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Preface

ISTA Method Validation Reports is an ISTA publication initiated by the Seed Health Committee in 2003. It contains reports of method validation studies which support proposals for new or modified methods to be included in the International Rules for Seed Testing. Publication will coincide with announcements of rules proposals to be voted on by the ISTA Membership at the next Ordinary Meeting.

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Proposal for a new method for detecting *Pseudomonas savastanoi* pv. *phaseolicola* on bean seeds

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Summary

The suitability of semi-selective media MSP, MT and m-KB for the detection of *Pseudomonas savastanoi* pv. *phaseolicola (Psp)* in bean (*Phaseolus vulgaris* L.) seeds was evaluated in a comparative test with seven laboratories organised by the International Seed Health Initiative for Vegetables. Four naturally infected seed lots were used with five sub-samples of 1,000 seeds each, giving a total of 20 sub-samples per laboratory. For each sample and medium the numbers of 'suspect' and 'other' colonies were recorded. Suspect colonies isolated from the media were confirmed by a pathogenicity test. The results indicate that the selective medium MSP exhibited the highest number of confirmed positive colonies of *Psp* followed by MT and m-KB. The highest number of positive sub-samples was found in the combination of MSP and MT media. MT gave the best reproducibility and repeatability dispersion for detecting the presence or absence of *Psp*. The combination of MSP/MT showed best reproducibility of all the combinations but gave a slightly lower repeatability value compared to MT/m-KB. m-KB in combination with MSP however gave the poorest within laboratory dispersion (repeatability). An advantage of MT is that seed-borne *Xanthomonas axonopodis* pv. *phaseoli* can also be detected. For routine seed testing of bean, a combination of the complementary semi-selective media MSP and MT is recommended.

Introduction

Pseudomonas savastanoi pv. *phaseolicola* (*Psp*) (syn. *Pseudomonas syringae* pv. *phaseolicola*) (Gardan *et al*., 1992), the causal agent of halo blight is a serious seed-borne pathogen of bean (*Phaseolus vulgaris* L.) that can reduce yield and quality. Contaminated seed is a source of primary inoculum (Taylor, 1970) and seed health testing plays a vital role in preventing outbreaks of this disease.

ISTA Working sheets

At present ISTA has two different working sheets published for the detection of *Psp* in bean seeds. In Working sheet No. 65 (Van Vuurde and Van den Bovenkamp, 1987) the bacteria are extracted from the bean seeds by a stationary soaking for 6 hours followed by a surface extract sampling and a direct immuno-fluorescence microscopy screening. The samples that appear positive are re-incubated for another 18 hours, sampled and plated onto KB medium. Positive samples produce diffusible blue or blue-green fluorescent pigment on this medium and are confirmed by either an immuno-fluorescence or a pathogenicity test.

An alternative method is described in working sheet No. 66 (Jansing and Rudolph, 1996) where bean seeds are subjected to a stationary soaking for 20 hours before the soak is sub-sampled and centrifuged. The pellet is re-suspended, pre-screened by immuno-fluorescence, and plated on MSP medium. Suspect colonies are identified by the detection of phaseolotoxin

using different media, *Escherichia coli* nutrient medium and modified WATANABE medium, which can discriminate between *Psp* and *Pseudomonas syringae* pv. *syringae* (*Pss*).

In general, the most common methodologies used by seed health testing laboratories for the detection of bacteria in seeds are based on a seed wash-dilution plating assay. These plating assays either use a combination of a non-selective and selective medium or a combination of two selective-media.
Seed health laboratories, from the seed testing and governmental agencies, selected components from both ISTA working sheets as they relate to a seed wash-dilution plating assay. Assay components include different selective-media, extraction methods and means to identify the target bacteria were key items to the development of this new proposed method for *Psp*.

Different types of media
KB medium was used in working sheet No. 65 and MSP in working sheet No. 66. KB is a non-selective medium. MSP is an excellent selective medium for *Psp* but cannot be used to differentiate *Psp* from other *Pseudomonads*. Instead of KB, a more selective KB + cephalexin medium (m-KB) (H. Koenraadt, pers. comm.) was used in the validation test.
MT (Goszczynska and Serfontein, 1998) medium can identify three major bacterial seed-borne pathogens of bean (*Psp*, *Xanthomonas axonopodis* pv. *phaseoli* (*Xap*) and *Pss*). This unique feature allows seed testing laboratories to test for more than one pathogen in bean during the same test.

Extraction methods
The extraction procedures in the two *Psp* working sheets are similar, using a refrigerated seed soak. The major difference between the two is a centrifugation step in working sheet No. 66. This was intended to concentrate the target bacteria to assist their detection. However, this practice can decrease detection of Psp especially if a seed lot contains many saprophytes. The decision was made not to include the laborious centrifugation step based on the fact that this step is seldom used by the seed testing industry.

Identification of colonies
There are several options to confirm the identity of colonies of *Psp* (IF, PCR or a pathogenicity test). In this study, a pathogenicity test (working sheet No. 65) was used to confirm suspect colonies as it has a number of advantages compared to the phaseolotoxin assay (working sheet No. 66), IF (working sheet No. 65) and PCR. A pathogenicity test gives reliable results and is easy to perform in routine laboratories compared to the phaseolotoxin test which is labour-intensive, requires a special medium, a high temperature incubator (37°C) and a reference strain of *E. coli*. There is also a possibility of obtaining false negative results since phaseolotoxin-deficient strains have been identified in Spanish bean production areas (Rico *et al.*, 2003). One disadvantage of the pathogenicity assay is the timeframe of this test which is longer than IF and PCR (Prossen *et al.*, 1993). However, the IF test is not specific and requires an additional confirmatory method and the current PCR test does not detect some non-toxogenic isolates of *Psp* are not detected (Rico *et al.*, 2003).

Aim of the test
In this validation study the use of MSP, m-KB and MT media for the detection of *Psp* was evaluated by seven seed health testing laboratories from the Netherlands, United States, France and South Africa, all with practical experience in detecting this pathogen, in order to determine which medium would be a good second medium to MSP.

Materials and Methods

Seed samples
Four bean seed lots, with variable levels of natural infection were selected by Naktuinbouw; P14207, P14209, Pmix (P14210 & P14211; 1:10), and P14210. For each lot the number of infected sub-samples from five sub-samples of 1,000 seeds tested and was 0, 1, 3 and 5 respectively.

For the comparative test each seed lot was divided into 35 sub-samples of 1,000 seeds based on weight. Each sub-sample was placed in a plastic Ziploc bag and coded randomly before being sent to participating laboratories to ensure a blind comparative test. For each seed lot and media five replicates (sub-samples) of 1,000 seeds were sent to each of the seven laboratories for testing.

Media
MSP medium (Modified Sucrose Peptone agar; Mohan and Schaad, 1987) was prepared by dissolving 20.0 g/l sucrose, 5.0 g/l Proteose peptone #3, 0.5 g/l K_2HPO_4, 0.25 g/l $MgSO_4$ * $7H_2O$, with 20.0 g/l of agar in distilled water. The medium was autoclaved for 15 min at 121°C. After cooling to 50°C the following ingredients were added: 200 mg/l cycloheximide, 80 mg/l cephalexin, 10 mg/l vancomycin and 1 ml/l bromothymolblue (15 mg/ml; 95 % ethanol).

KB medium (Kings Medium B; King *et al.*, 1954) was formulated using 20.0 g/l Proteose peptone #3, 1.5 g/l K_2HPO_4, 1.5 g/l $MgSO_4$ * $7H_2O$, 15 ml glycerol and 15.0 g/l of agar in distilled water. It was autoclaved for 15 min at 121°C.

m-KB medium (Modified Kings Medium B; King *et al.*, 1954) was prepared by dissolving 20.0 g/l Proteose peptone #3, 1.5 g/l K_2HPO_4, 1.5 g/l $MgSO_4$ * $7H_2O$, 15 ml glycerol and 15.0 g/l of agar in distilled water. The medium was autoclaved for 15 min at 121°C. After cooling to 50°C, 50 mg/l of cephalexin was added.

MT medium (Milk Tween agar; Goszczynska and Serfontein, 1998) was prepared by dissolving 10.0 g/l Proteose peptone #3, 0.25 g/l $CaCl_2$, 0.5 g/l tyrosine and 15.0 g/l of agar in distilled water. The medium was autoclaved for 15 min at 121°C. After cooling to 50°C the following ingredients were added: 200 mg/l cycloheximide, 80 mg/l cephalexin, 10.0 mg/l vancomycin and 10 ml/l Tween 80 (autoclaved separately) and 10.0 g/l skim milk powder (autoclaved separately).

In order to reduce the chance of introducing variability in the comparative test, antibiotics and other critical materials such as skim milk powder were prepared and distributed by Naktuinbouw, the Netherlands.

Seed washing assay
Sub-samples of 1,000 seeds were soaked overnight at 5°C in 600 ml of saline (0.85%). A tenfold-dilution series (10^0, 10^{-1}, 10^{-2}, 10^{-3}) was prepared from each seed soak extract and 100 µl of each dilution and the undiluted extract were spread in duplicate on MSP, m-KB and MT media. A sterility check was done by preparing a dilution series from a sample of the extraction medium, containing no seeds, and plating it on each of the media as for samples. Positive control plates were prepared by spreading 100 µl of serial tenfold-dilutions of suspensions of a pure culture of two known *Psp* strains (Psph-1 and Psph-4 from Naktuinbouw) on each of the media. All plates were incubated at 28°C for 4 days and then examined for circular, raised globose, glistening and light yellow colonies on MSP medium with a less dense centre. The medium around the colony turns light yellow after 3 days (*Pss* looks identical to *Psp* on this media). On m-KB medium, colonies of *Psp* are circular, flat, blue and green fluorescent after 4 days. MT medium showed colonies of *Psp* as creamy white, flat and circular, 4.5-5 mm in diameter. Many colonies show a pale blue fluorescent pigment under UV light. *Psp* has no clear zone around the colony while *Pss* does.

The numbers of suspect *Psp* and all 'other' colonies present on each plate were recorded. Colonies were considered suspect if they appeared similar to colonies of the reference strains (Psph-1 and Psph-4 from Naktuinbouw).
If present, up to five suspect colonies from each sub-sample were sub-cultured to sectored plates of KB, which were then incubated at 27°C for 2-4 d. Sub-cultured isolates were compared visually with the reference strain and were confirmed by pathogenicity testing (Fenwick and Guthrie, 1969; Van Vuurde and Van den Bovenkamp, 1989).

Data analysis
General introduction
For each combination of laboratory, medium, seed lot, sub-sample and dilution the number of suspect (*Psp*) and 'other' colonies were recorded.
For each sample the best 'countable' dilution plates (i.e. with the number of colonies between 1 and 150) were tabulated. The results of the pathogenicity test were used to calculate the number of confirmed positive colonies as the number of suspect colonies multiplied by the proportion of pathogenicity positive colonies. For example, if 100 suspect colonies are recorded on the MT medium and five are tested in the pathogenicity test, four positive in the confirmation test are equivalent to 80 (100 * 4/5) confirmed positives.

The number of suspect and the total number of confirmed positives were analysed in two different generalised linear modelling facilities of GenStat (Payne *et al.*, 2003), a binomial model (i.e. data in terms of either a positive or negative result), and a Poisson model (i.e. count data for the number of *Psp* and 'other' colonies detected).

Binomial model
The binomial model was specified as having a binomial error distribution with a complementary log-log link function. The effects of both seed lot and laboratory were tested against the mean deviance of samples within laboratory, under the assumption that the mean deviance ratio by approximation follows an F-distribution. The predictions (based on the model) and standard errors were calculated taking the mean deviance of the samples within laboratory as the dispersion factor. For the binomial data no over-dispersion occurred at the level of the residuals. So the effect of media and interaction with media were tested according

to the model assumption that the deviance (of these effects) follows a Chi-squared distribution. The standard errors are based on the binomial distribution with a dispersion factor of one.

<u>Poisson model</u>
The model for the count data was specified as having a Poisson error distribution with a log-link function and the dilution was accounted for by an offset term (the natural log of the dilution). The effects of both seed lot and laboratory were tested against the mean deviance of the samples within laboratories. The effect of medium was tested against the lot x laboratory x medium term in the model. The predictions (based on the model) and corresponding standard errors were calculated. The standard errors are based on the dispersion factor that was set to the mean deviance of the sample within laboratory or the lot x laboratory x medium respectively.

