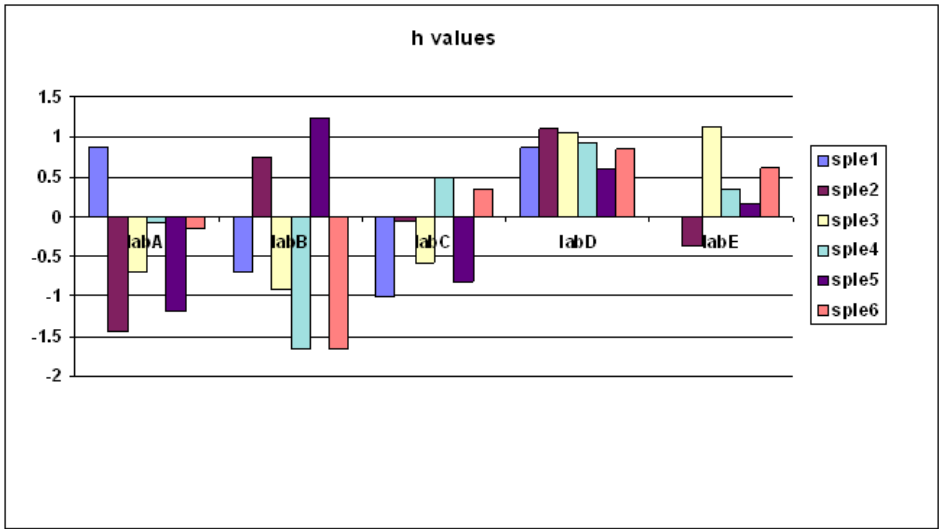
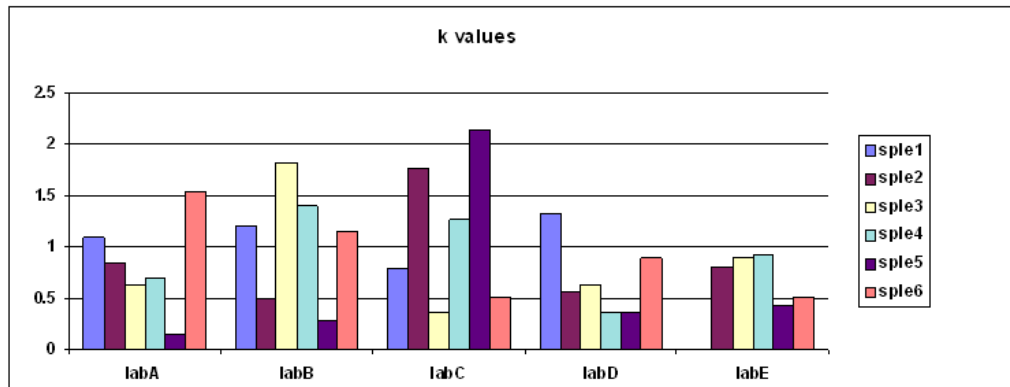


Figure 1: h values for the radicle emergence of six lots of maize assessed at 13 °C in five laboratories in each of two runs.

a) 13 °C Run 1



b) 13 °C Run 2

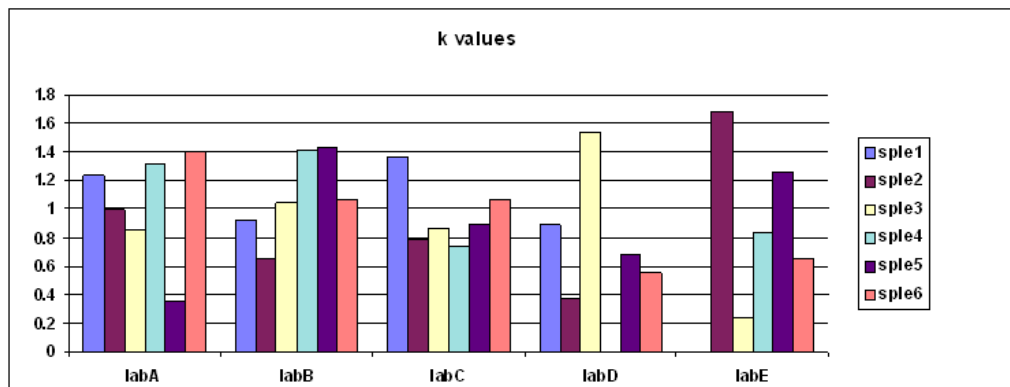
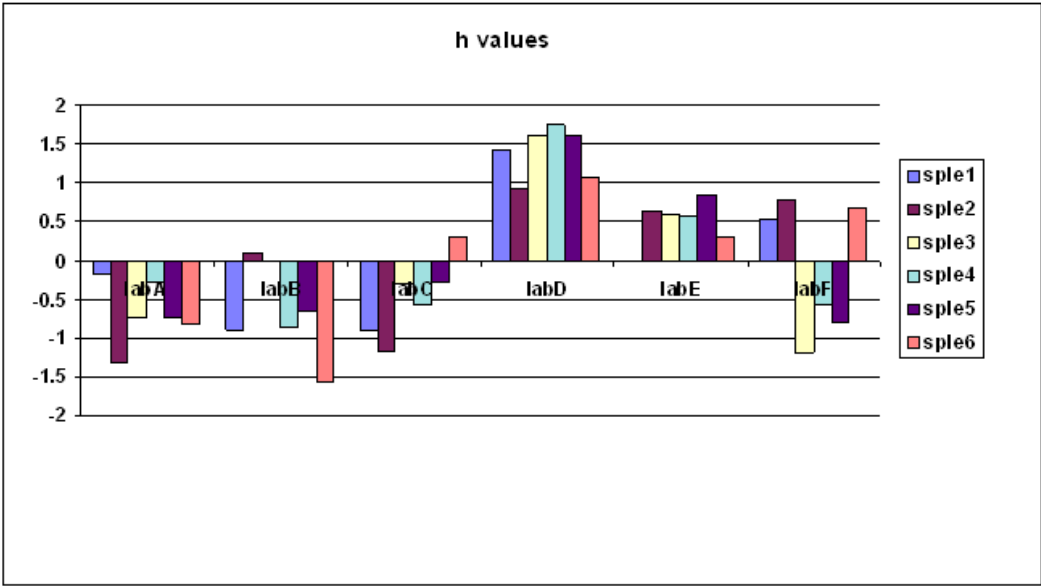


Figure 2: k values for the radicle emergence of six lots of maize assessed at 13 °C in five laboratories in each of two runs.

a) 20 °C Run 1



b) 20 °C Run 2

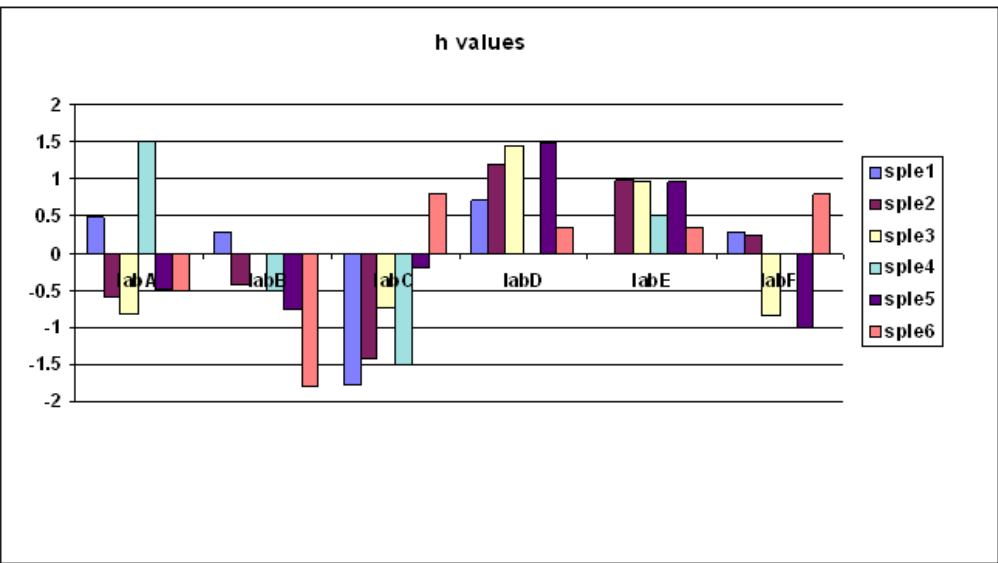
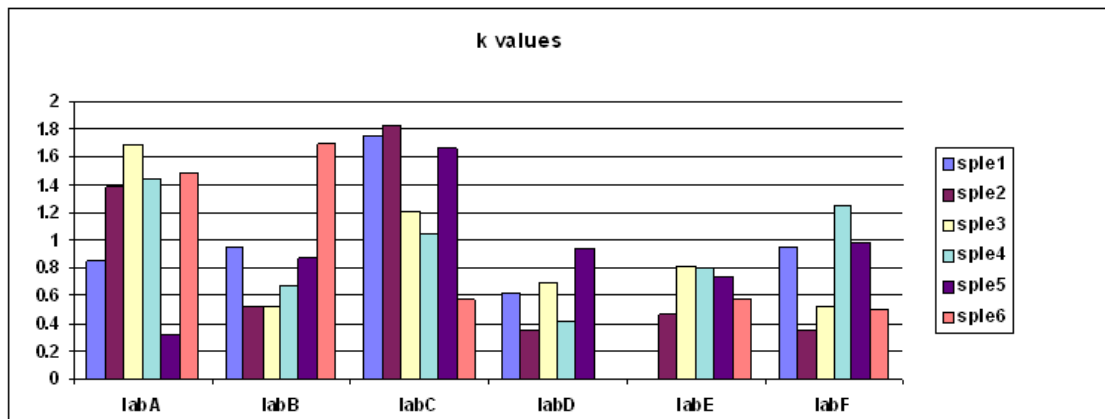