The authors are aware that the analysis for positive colony counts cannot strictly be considered Poisson because the data is a multiple of a binomial result (number of confirmed colonies of those that were tested (5) by the (Poisson) number of colonies found on the plate) however it is felt that the conclusions from the analysis are valid.

<u>Repeatability and reproducibility</u>
The repeatability (within laboratory variability) is equivalent to the mean deviance of the samples within laboratories (LSF) value. The reproducibility dispersion (between laboratory variability) is based on the between laboratory dispersion plus the within laboratory dispersion. In practice this is equivalent to the deviances of the laboratory, LSF, lot x laboratory, in total, divided by the degrees of freedom of all three.

Results

All laboratories were able to detect *Psp* on the infected seed lots, P14209, P14210 and Pmix. Prior tests on the 'healthy' seed lot (P14207) tested negative for *Psp.* However, one laboratory detected and confirmed colonies of *Psp* in two sub-samples during the comparative test.

The numbers of confirmed positive sub-samples were counted. The results indicate that the selective medium MSP exhibited the highest number of confirmed positive colonies of *Psp* followed by MT and m-KB. The highest number of confirmed positive sub-samples were found in the combination of MSP and MT media (Table 1).

The two positive isolates included in the test were recovered on all media in five laboratories. However, some laboratories had very low recovery of isolate Psph-4 and used their 'in-house' isolate in addition. Two laboratories decided to use their 'in- house' isolate which was recovered on all media.

Binomial model
A summary of the statistical effects of the analysis performed on the binomial data, presence and absence, are presented in Table 2. A significant media effect was found together with a lot effect for suspect and confirmed colonies as expected. In addition, the laboratories exhibited significant differences for the number of suspect colonies. The interaction between

lots and media was significant for the confirmed colonies while the laboratory media interaction was significant for suspect and confirmed colonies. On the other hand, the interaction between lots and laboratories was not significant.

The proportions of suspect and confirmed colonies for each medium are summarised in Figure 1. The most suspects were found on MSP and m-KB followed by MT while the highest proportion of confirmed colonies was scored on MSP followed by MT.

In Figure 2 the proportions of suspect colonies for each laboratory and medium are presented. Laboratory 1, 4, 5, 6 and 7 recorded similar results for the media MSP and MT. Laboratories 3, 4, 6 and 7 scored high counts for m-KB medium which indicated that this medium was less selective. An exception was laboratory 2 who counted a high score of suspects, which were confirmed positive, on MSP. All laboratories, except laboratory 1, found a significant difference, either higher or lower, for m-KB medium compared to the two other media.

The proportions of suspect and confirmed colonies for each seed lot are summarised in Figure 3. These results confirm the starting point with different levels of the lots as detected in the pre-tests. P14207 was negative in most cases while P14210 was heavily infected. P14209 and Pmix were both lightly to mildly infected but Pmix contained more non-Psp colonies than P14209.

In Table 3 the reproducibility dispersion (between laboratory variability) and the repeatability dispersion (within laboratory variability) for the confirmed positive colonies based on the binomial data are presented. Medium MT gave the best reproducibility dispersion and repeatability dispersion for detecting the presence or absence of *Psp*. Looking at the different combinations of media the best reproducibility is scored for MSP and MT and the best repeatability for m-KB and MT.

Poisson model

Table 4 contains the statistical effects for the different factors tested in this comparative test. For both, suspected and confirmed positive colonies, a significant effect was detected for the different lots, media and laboratories. Further analysis showed the interactions between lots and media and laboratories and media were also significant.

In Figure 4, the natural logarithm of the counts for laboratories on each medium is shown. Laboratory 1, 2, 3, 4, 6 and 7 scored fewer colonies on MT in comparison to MSP. More suspect colonies were recorded on m-KB medium compared to the other media by laboratory 3, 4, 6, and 7 which is in agreement with the results of the binomial data.

In Figure 5, the natural logarithm of the counts of suspect, confirmed and ‘other’ colonies for media is shown. Most suspect and confirmed colonies were recorded on MSP. The number of other colonies was higher for m-KB for which it is known to be less selective.

Discussion

All participants detected *P. savastanoi* pv. *phaseolicola* in the three infected bean seed lots P14209, P14210 and Pmix. One laboratory detected and confirmed the presence of *Psp* in the negative seed lot P14207 in two sub-samples. In the pre-test, a relatively small number of

seeds (5,000) were tested. In the more extensive comparative test, many more seeds (35,000) from the "negative" seed lot were tested. It is possible that a low level of infection existed in the lot and that the number of contaminated seeds is lower than 1 out of 5,000 but higher than 1 out of 35,000.

The results of the binomial data (presence / absence) analysis and the Poisson data (counts) analysis both show a significant effect for the seed lots, media and for the laboratory x media interaction of suspect and confirmed positive colonies. The effect of laboratory was significant in both data analyses for suspect colonies but was only significant in the Poisson analysis for confirmed positive colonies. An explanation could be that not all laboratories are experienced with all three types of media tested in this comparative test. When laboratories are unfamiliar with a medium it is not unusual for them to have difficulties in identifying how the organism grows on the new medium.

A significant lot x media effect was found when analysing the binomial data of the confirmed positive colonies but not the suspect colonies. Whereas for the counts the effect of both, suspects and confirmed positives were significant. This lot x media interaction is not unexpected as the micro flora associated with bean seed will vary from one lot to another and the different media will control this micro flora in a different way.
The interaction between lot and laboratories was not significant which means that all laboratories had a similar infection level for each lot. In addition, the scored infection levels were in agreement with the results obtained at the pre-tests of the seed lots.
The least number of false positive suspect colonies was scored on MT. This implies that suspect colonies were easier recognised on MT. For all seed lots the number of 'other' colonies were lowest on MSP.

The dispersions based on the binomial analysis showed that MT performed better in different laboratories and within laboratories due to the lower reproducibility dispersion and repeatability dispersion. Reliance on one semi-selective medium could be dangerous because a medium might not be able to detect all isolates of an organism. Bacterial seed contamination levels vary within seed lots along with saprophyte contamination levels which also play an important role on a medium detecting the target organism. The combination of MSP/MT showed best reproducibility of all the combinations but gave a slightly lower repeatability value compared to MT/m-KB. m-KB in combination with MSP however gave the poorest within laboratory dispersion (repeatability).

Overall MSP has shown benefits in its selectivity and produced more confirmed positive *Psp colonies* than MT or m-KB. MT has the ability to discriminate *Psp* from *Pss* and for routine bean testing it is also used to detect *Xanthomonas axonopodis* pv *phaseol*i (Sheppard *et al*, 2007). Statistically the combination of MSP and MT is better in terms of reproducibility of results between laboratories and has a reasonable repeatability compared to the other combinations.

Media preparation
To prevent introducing variability in the comparative test due to the ingredients of the test media, one laboratory prepared and distributed the individual ingredients. In particular the source of milk powder is critical for MT medium. The quality of milk powder is vital to develop the hydrolysis of starch in MT medium. Two milk sources that work well are Oxoid and Sigma brands.

Cycloheximide was widely used to control fungal contamination in semi-selective media for the detection of bacteria. However in recent years the availability of cycloheximide has been in question while it is expensive and very toxic. Nystatin was identified by seed health testing laboratories as a suitable alternative for controlling fungal contamination (Asma, 2005).

With regard to the pathogenicity test, some laboratories reported that plants became etiolated and the cotyledons dropped off too quickly. Reducing the germination temperature from 25°C (Van Vuurde and Van den Bovenkamp, 1987) to a temperature of 18-20°C has proved successful in some laboratories (C. Kurowski, pers. comm.)

Conclusions and Recommendations

All laboratories were able to detect *P. savastanoi* pv. *phaseolicola* from the contaminated seed lots and all three media used within this comparative test were suitable for the detection of *Psp* on bean seed.

The highest number of positive sub-samples and the lowest number of false-positive suspect colonies were recorded on MSP. Overall when using a combination of media laboratories detected the highest number of positive sub-samples on a combination of MSP and MT.
With regard to the analysis of repeatability and reproducibility, dispersion values of the binomial data show that MT performed the best for detecting the presence of the bacteria looking at both, repeatability and reproducibility. In addition MSP/MT gave the best reproducibility of the combined media. Based on this and the ability of MT to identify more than one species it It is therefore recommended that the two complementary semi-selective media MSP and MT are used for routine testing of *Psp*.

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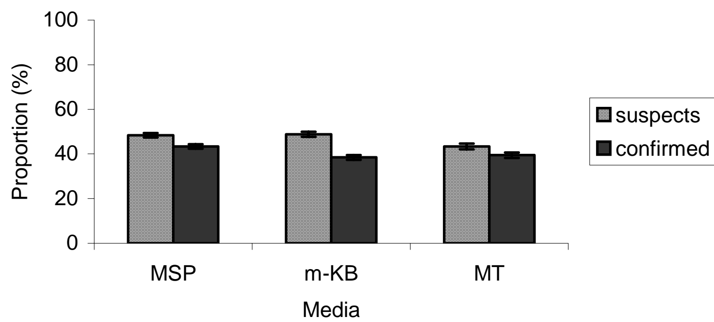

Figure 1 Proportions of suspect and confirmed *P. savastanoi* pv. *phaseolicola* colonies detected on each medium for all laboratories and seed lots[1].

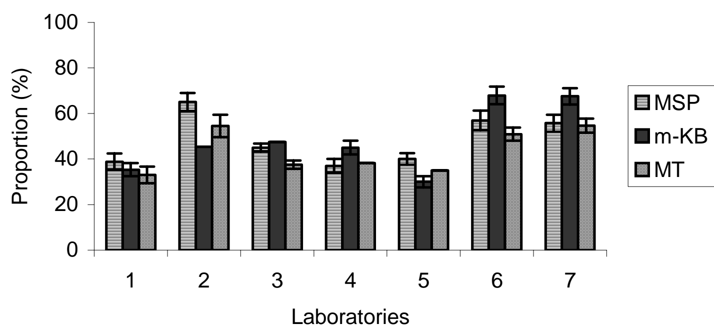

Figure 2 Proportions of suspect *P. savastanoi* pv. *phaseolicola* colonies detected in bean seed extract for each laboratory and medium[1].

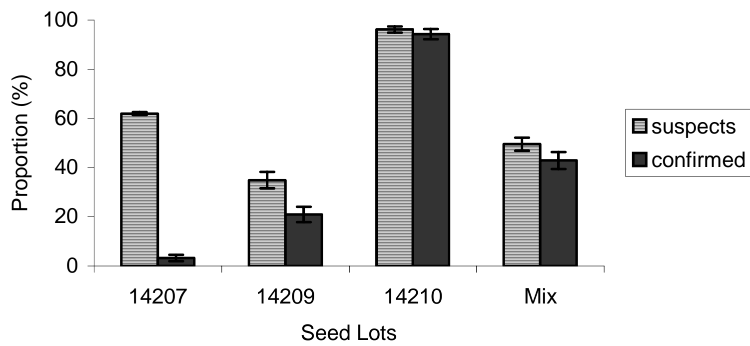

Figure 3 Proportions of suspect and confirmed *P. savastanoi* pv. *phaseolicola* colonies detected in bean seed extract for each seed lot[1].

[1] Note error bars are equivalent to standard errors predicted.

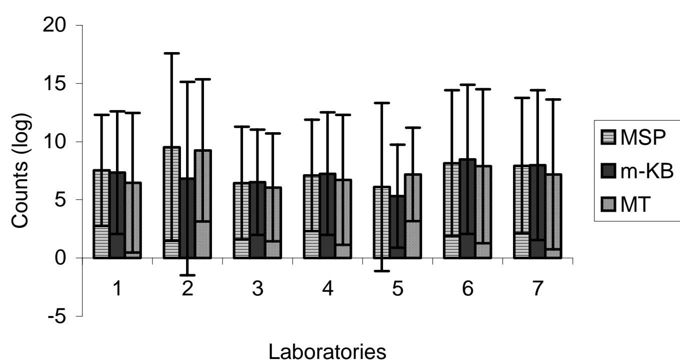

Figure 4 Natural logarithm of the counts (CFU/ml) of suspect *P. savastanoi* pv. *phaseolicola* colonies detected in bean seed extract for each laboratory and medium[2].

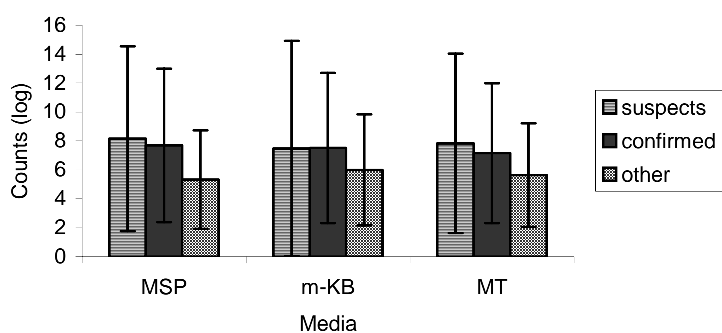

Figure 5 Natural logarithm of the counts (CFU/ml) of suspect and confirmed *P. savastanoi* pv. *phaseolicola* colonies and other colonies detected in bean seed extract for each medium[2].

[2] Note error bars are equivalent to standard errors log transformed

Table 1: Total number of confirmed sub-samples for each medium and media combination.

Media	Number of confirmed positive sub-samples
MSP	63
MT	58
m-KB	56
MSP/MT	72
MSP/m-KB	69
MT/m-KB	68

Table 2: Determination of statistical significant differences for lot-, laboratory- and media-effects and their interaction for binomial (presence / absence) results (P<0.05).
Effect of lot and lab is tested against the mean deviance of samples within laboratories (LSF) under the assumption that the mean deviance ratio by approximation follows an F-distribution. For the binomial data no over-dispersion occurred so the effect of media and interaction with media was tested with the deviance (of these effects) which follows a Chi-squared distribution.

Factor	Df	Binomial Suspect colonies Deviance Ratio	Binomial Confirmed colonies Deviance Ratio
Lot effect	3/112	49.93*	57.64*
Laboratory effect	6/112	2.96*	1.22
Lot.Laboratory effect	18/112	1.57	1.50

* statistical significant differences compared to the F-value

Factor	Df	Binomial Suspects Deviance	Binomial Confirmed Deviance
Media effect	2	8.08*	9.37*
Lot.Media	6	12.51	22.25*
Laboratory.Media	12	46.55*	21.91*
Lot.Laboratory.Media	36	23.72	33.45

* statistical significant differences compared to the Chi-value

Table 3: Reproducibility dispersion and repeatability dispersion for each medium (based on the presence / absence data) of the confirmed positive *P. savastanoi* pv. *phaseolicola* colonies detected in bean seed extract for each medium and all laboratories and seed lots.

Medium	Reproducibility dispersion	Repeatability dispersion
MSP	1.42	1.48
MT	1.39	1.35
m-KB	1.59	1.49
MSP/MT	1.45	1.44
MSP/m-KB	1.46	1.52
MT/m-KB	1.53	1.42

Table 4: Determination of statistical significant differences for lot-, laboratory- and media-effects and their interaction for the counts (P<0.05). Effect of lot and lab is tested against the mean deviance of samples within laboratories (LSF) under the assumption that the mean deviance ratio by approximation follows a F-distribution. Media and interaction with media is tested against the mean deviance of lot.laboratory.media for the count-data.

Factor	Df	Counts Suspects Deviance Ratio	Counts Confirmed Deviance Ratio
Lot effect	3/112	59.16*	65.40*
Laboratory effect	6/112	3.81*	3.94*
Lot.Laboratory effect	18/112	0.70	0.83
Media effect	2/36	22.03*	31.28*
Lot.Media	6/36	2.49*	6.63*
Laboratory.Media	12/36	7.87*	10.75*

* statistical significant differences compared to the F-value

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Proposal for a new method for the detection of Pea Seed-borne Mosaic Virus and Pea Early-Browning Virus in *Pisum sativum* L. seed using ELISA

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Summary

There is currently no internationally accepted protocol for the detection of PSbMV and PEBV in pea seed. An international comparative test was organised to evaluate the use of DAS-ELISA for the detection of both PSbMV and PEBV in seed extracts. In this comparative test pea seed flour was used as reference material. Each of seven laboratories received 100 samples of pea flour, a description of the protocol, critical ELISA compounds such as ELISA microtiter plates, antisera and purified PSbMV and PEBV. Analysis of the data revealed that all laboratories were able to detect PSbMV and PEBV in the medium and heavily infested samples. Most laboratories were able to detect PSbMV and PEBV with the prescribed DAS-ELISA protocol at both high and low A405 values. Low virus titres were used to discriminate whether the DAS-ELISA protocol was robust in all laboratories. Some laboratories that found high background values in ELISA readings had difficulty in detecting virus in samples with low virus titers. The intensity of washing between the different ELISA steps is known to influence the amount of background in the test. Describing washing procedures in more detail may help to resolve this problem. It is recommended that PSbMV and PEBV are determined using the same seed extract taken from a sub-sample of 100 seeds ground to a fine flour and mixed with extraction buffer followed by DAS-ELISA. PSbMV and PEBV tested individually using a separate sub-sample from the seed extract and a separate microtitre plate. Testing 2000 seeds (20 x 100 seed sub-samples) per seed lot would give a 95% probability that a 0.15% infestation of either PSbMV or PEBV is detected.

Introduction

Pea seed-borne mosaic virus

Pea seed-borne mosaic virus (PSbMV) is a well-characterized member of the Potyviridae (Hampton and Mink, 1975). It has a wide experimental host range but is of economic importance in three crops, pea (*Pisum sativum* L.), lentil *(Lens culinaris* Medik.) and broad bean (*Vicia faba* L.*)* in descending order of importance (Khetarpal and Maury, 1987), because of the significant loss it causes in crop yield and quality. PSbMV is transmitted mechanically by seed and by aphid vectors either semi-persistently or non-persistently. Good control can be achieved if virus-free seed is planted (Hampton *et al.*, 1993; Shukla *et al.*, 1994). PSbMV has a worldwide distribution (EPPO, 2005) and seed treatment is not possible.

Pea early-browning virus

Pea early-browning virus (PEBV) is a member of the tobravirus genus named after the tobacco rattle virus (TRV) (Harrison *et al.*, 1971; Harrison, 1973). PEBV is the cause of the early-browning disease of peas. Natural infection with PEBV has been reported mainly in legume species, including pea (*Pisum sativum* L.), faba bean (*Vicia faba major*, var. equina and var. minor), French bean (*Phaseolus vulgaris* L.), lupin (*Lupinus* spp.), and alfalfa (lucerne) (*Medicago sativa* L.) (Boulton, 1996).

Like the other members of the tobravirus genus, PEBV is transmitted in soil by species of free-living plant parasitic nematodes of the family *Trichodoridae*, in particular by *Trichodorus* spp. and *Paratrichodorus* spp. These nematodes prefer free-draining sandy or light clay soils. PEBV is seed-borne in pea and faba bean. The incidence of PEBV in a crop grown on soil not previously carrying the virus will depend on the amount of the initial seed

infection and on its transmission to the progeny plants, and possibly between plants, if vector nematodes are present (Bos and Van der Want, 1962). The virus is not spread above ground by, for example, foliar contact or insect vectors and a low percentage of seed infection would be unlikely to result in serious crop damage. PEBV is present in Europe and North Africa (EPPO, 2005) and seed treatment is not possible.

Detection methods for PSbMV and PEBV

PSbMV and PEBV are seed transmissible viruses of pea and therefore the detection of these viruses in seeds of pea is an important tool in disease-control strategies. Several serological methods for the detection of PSbMV and PEBV have been described, such as dot-immuno-binding (Lange *et al*., 1989), enzyme-linked immunosorbent assay (ELISA) (Van Vuurde and Maat, 1985; Hamilton and Nichols, 1978; Kumari and Makkouk, 1993) and disperse-dye immuno assay (Van Vuurde and Maat, 1985). The ELISA is widely used for the detection of plant viruses (Clark and Adams, 1977) and although relatively robust there are only a few internationally accepted ELISA protocols used to test viruses in seed.

Using ELISA Van Vuurde and Maat (1985) showed that it was possible to detect one PEBV-infected seed in a sub-sample of 100 seeds. PSbMV-infected seeds were also easily detected in a sub-sample size of 100 (Van Vuurde, Personal Communication). ELISA is also used in several seed testing laboratories to test for the presence of PSbMV and PEBV in flour, produced by grinding sub-samples of 100 seeds, normally 20 sub-samples of 100 seeds are tested. Testing 2,000 seeds in a seed lot gives a 95% probability of detecting an infection level of 0.15 %. Over 20 years using ELISA and a working sample size of 2000 seeds seed companies involved in this comparative test have seen no PSbMV- and/or PEBV-related complaints, either nationally or internationally. Based on this information a working sample size of 2000 seeds is used in the comparative test.

For many seed-borne pathogens it has been demonstrated that disease transmission from an infected seed to the seedling is dependant on many variables including climatic conditions, varietal resistance and not least the location of the pathogen within the seed. In general, the localisation of a virus in the embryo leads to higher levels of disease transmission than when the virus is in the seed testas. Maury *et al*. (1987) showed that by removing the testas from the seeds and testing only the embryos a more accurate prediction of seed transmission could be obtained but this is not a practical approach for routine seed testing. Studies have shown that PSbMV can be present in the testas and not in the embryo (Maury *et al*., 1987, Wang and Maul, 1992, Wang and Maul, 1994). Even when the virus is present a comparison of pea varieties revealed that the seed transmission of PSbMV varied from 0-74% (Wang et al., 1993).

The use of reference materials in comparative testing

A limited number of comparative tests for the detection of seed-borne pathogens have been organized in the past. One important bottleneck has been the lack of appropriately infected samples and/or stable reference material. Using naturally contaminated seed lots with a low incidence of infected seeds and an uneven amount of virus per infected seed makes it difficult to provide individual laboratories with identical samples. The laboratory at Naktuinbouw, the Netherlands Inspection Service for Horticulture, has tackled this problem by using fine flour ground from seeds with variable loads of viral contamination and for more than 5 years flour of healthy and virus-contaminated seed lots have been used as negative and positive controls.

Researchers at Naktuinbouw found a relatively even distribution of virus in finely ground flour. Also, the amount of ELISA-detectable virus was relatively stable in time irrespective of the storage temperature. These findings suggested that flour could be a reliable reference material and had the advantage that laboratories were able to test identical samples. The predictable distribution of the virus in flour and its stability has been confirmed in several national comparative tests for the detection of squash mosaic virus (SqMV), PSbMV and PEBV in the framework of Naktuinbouw's activities in accrediting laboratories (H. Koenraadt, personal communication).

The use of reference antisera in comparative testing

Antisera against viruses are available from numerous suppliers but they are known to vary in their quality. For detecting viruses in high-quality seed lots the specificity and affinity/avidity of antibodies are critical since low titers of virus have to be detected. In addition to antiserum variation, inter-laboratory variation and the lack of reference material often made it difficult to analyse comparative test results hindering the development of internationally accepted standard methods.

To overcome the problem of antisera of differing quality confounding the results of the comparative test, a single antiserum of PSbMV and PEBV have been used.

The comparative test for detecting PSbMV and PEBV

As already indicated the use of reference material and antiserum are critical factors for the success of a comparative test. The quality of microtiter plates can also lead to confounding results making the comparative test difficult to evaluate. Therefore, microtiter plates were also sent to participating laboratories along with samples of flour and antiserum.

The aim of the comparative test was to evaluate whether several laboratories were able to detect PSbMV and PEBV in contaminated samples when following a prescribed DAS-ELISA protocol. The final objective was to obtain a reliable and internationally accepted ELISA protocol for the detection of PSbMV and PEBV in seed lots. This comparative test is based on testing whole seeds with an infestation of 0.15% .

Material and Methods

Seeds

Pea seeds were obtained from the naturally contaminated seed lot collection of Naktuinbouw. The seed lots were stored at 4ºC and low humidity. The collection numbers were: ZZB94 (PSbMV contaminated), ZZB95 (PSbMV contaminated), ZZB96 (healthy control), ZZB135 (PEBV contaminated) and sample 02-F-6926 (PSbMV contaminated).

Pre-test and design of comparative test

Several seed lots of pea were tested for the presence of PSbMV and PEBV using ELISA. Different ratios of the healthy and PSbMV/PEBV-contaminated seed lots (1 PSbMV or PEBV-contaminated seed per 100 seeds per sample) were ground in a grinder (Retsch-Grindomix GM200) at 10,000 rpm for 20 seconds to obtain a fine flour. The flour was transferred to plastic bags. The grinder was cleaned thoroughly between each sample using a fine brush and a vacuum cleaner to prevent cross-contamination. A total of 100 samples were

selected by the organiser as recommended by the ISTA Statistics Committee with the objective of including mostly healthy samples and samples that gave low to medium A405 values in ELISA. To obtain samples with low A405 values some contaminated flour samples were diluted with healthy flour. Only a few strongly positive samples were selected since they were considered as being less discriminative for assessing the ability of a laboratory to run the ELISA protocol.