Figure 3: h values for the radicle emergence of six lots of maize assessed at 20 °C in six laboratories in each of two runs.

a) 20 °C Run 1



b) 20 °C Run 2

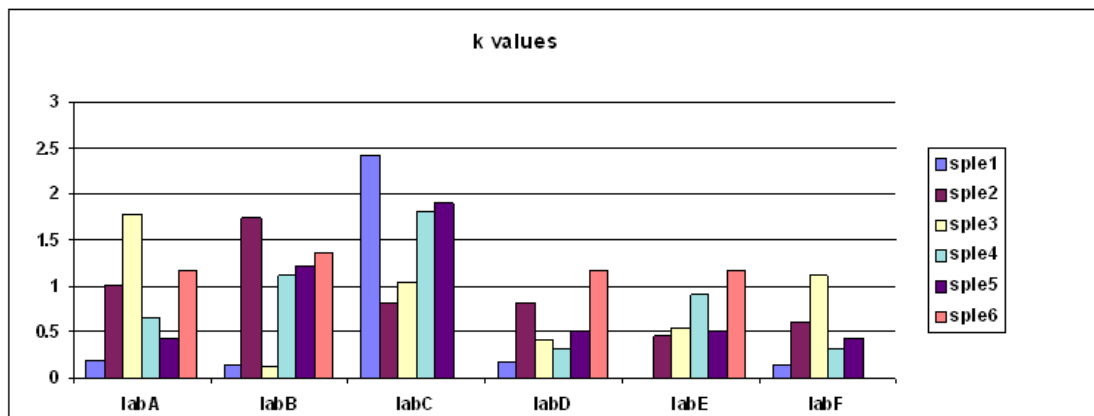


Figure 4: k values for the radicle emergence of six lots of maize assessed at 20 °C in six laboratories in each of two runs.

Appendix I: Data used for relationships in Table 4 (main text).

Table A: Counts of radicle emergence * (RE; 2 mm radicle) of 9 lots of F1 hybrid maize after 6 d at 13 °C and 66 h at 20 °C, alongside cold test germination(ex Pioneer Hi-Bred laboratory) and field emergence (%) (Iran)

Highlighted lots selected for comparative tests

Seed lot		RE (%) after:				Cold test (% normal seedlings)	Field emergence (%)
		48 h 20 °C		66 h 20 °C	6 d 13 °C		
7	60	81	89	96	90		
8	14	63	97	97	90		
1	53	89	86	90	89		
2	5	43	37	94	78		
3	24	51	41	76	84		
9	12	43	20	88	76		
4	66	85	92	94	82		
5	0	7	0	82	78		
6	73	95	96	96	92		

* Determined at 13 °C and 20 °C in Aberdeen on two replicates of 25 seeds in two runs i.e. a total of 100 seeds.

Appendix II: ANOVA tables

Table B: Radicle emergence at 13 °C

Source	DF	SS	MS	F	
Model		34	178911.9417	5262.1159	64.15
Error		205	16816.3542	82.0310	
Corrected total		239	195728.2958		

Source	DF	Type 1 SS	MS	F	
Lot	5	163344.5208	32668.9042	398.25	<0.0001
Lab	4	5329.4833	1332.3708	16.24	<0.0001
Run (lab)	5	1261.5208	252.3042	3.08	<0.0106
Lot x lab	20	8976.4167	448.8208	5.47	<0.0001

Source	DF	Type 111 SS	MS	F	
Lot	5	163344.5208	32668.9042	398.25	<0.0001
Lab	4	5329.4833	1332.3708	16.24	<0.0001
Run (lab)	5	1261.5208	252.3042	3.08	<0.0106
Lot x lab	20	8976.4167	448.8208	5.47	<0.0001

Table C: Radicle emergence at 20 °C

Source	DF	SS	MS	F	
Model		40	127896.1429	3197.4036	52.56
Error		239	14539.0000	60.8326	
Corrected total		279	142435.1429		

Source	DF	Type 1 SS	MS	F	
Lot	5	91954.20952	18390.84190	302.32	<0.001
Lab	5	15117.92000	3023.58400	49.70	<0.001
Run (lab)	6	719.0000	113.83333	1.97	<0.0707
Lot x lab	24	20105.01333	837.70899	13.77	<0.001

Source	DF	Type 111 SS	MS	F	
Lot	5	92761.72000	18390.84190	304.97	<0.001
Lab	5	15117.92000	3023.58400	49.70	<0.001
Run (lab)	6	719.0000	113.83333	1.97	<0.0707

Lot x lab	24	20105.01333	837.70899	13.77	<0.001
-----------	----	-------------	-----------	-------	--------