0.45-0.55 g of flour from each ground sample was transferred to a labelled tube. Ten labelled tubes were prepared per sample to obtain enough material for all participating laboratories plus an additional pre-test. In the pre-test one set of samples was analysed at Naktuinbouw using the method described prior to the samples being sent to the laboratories. In the interim period the samples were stored at 4ºC in air-sealed plastic bags. After the results were confirmed in the pre-test the samples were sent by express mail to seven laboratories: Agdia, Elkhart (IN) USA; ISDA – Plant Industry Lab, Boise (ID) USA; Washington State University, Prosser (WA) USA; SNES-GEVES, Beaucouze, France; Naktuinbouw, Roelofarendsveen, the Netherlands; Nunhems NL, Haelen, the Netherlands; and Seminis Seeds, Enkhuizen, the Netherlands. Purified PEBV and PSbMV obtained from Plant Research International (PRI) based in Wageningen in the Netherlands was included in each package as positive controls along with the antisera and microtiter plates.

Test method

Antisera (coating and conjugates) for detecting PSbMV and PEBV were purchased from PRI. The PEBV and PSbMV polyclonal antisera were raised in rabbits against purified virus of isolates E-116 and E-210, respectively. Although microtiter plates were supplied by the test organiser, to further minimise microtiter plate effects, extracts from each sample were tested in two different plates for each virus.

ELISA buffers

Buffers were made up as follows:

Coating buffer: 1.59 g Na_2CO_3 plus 2.93 g of $NaHCO_3$ were dissolved in 1 litre of de-ionised/distilled water and adjusted to pH 9.6 if necessary. Extraction Buffer (0.05 M): 8.0 g NaCl, 1.0 g KH_2PO_4, 14.5 g $Na_2HPO_4.12H_2O$ 20.0 g PVP (ELISA grade mol. wt. 10,000) were dissolved in 998.5 ml of de-ionised/distilled water. 1.5 ml Tween 20 was added and the pH checked and adjusted to pH 7.4 if necessary. Conjugate Buffer (0.05 M): 8.0 g NaCl, 1.0 g KH_2PO_4, 14.5 g $Na_2HPO_4.12H_2O$, 5.0 g BSA (ELISA grade, e.g. BSA fraction 5) was dissolved in 998.5 ml of de-ionised/distilled water. 1.5 ml Tween 20 was added and the pH checked and adjusted to pH 7.4 if necessary. Substrate Buffer: 97 ml diethanolamine plus 15 ml HCl (32%) was mixed with 888 ml of de-ionised/distilled water. The pH was adjusted to pH 9.6 if necessary.

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

Coating of ELISA plates

60 µl of each of PSbMV and PEBV coating serum was added to 60 ml coating buffer (1:1000 dilution). Plates were coated with either 180 µl PSbMV coating buffer or PEBV coating buffer per well. Plates were covered with lid or wrap with plastic foil to minimise evaporation and incubated overnight at 4°C.

Extraction of virus from flour and incubation of extracts.
5 ml of extraction buffer was added to each tube containing 0.5 g flour and vortexed for 15 seconds then allowed to settle for 5 minutes. The coating buffer was removed from plates and immediately rinsed using washing buffer to remove residues. Plates were rinsed three times or a suitable washing device was used. Immediately after rinsing 180 µl of each seed extract was added per well. Purified virus was diluted in extraction buffer to give five positive controls at various dilutions (100 fold to 62500 fold). Plates were then covered and incubated overnight at 4°C.

Incubation of conjugate
60 µl of each of PSbMV and PEBV antiserum was added to 60 ml conjugate buffer (1:1000dilution). The flour extract solution was removed from plates and immediately rinsed using washing buffer to remove residues of seed extracts. Plates were rinsed three times or a suitable washing device was used. Immediately after rinsing 180 µl of either PSbMV or PEBV diluted conjugate was added per well. Plates were then covered and incubated at 37°C for three hours.

Addition of substrate to ELISA plates
Substrate solution was prepared by adding 10 mg para-nitrophenol phosphate to 20 ml of substrate buffer. The diluted conjugate was removed from plates and immediately rinsed using washing buffer to remove residues. Plates were rinsed three times or a suitable washing device was used. Immediately after rinsing 180 µl of substrate solution was added per well and plates incubated for two hours at 20°C.

Interpretation of the data
Based on the origin of the samples and pre-test results from Naktuinbouw, samples were expected to give positive or negative results. An overview of the composition of each sample is provided in Table 1.

Laboratories were asked to submit the measured extinction values (A405) for each sample (x100) and plate (x 4) . In addition, they were asked to state whether a sample was positive of negative for each virus. As a guideline a negative-positive threshold of 2.5 times the extinction value (or A405) of healthy samples was recommended. An evaluation of the threshold was not an objective of this comparative test.

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

<u>Repeatability and reproducibility</u>
The repeatability and the reproducibility are computed on the data scale for a nominal proportion π equal to 0.05 using the formulae:

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

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

where:

- J is the number of laboratories
- n_{ij} is the denominator for the false-positive or false-negative proportions in laboratory j
- $\hat{\sigma}^2_{Laboratory}$ is an estimate of the laboratory variance after fitting a Generalized Linear Mixed Model (logistic link)(Laffont, 2006).

Results

The pre-test data obtained at Naktuinbouw agreed with the expected results from the prepared samples with the exception of sample 49 where no virus was found.

PSbMV
All laboratories were able to detect PSbMV in samples with large amounts of detectable PSbMV (samples 7, 8, 21, 31 and 78), Figure 1b. Figures 1a. 1b and 1c show clearly the low positive samples with a limited amount of detectable virus (e.g. samples 2, 23, 26, 30 and 60). These samples were at and even below the limit of detection for one or several laboratories. The limit of detection for PSbMV varied among laboratories. For example, Laboratories 1 and 6 had significantly higher A405 values than Laboratories 2 and 5.

The predicted percentage of false-positive and false-negative samples are shown in Tables 2 and 3 for PSbMV. For the proportion of false-positive samples of PSbMV a significantly higher amount was scored by Laboratory 2 (Figure 3, Table 2). Laboratory 4, and Laboratory 7 scored a significantly higher percentage of false negatives (Figure 4, Table 3).

PEBV
PEBV could also be detected in most samples by the participating laboratories. The amount of detectable virus varied widely among the samples. In samples 27, 53, 57 and 90 the amount of detectable virus was small while there were large amounts of PEBV present in samples 18, 20, 45, 72 and 99 (Figure 2a, 2b and 2c). Like PSbMV the sensitivity varied among laboratories. Laboratories 6 and 7 found higher A405 values than Laboratories 2 and 5 (Figure 2b).

For PEBV the number of false-positive results was small; Laboratories 2 and 5 had one false-positive result each (Figure 5). A statistical analysis revealed no significant differences for false-positive PEBV samples (Table 4). Laboratories 6 and 7 had two false-negative results each while Laboratory 4 found many (14) false-negative samples. The statistical analysis revealed that laboratory 4 was significantly different (Table 5).

Diluting the purified viruses did not give the expected A405 values (data not presented) indicating that the purified viruses were not stable in time.

The reproducibility dispersion (between laboratory variability plus within laboratory variability) and the repeatability dispersion (within laboratory variability) for both PEBV and PSbMV based on the binomial data are presented in Table 6. PEBV gave a better reproducibility and repeatability than PSbMV.

Discussion

PSbMV was present in 20 samples out of 100. Most laboratories were able to detect the virus in samples with high virus loads. Laboratories 4 and 7 were unable to detect the virus in samples 2, 10, 26, 30 and 60 with a low virus load (Figure 1). This statistically significant lower sensitivity (Table 3, Figure 4) appeared to be caused by relatively high backgrounds found in all samples in these laboratories. None of the laboratories was able to detect PSbMV in sample 49. The origin of this sample was a PSbMV-contaminated seed lot, either the virus

was not present in the sample in the pre-test and the comparative test or was below the detection limit of even laboratories that were able to detect very low virus loads. Data from this sample could be arbitrarily scored as a real negative or a false negative. Out of 700 test results 16 were false positive for PSbMV in this comparative test. (Table 2 and Figure 3).

PEBV was present in 19 samples out of 100. Most laboratories were able to detect the virus in samples with high virus loads. Only Laboratory 4 was unable to detect the virus in samples 27, 53, 57, 71 and 90 with low virus loads (Figure 2). The reason for the statistically significant high number of false negatives in this laboratory (Table 5, Figure 6) was the relatively high backgrounds it found in all samples. Two false-positive results were found for PEBV in this comparative test. There were no statistically significant differences between the laboratories (Table 4).

With regard to high backgrounds found in the PSbMV ELISA, more rigorous washing might solve this problem. The arbitrarily chosen positive-negative threshold in this comparative test was a function of the background and therefore laboratories with high backgrounds obtained high absolute thresholds, which masked the presence of PEBV and/or PSbMV.

Earlier work has already shown that PSbMV and PEBV are homogenously distributed in flour of finely ground seeds and stable over time(Koenraadt, not published). As the composition of each sample was known in time, it is therefore easy to determine which of the reported results are correct (true negative or positive) or incorrect (false positive or false negative). However a disadvantage of using flour as the starting point in the comparative test is that the grinding step, a potential critical factor in seed testing, is not evaluated for the participating laboratories.

The use of a purified virus solution as a positive control was not a success. Purified PSbMV was especially not suitable as reference material because of the apparently low stability of the antigen. Most laboratories were not able to detect PSbMV after diluting the purified virus. Apparently the antigens are more stable in dry flour than in the purified virus solution. In this comparative test the organiser provided the antisera obtained from PRI.

It should be noted that one of the participating laboratories Agdia used both the PRI and their own antisera in the comparative test and obtained similar results (data not presented).

Overall the DAS-ELISA combined with grinding of sub-samples has proven to be an effective method for detecting PSbMV and PEBV in pea seed. Measures to determine the repeatability and reproducibility for the type of data presented are few and very much under development. Using false negative and false positives rates from individual laboratories and individual viruses shows smaller repeatability and reproducibility values for PEBV than PSbMV.

An important question is whether this DAS ELISA format is a suitable tool to help minimise the risk of PEBV and PSbMV transmission through seed. In the case of naturally infected seed lots the A405 ELISA readings in general were high, even when there was only one virus-contaminated seed per sub-sample of 100 seeds. Low A405 readings, obtained through dilution of virus-contaminated flour with healthy flour, were used to distinguish between laboratories in their ability to run the ELISA protocol, although high background readings

were a problem for one or two laboratories in general laboratories were able to detect low titres of viruses. Therefore it can be assumed that 100 seeds per sub sample is a safe choice.

Recommendations about sample size may be somewhat arbitrary for a host-pathogen combination depending on the epidemiological information available. By testing 2,000 seeds per sample there is a 95% probability that a seed lot with a contamination level of 0.15% is detected. In general, a high number of seeds (say 30,000 corresponding to a 95% probability of detecting 1 contaminated seed in 10,000 healthy seeds) are tested per seed lot when pathogens represent a high phytosanitary risk, as in the case of *Xanthomonas campestris* pv. *campestris*. A rapid spread of PEBV and PSbMV from contaminated seeds is only possible when suitable vectors of the viruses are present. Phytosanitary risk for both PEBV and PSbMV are rated as moderate (Crop Protection Compendium, 2005) and in the authors opinion the sample size choice of 2,000 seeds is reasonable.

Conclusions and Recommendations

Most laboratories were able to detect PSbMV and PEBV with the prescribed DAS-ELISA protocol at both high and low A405 values. Some laboratories that found high background values in ELISA readings had difficulty in detecting virus in samples with low virus titers. The intensity of washing between the different ELISA steps is known to influence the amount of background in the test. Describing washing procedures in more detail may help to resolve this problem. The use of PEBV and PSbMV contaminated flour is useful for comparative testing for two reasons. Firstly, the extraction of PEBV and PSbMV from flour is more efficient than extraction of virus from whole seeds (van Vuurde, personal communication). Secondly, the use of flour reduced sampling variability in comparative testing where previously, when using whole seeds this was the limiting factor. Although data that proves the extraction efficiency of virus from fine flour has not been published, the fact that the distribution of virus in ground flour is more homogeneous has been corroborated by work at Naktuinbouw under the framework of their national comparative (proficiency) test programme. Testing ground fine flour for virus has reduced sampling variability and has proven significant in uniformity of testing between laboratories in the Netherlands.

The use of a purified virus solution of PSbMV and PEBV as positive controls is limited since their stability, especially that of PSbMV, was not sufficient.

Determining threshold values was not an objective of this comparative test. In this study the threshold used was a function of the background. Reducing the background through additional washing, alternative threshold calculations, or through subtracting the background from all the readings for instance, could be useful in increasing the probability of detecting samples with low virus loads.

It is recommended that PSbMV and PEBV are determined using the same seed extract taken from a sub-sample of 100 seeds ground to a fine flour and mixed with extraction buffer followed by DAS-ELISA. PSbMV and PEBV are tested individually using a separate sub-sample from the seed extract and a separate microtitre plate.

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Table 1 List of PSbMV- and/or PEBV-contaminated samples in the comparative test

Sample designation*	PEBV	PSbMV	PEBV+PSbMV**
2		ZZB94	
4	ZZB135		
5		6926	
7		ZZB95	
8		6926	
10		ZZB95	
16			6926-9 + ZZB135
18	ZZB135		
20	ZZB135		
21		ZZB95	
22	ZZB135		
23		ZZB94	
26		6926	
27	ZZB135		
30		ZZB95	
31		6926	
38	ZZB135		
39		ZZB95	
43			6926-9 + ZZB135
45	ZZB135		
47		6926	
49			ZZB94**
51	ZZB135		
53	ZZB135		
57	ZZB135		
60		ZZB94	
61	ZZB135		
63		6926	
71	ZZB135		
72	ZZB135		
74		6926	
78		6926	
84	ZZB135		
85	ZZB135		
88		ZZB95	
90	ZZB135		
98		ZZB95	
99	ZZB135		

* Only virus-contaminated samples are listed. Other samples consisted from seeds of the healthy seed lot ZZB96.

** Samples were mixtures of flour.

*** Sample 49 originated from a PSbMV contaminated seed lot but none of the laboratories were able to detect the presence of virus in flour of this particular sample. Therefore this sample was regarded as "healthy" for statistical analysis.

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

Laboratory	Proportion	Standard Error	Significant differences
1	0.0	0.0	a
2	0.19	0.04	b
3	0.0	0.0	a
4	0.0	0.0	a
5	0.01	0.01	a
6	0.0	0.0	a
7	0.0	0.0	a

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

Laboratory	Proportion	Standard Error	Significant differences
1	0.15	0.08	a
2	0.0	0.0	a
3	0.06	0.05	a
4	0.65	0.12	b
5	0.0	0.0	a
6	0.25	0.10	a
7	0.95	0.05	c

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

Laboratory	Proportion	Standard Error	Significant differences
1	0.0	0.0	a
2	0.01	0.01	a
3	0.0	0.0	a
4	0.0	0.0	a
5	0.01	0.01	a
6	0.0	0.0	a
7	0.0	0.0	a

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

Laboratory	Proportion	Standard Error	Significant differences
1	0.0	0.0	a
2	0.0	0.0	a
3	0.0	0.0	a
4	0.74	0.10	b
5	0.0	0.0	a
6	0.11	0.07	a
7	0.11	0.07	a

Table 6: Reproducibility dispersion and repeatability dispersion for PEBV and PSbMV (based on the binomial data, $\pi = 0.05$) detected in pea seeds for all laboratories and samples.

Pathogen	Reproducibility dispersion	Repeatability dispersion
PEBV	0.00743	0.00152
PSbMV	0.01114	0.00732

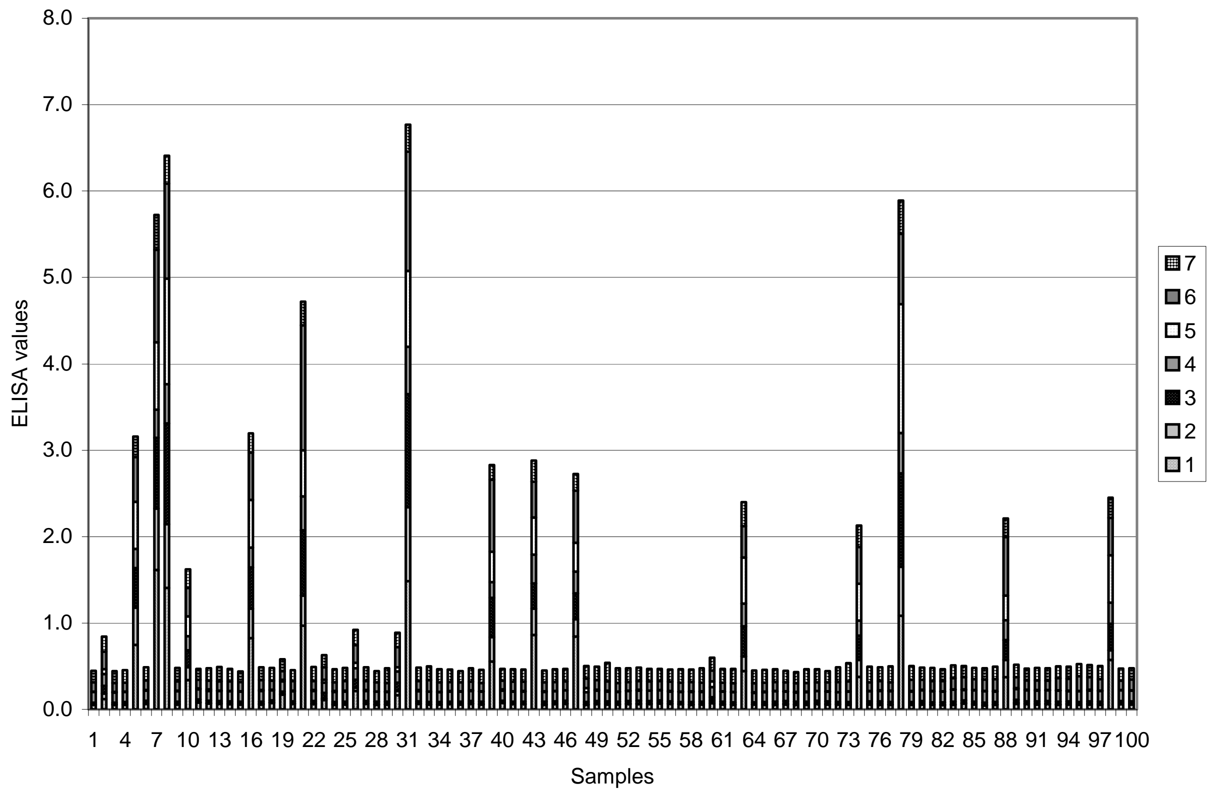

Figure 1a. Cumulative A405 ELISA values per sample of the seven laboratories in the comparative test for the detection of PSbMV.

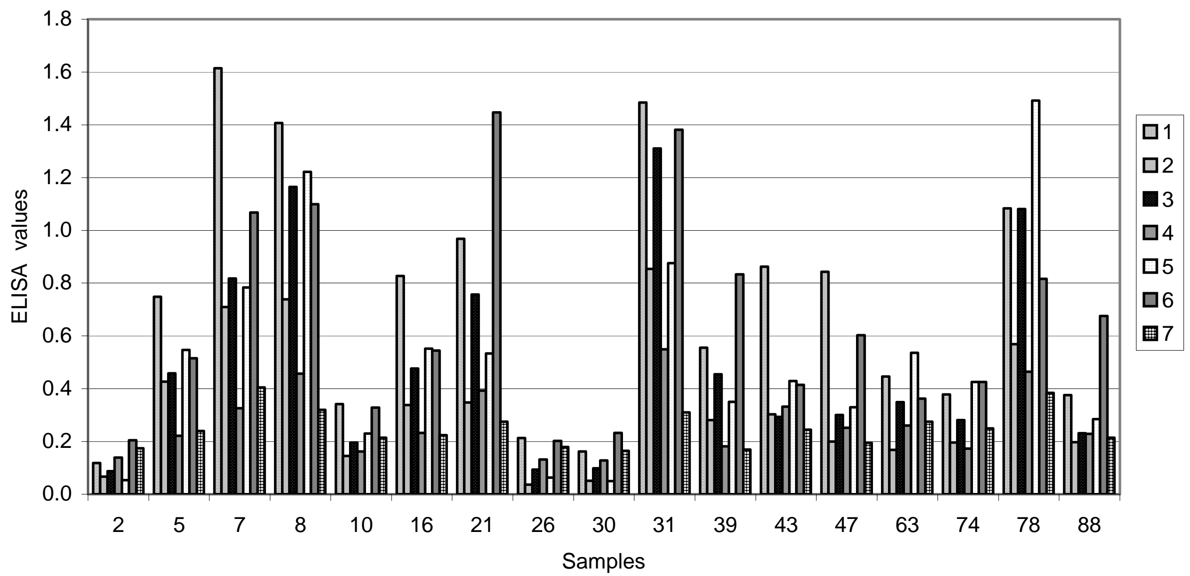

Figure 1b. A405 ELISA values of PSbMV-positive samples for each laboratory.

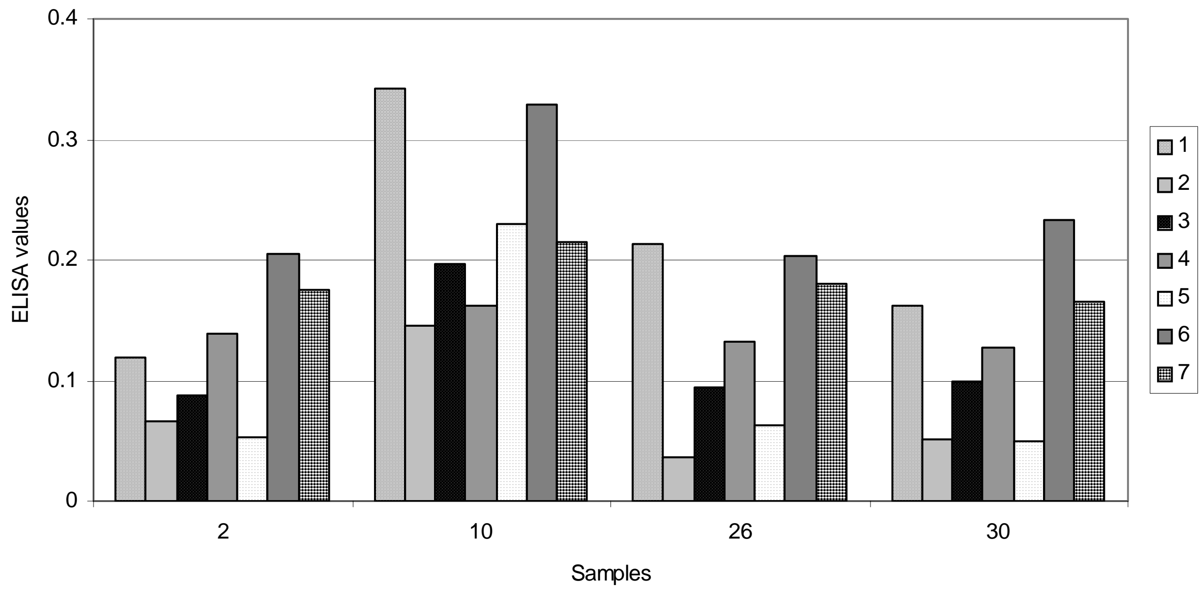

Figure 1c. Detail of PSbMV-positive samples with relatively low cumulative A405 values (<2) for each laboratory.

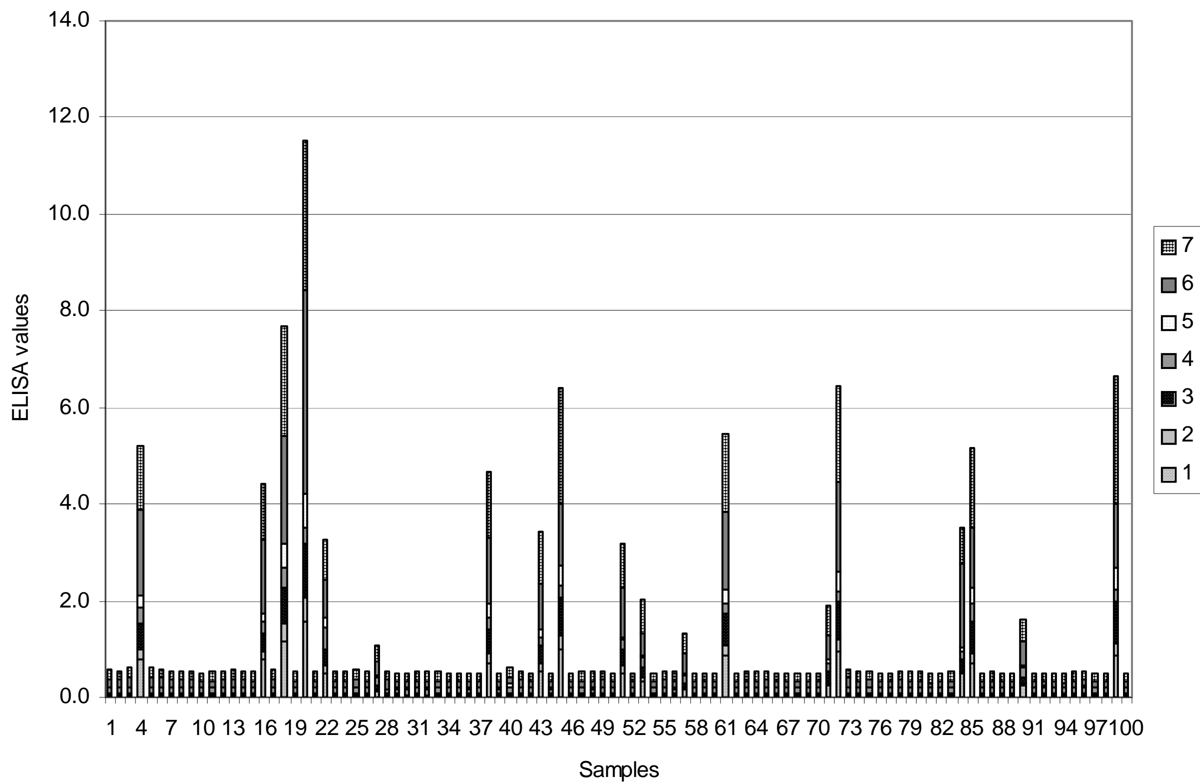

Figure 2a. Cumulative A405 ELISA values per sample of the seven laboratories in the comparative test for the detection of PEBV.

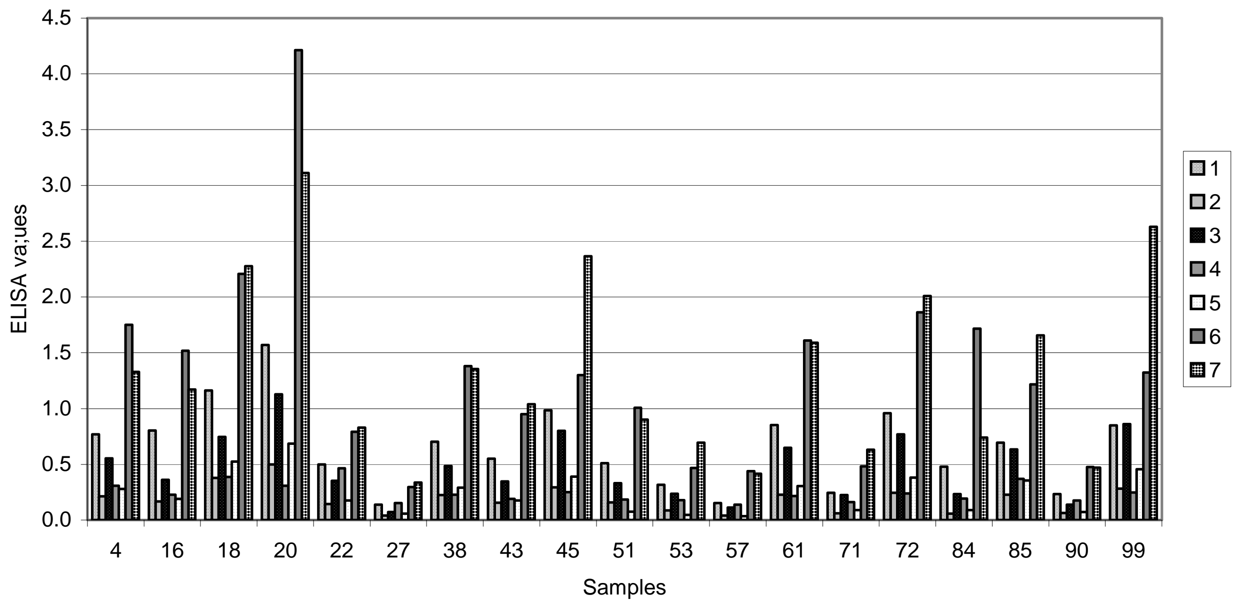

Figure 2b. A405 ELISA values of PEBV-positive samples for each laboratory.

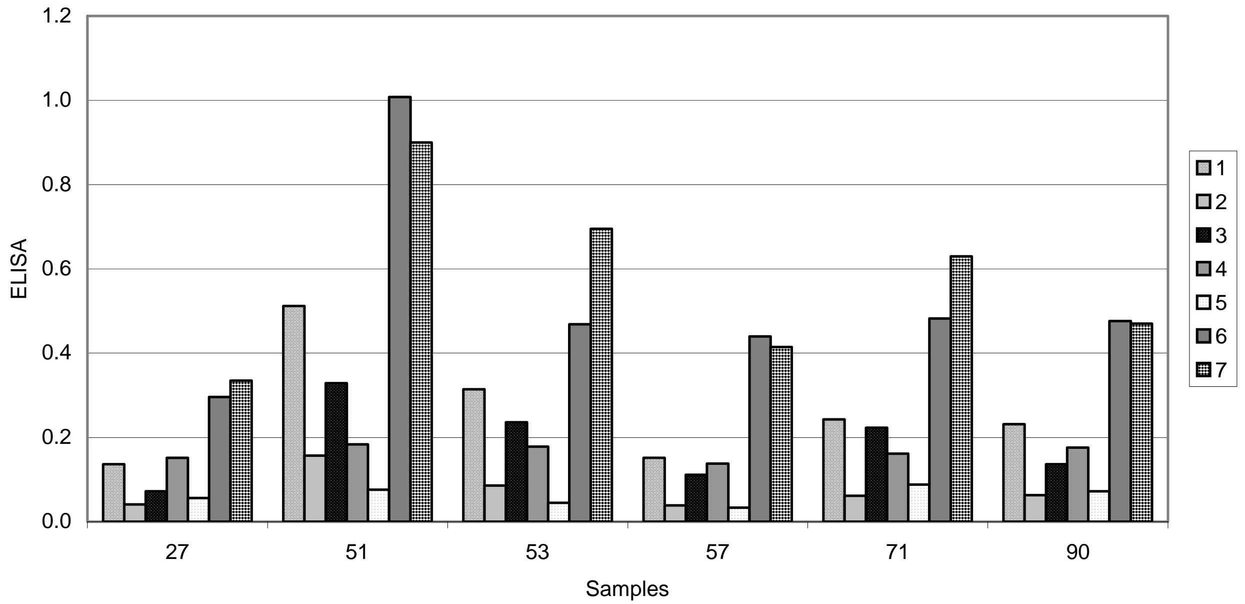

Figure 2c. Detail of PEBV-positive samples with relatively low cumulative A405 values (<2) for each laboratory.

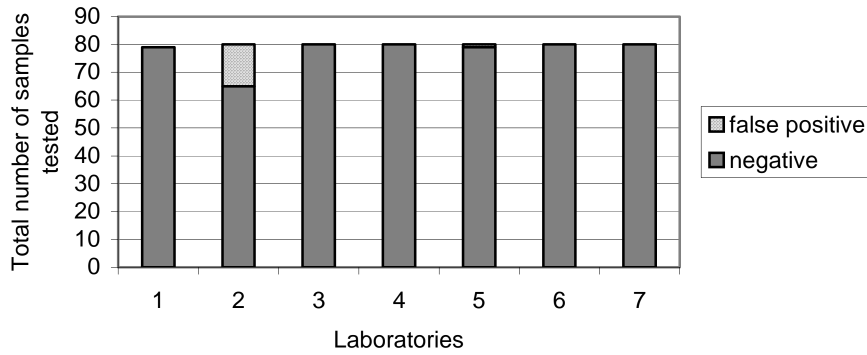

Figure 3. Number of negative samples in relation to the number of false positives scored by each laboratory for PSbMV.

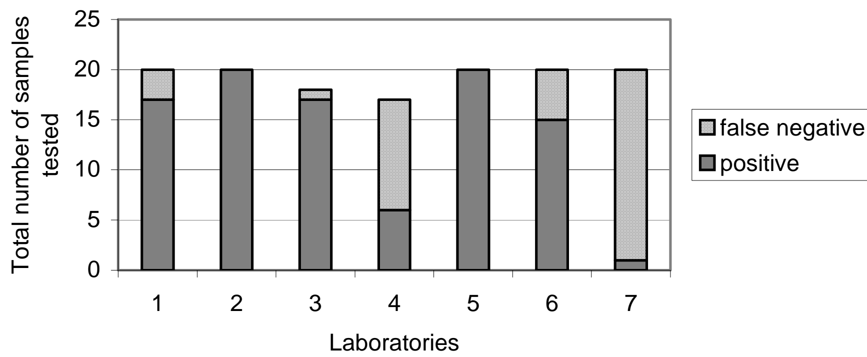

Figure 4. Number of positive samples in relation to the number of false negatives scored by each laboratory for PSbMV. Note that laboratory 3 and 4 did not give a positive-negative score for all the samples.

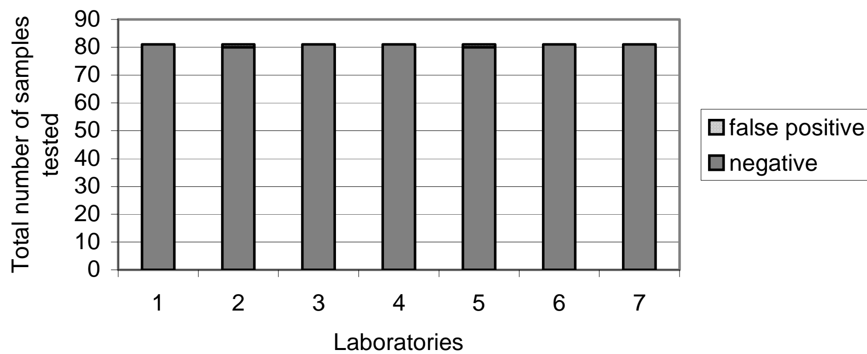

Figure 5. Number of negative samples in relation to the number of false positives scored by each laboratory for PEBV.

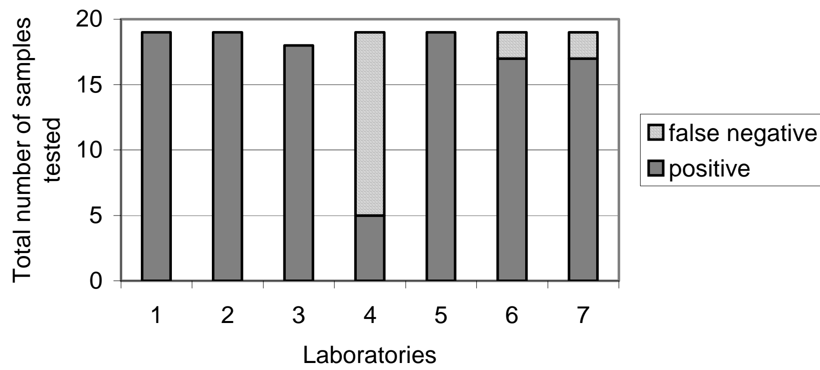

Figure 6. Number of positive samples in relation to the number of false negatives scored by each laboratory for PEBV. Note that laboratory 3 did not give a positive-negative score for all samples.

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Modification to Method 7-013 *Ustilago nuda/Hordeum vulgare*: replacement of lactophenol with lactic acid solution

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Summary

ISTA Method 7-013 for the detection of *Ustilago nuda* in barley has since the mid-eighties used lactophenol as a clearing agent to help identify mycelium of *U. nuda* in the scutellum area of the barley embryo. In recent years a number of laboratories have requested the removal of lactophenol due to the risks involved with using phenol. A study using two laboratories was organised in 2006 to determine whether equivalent results were produced when lactophenol (1 part glycerine: 1 part lactic acid: 1 part phenol) was replaced with a lactic acid solution (1 part glycerine: 1 part lactic acid: 1 part water). Twenty-four sub-samples (3 seed lots x 4 sub-samples x 2 methods) were sent to each of two laboratories. There was no significant effect on the test results when lactophenol was replaced with a lactic acid solution.

Introduction

For many years, the successful detection of *Ustilago nuda* in barley seed has been made using ISTA method 7-013. Lactophenol is used in this method as a clearing agent making *U. nuda* mycelium easily visible in the scutellum area of the embryo. In recent years a number of laboratories have requested the removal of lactophenol due to the risks involved with using phenol. Indeed in some countries the use of phenol in seed health laboratories is not permitted. This need was re-confirmed in the 'Five Year Review of Official Methods Introduced in 2001 to Chapter 7, ISTA International Rules for Seed Testing' (Cockerell and Koenraadt, 2006). In 2004 the ISTA –SHC Ustilago Working Group agreed to organise an experiment to determine whether lactophenol (1 part glycerine: 1 part lactic acid: 1 part phenol) could be replaced with a lactic acid based solution (1 part glycerine: 1 part lactic acid: 1 part water) already being used in some laboratories. A study using two laboratories, with many years experience using the ISTA Method 7-013, and still using lactophenol was organised in 2006 to determine whether equivalent results were produced when a lactic acid solution was used.

Materials and Methods

Seed Sample Preparation
For this experiment it was important to try to use seed samples with greater than 0.5% infection to ensure the chance of a sub-sample giving zero infected embryos was kept to a minimum as zero results are non-informative in this case. To ensure results generated provided sufficient evidence for the comparison of the two reagents three seed lots x four sub-samples x two methods were tested by two laboratories.

Three seed stocks were identified, two winter, and one spring barley. Two of the three seed stocks had infections levels greater than required and were mixed with healthy seed of the same variety to bring the infection level to within a 0.5% - 1.5% range. The third seed stock

required no dilution. Each seed sample was mixed thoroughly by passing through a soil divider six times before being divided into 24 sub-samples of 120g.

Sub-samples were labelled with a seed lot reference A, B or C; a method designation either lactic acid solution or lactophenol; and a number randomly assigned between 1 and 72.

Twenty-four sub-samples (3 seed lots x 4 sub-samples x 2 methods) were sent to each of two laboratories the Official Seed Testing Station for Scotland, Scottish Agricultural Science Agency and the Austrian Agency for Health and Food Safety, Institute for Seed. An extra set of samples were held at SASA should they be required.

Preparation of Reagents
The Lactic acid solution was prepared as per Table 1 and lactophenol was prepared as per Method sheet 7-013 (Anon, 2006).

Table 1. Lactic Acid Solution

Compound	ml/l
Glycerine	333.3
Lactic Acid (90% pure, minimum assay 88% purity)	333.3
Water	333.3

Procedure
At each laboratory the 12 sub-samples marked “lactophenol” were tested using the ISTA Method 7-013 and for the 12 sub-samples marked “lactic acid” the ISTA Method 7-013 was followed but the lactophenol was replaced with the lactic acid solution.

The number of embryos examined and the number of infected embryos found per sub-sample were recorded.

Data analysis
A binomial distribution was assumed in the preparation of the test plan (sub-sample-to-sub-sample variability) and the analysis of the results. A generalised linear model was therefore used to analyse the results. The specific model used was a linear logistic model (binomial distribution with the link function as the logistic function) (McCullagh and Nelder, 1989) and analysed using Genstat for Windows (Eighth Edition). Deviance ratios are based on a dispersion parameter with value 1.

Results

The analysis shows that data is consistent with binomially distributed data with no evidence of overdispersion. The seed lots had clearly different proportions and accounted for most of

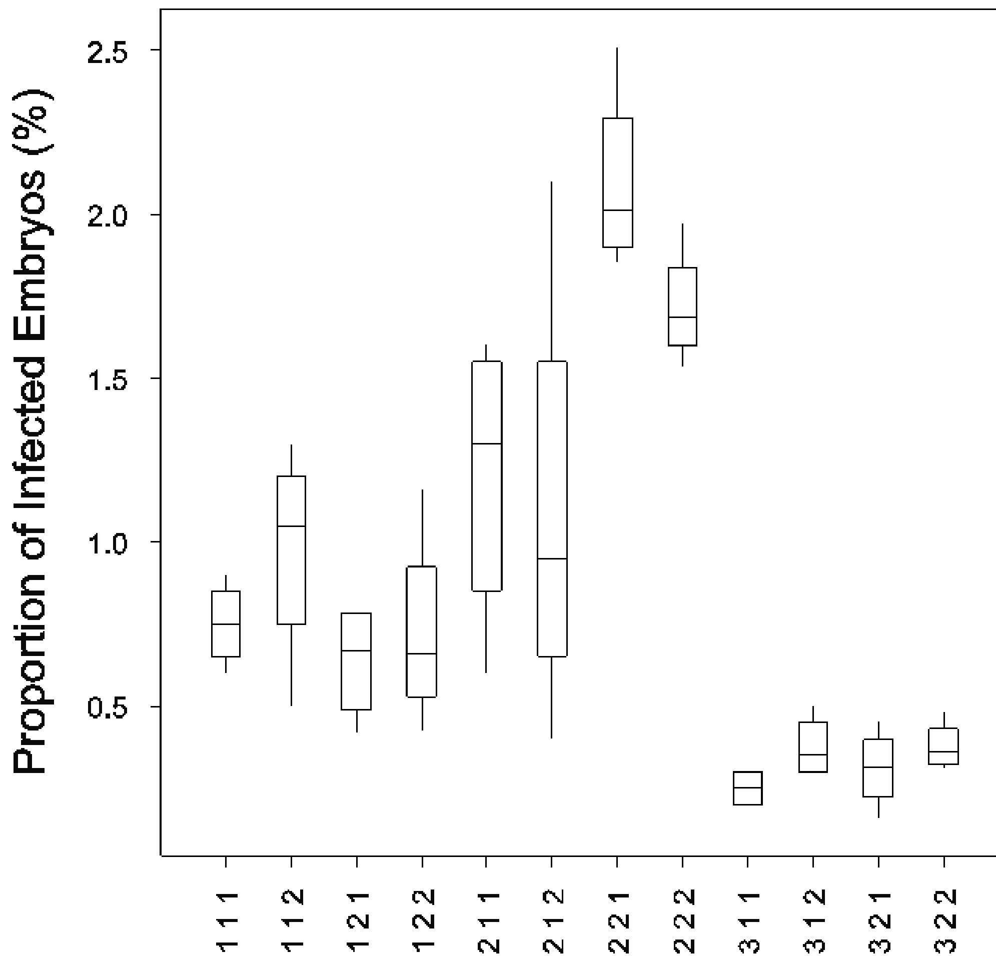

Figure 1 Boxplot showing three-way plot of lot, laboratory and method means. (label can be read as follows e.g. 321 equals Sample 3, Laboratory 2, Method 1(lactophenol)

Table 2. Determination of statistical significant differences for lot-, laboratory- and method-effects and their interaction (P<0.05).

Factor	d.f.	deviance	mean deviance	deviance ratio*	approx chi pr
Sample	2	235.019	117.510	117.51	<.001
Lab	1	7.871	7.871	7.87	0.005
Method	1	0.241	0.241	0.24	0.623
Sample.Lab	2	14.522	7.261	7.26	<.001
Sample.Method	2	5.430	2.715	2.71	0.066
Lab.Method	1	0.670	0.670	0.67	0.413
Sample.Lab.Method	2	0.030	0.015	0.02	0.985
Residual	36	41.098	1.142		
Total	47	304.882	6.487		

**Ratios are based on dispersion parameter with value 1*

the variability, Table 2. Lot 2 (B) showed the most variability, especially between laboratories and within laboratory 1, Figure 1. There was also evidence for difference between the two laboratories and also an interaction with the lot, Table 2. There was no evidence for differences between the methods in mean proportions, neither overall nor within laboratory or lot, Table 2.

Discussion

Sample 2 (B) showed a great deal of variability both within laboratories and between laboratories compared with samples 1(A) and 3(C). Sample 3 (C) showed least variability between laboratories. It is possible that the variability in sample 2 (B) was related to the dilution of the seed lot with healthy seed to provide a target seed infection or an inherent variability in the original stock whereas Sample 3 (C) was not diluted with health seed. There was no significant difference between laboratories for Samples 1 or 3.

Replacement of lactophenol with the lactic acid solution had no significant affect on the results. Laboratory 1 commented that it was difficult to determine when embryos were clear at the boiling stage and Laboratory 2 commented that the embryos were still rather dark and less transparent after boiling than with the lactophenol. However if the embryos were left for at least 1 hour, preferably 2-3 hours the scutellum became very clear and equivalent to the lactophenol method. Using both a longer boiling time and extended waiting time before examination facilitated the ability to observe the Ustilago nuda mycelium in the embryo scutellum.

Conclusion and Recommendation

Replacing lactophenol with a lactic acid solution did not affect the results of the test.

It is recommended that lactophenol be replaced by the lactic acid solution described to avoid the use of phenol in testing for *Ustilago nuda* in *Hordeum vulgare*. The boiling time should be extended to 5 minutes and the advice that "the scutellum becomes more transparent if embryos left in glycerol for 1-2 hours making examination easier" be added to the Method Sheet.

Acknowledgements

The authors would like to thank Adrian Roberts, BioSS for his advice on statistical design of the experiment and to the seed analysts at the OSTS, Scotland and the Institute for Seed, Austria for completing the testing.

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Method for the detection of *Microdochium nivale* on *Triticum* spp.

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Summary

Three agar methods commonly used by seed testing laboratories for the detection of *Microdochium nivale* on *Triticum* spp were compared to determine whether the methods are equivalent to each other in terms of detection, repeatability and reproducibility. The objective was to have a recognised standardised method(s) listed in the ISTA International Rules for Seed Testing. The methods were also compared to determine whether they were suitable for further validation studies for the *Fusarium* spp previously considered as *Fusarium roseum*. The three methods varied in their detection levels, repeatability and reproducibility for both *M. nivale* and *F. roseum*. Method 1 (seed pre-treated in 1%NaOCl and incubated on PDA or MA with streptomycin sulphate at 20ºC in the dark for seven days) showed the best characteristics for detection of both *M. nivale* and *F. roseum*. Method 2 (seed pre-treated in 1%NaOCl and incubated on PDA or MA with streptomycin sulphate at 22ºC in alternating light (NUV)/dark for five days) was poorest overall for *M. nivale* testing. Although Method 3 (seed pre-treated in 1%NaOCl and incubated on PDA or MA with streptomycin sulphate at 5ºC in the dark for seven days followed by incubation at 20ºC in alternating light (NUV)/dark incubation for a further three days) showed better reproducibility than Method 1 for *M. nivale*, detection levels were lower. In addition, the ease of *M. nivale* identification by this method was considered more difficult than by Method 1 and additional incubation was often required before laboratories could evaluate the colonies. Method 3 was not suitable for *F. roseum* testing. From the results of this comparative test and the comments provided by the participants, it is recommended that Method 1 should be accepted as an ISTA Validated Method for the detection of *M. nivale*. It is also recommended that Method 1 should be considered for further validation studies in to routine testing of individual *Fusarium* spp that affect the quality of *Triticum* spp.

Introduction

By the early 1960's the agar plate test was widely adopted to detect seed-borne fungi of cereals, and was used in early comparative health tests sponsored by the Plant Disease Committee of the International Seed Testing Association (ISTA), Hewett (1965). An agar plate method for the detection of *Microdochium nivale* (*Fusarium nivale*) was first published by ISTA in working sheet S.3. No.33 (Anon., 1964). Two further methods were also described in the working sheet, the blotter test and a brickstone or sand test. These tests involved assessing seedlings for disease (blight) symptoms but it was not always possible to differentiate symptoms of *M. nivale* from other seed-borne fungi including *Leptosphaeria nodorum* (*Septoria nodorum*) and other *Fusarium* spp. The agar plate test was able to distinguish between pathogens. Ponchet, (1960) described the differences in appearance of *M. nivale* and *L. nodorum* on malt agar (MA) and Hewett (1965) noted that these differences were also obvious on potato dextrose agar (PDA). Although, some laboratories still use a blotter test (Doyer method; Jorgensen, 1971) where blight symptoms are assessed and results expressed as a percentage of total seedlings examined (Karin Sperlingsson, Personal Communication), many laboratories use an agar plate test where there is a need to differentiate between the different fungi causing blight symptoms in the blotter test.

The agar plate method described in the early working sheet; PDA and an 8-day incubation at 22°C period in the light or dark, has been modified over the years (Hewett, 1965; Petitt *et al.*, 1993; Cockerell and Rennie, 1996; Champion, 1997; Crop Protection Compendium, 2006).

Media with fungicides added as described by Petitt *et al*., 1993 have not been used on a routine basis, although the addition of the antibiotic streptomycin is commonly used to control bacterial saprophytes, which may inhibit fungal growth. In Europe where *M. nivale* can cause severe seedling losses when untreated seed is sown, some of these methods have been used for more that 30 years.

In the 1940's *Fusarium roseum* was recognised as a single species by Snyder and Hansen (Leslie and Summerell, 2006). Although this was popular with diagnosticians the reality was that *F. roseum* was not a single species and the concept of splitting *F. roseum* in to different sections as suggested by Wollenweber and Reinking, 1935 has been carried through to today's understanding of *Fusarium* taxonomy (Booth, 1971a, Nelson, 1983 and Leslie and Summerell, 2006). However in some seed testing laboratories *F. roseum* is still commonly used to describe a group of *Fusarium* spp. The principal species being *F. graminearum*, *F. culmorum*, *F. avenaceum* and *F. arthrosporioides* (Champion, 1997). A standardised method for detecting and evaluating *F. roseum* has never been presented to ISTA with most laboratories testing for this using the same method as for *M. nivale* (with the base colour of carmine red being diagnostic for *F. roseum*) or a blotter method. It is recognised that the systematics and nomenclature of *Fusaria* has evolved since the term was first used and the name *F. roseum* subsumed so many different taxa that it is presently of little value and no longer used because the information content of the name is so low (Leslie and Summerell, 2006). However, the principal *Fusarium* species of *F. roseum* as described by Champion, 1997 can affect the emergence of untreated wheat seed. This effect is more prevalent in warm climates and more commonly these pathogens are associated with head blight.

At the ISTA Mycology Workshop in Ås, Norway, 2000 it was decided that three agar methods commonly used by seed testing laboratories for the detection of *M. nivale* would be compared to determine whether the methods are equivalent to each other. The objective was to have a recognised standardised method(s) listed in the ISTA International Rules for Seed Testing. The methods to be evaluated are currently used to assess the quality of seed for planting, and/or to make decisions on seed treatment. They are also being used to support regulatory programmes or voluntary seed health management programmes. As the early ISTA Working Sheet for *M. nivale* had never been revised and was out-dated, it was not included in the comparative test. In addition an evaluation was to be made on the suitability of the methods for *Fusarium* spp particularly those previously associated with *F. roseum*.

Materials and Methods

Seed Lots

Six naturally infected seed lots were chosen from a selection supplied by the French, Norwegian and Scottish Official Seed Testing Stations and evaluated at the Official Seed Testing Station for Scotland (OSTS). Samples chosen had either low levels of both pathogens; medium/high levels of both pathogens; or different levels of either pathogen.

Sample preparation

For each seed lot 54 sub-samples of 200 seeds were prepared according to the International Rules for Seed Testing (Anon., 2001). Each sub-sample was coded randomly. There was no relationship between successive codes and seed lots. Six sub-samples from each lot were sent

to the nine participating laboratories in Austria, Canada, Denmark, England, France, The Netherlands, Norway, Scotland and Switzerland.

Design
For each method and each seed lot, two sub-samples of 200 seeds were tested in 2 x 100 seed replicates. The number of seed lots and sub-samples tested per method was estimated to be sufficient for the level of discrimination required for the comparisons over nine laboratories.

Media
Depending on the experience of the laboratory either Potato Dextrose Agar (PDA) or Malt Agar (MA) was used, as both media produce similar colony types and sporulation (Ponchet, 1960; Hewett, 1965). Media was prepared according to manufacturers instructions and streptomycin sulphate was added after autoclaving at a rate of 100ppm to PDA and 50ppm to MA. Each laboratory was instructed to test the sustainability of their media using *M. nivale* reference colonies supplied by the OSTS, Scotland. Where the performance criteria was not met, i.e. less than 5 cm growth from a 0.75 cm plug in five days (from a reference culture no older than 28 days), the media was to be rejected.

Pre-treatment of seeds
For all three methods, the seeds were surface sterilised by immersing in a solution of sodium hypochlorite (1% available chlorine) for 10 minutes, then drained. Five seeds were placed equidistant around the perimeter of a 9.0 cm petri dish containing either PDA or MA.

Incubation
The time, temperature and light regime for incubation are given in table 1 for the different methods. NUV was specified as cool black lights with a wavelength of 360 nm.

Examination
After incubation, fungal colonies developing from the seeds on the plates were visually examined for mycelial characteristics. If necessary, plates were left in the light for 3-4 hours to allow *M. nivale* colonies to 'colour up' (see description). High power examination (x100 – x400) of spores could also be used where required. The number of *M. nivale* colonies found per replicate was recorded. *M. nivale* colonies from seed are relatively fast growing; aerial mycelium is white to very pale pink. Spores may be present, usually at colony margins, occurring as orange masses, (Nelson et al, 1983). From the reverse view, the colony colour is salmon pink (CMI Mycological Colour Chart, sheet 1, No. 41, Rayner, 1970) or colourless to light orange (Nelson *et al*., 1983). The colour is uniform throughout the entire colony. After incubation in the dark the reverse of the colony may be white. Conidia are curved, with blunt terminal cells that are difficult to distinguish from one another and the basal cell may be notched but never foot shaped; 1-3 septate; with conidia 10-30 x 2.5-5um; and chlamydospores are not observed (Booth, 1971b, Nelson *et al*., 1983).

To give an indication of a method's suitability for those *Fusaria* previously described as *F. roseum*, by seed testing laboratories, the number of *Fusarium* colonies with shades of carmine red at their bases was recorded: for the purposes of this comparative test these colonies are referred to as *F. roseum*. In addition, the total number of *Fusarium* colonies excluding *M. nivale* was also recorded: for the purposes of this comparative test these colonies are referred to as *Fusarium spp*. *Fusarium spp* therefore contains colonies referred to as *F. roseum* and *Fusarium* colonies that do not exhibit carmine red colouration.

Statistical Analysis
Initial evaluation of the seed lots showed the variability of the test results was consistent with that of a binomial distribution (data not presented). A generalised linear model was therefore used to analyse the results. The specific model used was a linear logistic model (binomial distribution with the link function as the logistic function) (McCullagh and Nelder, 1989) and analysed using Genstat for Windows (Sixth Edition). For spotting individual anomalous results, deletion residuals were used (McCullagh and Nelder, 1989). To set thresholds, it was assumed that these were approximately normally distributed. To compare the size of variability due to different factors, the method of generalised mixed models was used (Schall, 1991). This is an extension of generalised linear models, allowing the incorporation of random effects.
Unless otherwise stated, analyses were made on the full-expanded data set rather than a compressed version with accumulated results for each method-by-laboratory-by-sample combination.

Results

Data received
One of the nine laboratories was excluded from the analysis because the protocol was not followed. Complete results were received for *M. nivale*, but Laboratory 5 did not send results for *F. roseum* with Method 2 and Laboratory 9 sent no results for *Fusarium* spp. This left 576 results comprising 8 laboratories by 3 methods by 6 samples by 2 sub-samples by 2 replicates of 100 seeds. The actual number of seeds tested was sometimes less than 100 and this was taken in to account during the statistical analysis.

Overall analysis of the results
The variance components for all relevant experiment factors produced by the logistic random effects model are given in Table 2. Note the dispersion factor has been fixed at 1. This was reasonable as estimated dispersion factor for *M. nivale* was 0.85, for *F. roseum* 0.54 and 0.74 for *Fusarium* spp. The greatest source of variability was due to differences between samples and sub-sample to sub-sample variability was not negligible. There were also relatively large differences between laboratories for *Fusarium* spp.

When the sub-sample and replicate factors were removed from this model (leaving the factors sample, laboratory, method and their interactions), the dispersion factor for the residual increased to 1.30 for *M. nivale* (chi-squared test for extra-variation $p<0.001$), 1.34 for *F. roseum* ($p<0.001$) and 1.74 for *Fusarium* spp ($p<0.001$). This indicated that there was some degree of variability over and above that expected from random sampling between sub-samples and/or replicates from same sample at the same laboratory with the same method.

Outlier detection
Deletion residuals were calculated for the linear logistic model with factors sample, laboratory, method and their interactions. Table 3 shows those results with deviance residuals with size greater than 2.33 (corresponding to two-sided normal distribution 1% tail). Note that there were 4 results for each sample-by-laboratory-by-method combination.

Only laboratory 9 and sample 1 had no "outliers"; all three methods had outliers. Laboratory 4 had the most outliers especially for *Fusarium* spp. The majority of outliers had higher proportions of infected seed than expected. Some laboratory-by-method-by-sample combinations had several outliers indicating a more general problem with consistency (laboratory 4 method 1 sample 2, laboratory 4 method 3 sample 4, laboratory 4 method 3 sample 5 and laboratory 8 method 2 sample 4).

Consistency of results for each laboratory

Table 4 shows the level of variation left after accounting for sample and method for each laboratory. This is a measure of the consistency of the laboratory. The table shows the dispersion factor (the factor by which the variation exceeds the expected variation) along with the p-value (for a two-sided chi-squared test that this variability exceeds that expected for random sampling). The consistencies shown by laboratories 3, 4, 6 and 8 were less than expected. The results for laboratory 4 were particularly inconsistent compared to other laboratories. Results for *Fusarium* spp were generally less consistent.

Detection levels

To compare the detection levels given by each method, method-by-sample means have been calculated using the linear logistic model with terms sample, method and laboratory (with all interactions between them) and with an estimated dispersion factor. These are tabulated with standard errors in Tables 5, 6 and 7 and represented graphically in Figures 1, 2 and 3. All the factors in the model affected the results ($p<0.01$ for all). Note that Laboratory 4 was omitted for all three diseases, because of the inconsistency illustrated in Table 4 and laboratory 5 was omitted for *F. roseum* since it did not record *F. roseum* for method 2. Method 1 generally detected more *M. nivale* than method 3, which detected more than method 2. Methods 1 and 2 had similar levels of detection of *F. roseum* (with the exception of sample 3 – caused by a rogue result for laboratory 3 with method 2) and detected more than method 3.

Again methods 1 and 2 had similar levels of detection of *Fusarium* spp (with the exception of sample 3 – caused by a rogue result for laboratory 3 with method 2) and detected more than method 3.

Repeatability of each method

Table 8 shows the level of variation left after accounting for sample and laboratory for each method. This is a measure of the repeatability of the method. The table shows the dispersion factor along with the p-value (for a two-sided chi-squared test that this variability exceeds that expected for random sampling). Laboratory 4 was excluded due to its low level of consistency. Method 1 has acceptable levels of consistency for the three disease classifications. Method 3 was consistent for *M. nivale* and *F. roseum* but not *Fusarium* spp. Method 2 was inconsistent for all three classifications.

Reproducibility of each method

A measure of reproducibility is given by the sum of the variance components for laboratory, sub-sample and replicate. There was considerable sample-to-sample variation in variability so linear logistic random effects analyses were run for each method-by-sample combination. Again Laboratory 4 was excluded due to its low level of consistency. Table 9, 10 and 11 show the estimates of reproducibility for the three disease classifications and the laboratory-to-laboratory variation.

All three methods showed lower levels of reproducibility in *F. roseum* detection compared to *M. nivale*. Method 1 had the best reproducibility overall for *F. roseum*. All three methods had problems with sample 3, but the levels of reproducibility did not appear to be related to the *M. nivale* infection levels.

For *Fusarium* spp, Method 1 had the best reproducibility overall. Again all three methods had problems with sample 3, but the levels of reproducibility did not appear to be related to the *M. nivale* infection levels.

For *M. nivale* the reproducibility was more varied with Method 3 having the best reproducibility overall. The level of reproducibility did not appear to be related to the *Fusarium* spp infection levels.

Discussion

The results show that the three methods are not equivalent in terms of: detection by laboratories; repeatability; or reproducibility. In general Method 1 detected higher levels of *M. nivale* than Method 3 and both detected higher levels than Method 2. On the other hand, Methods 1 and 2 detected higher levels of *F. roseum* than Method 3. This was to be expected as Method 3 was designed to detect *M. nivale,* which has a lower optimum growth temperature than fungi belonging to *F. roseum*.

There was evidence that laboratories differed in their *M. nivale* detection rates for all three methods and the reproducibility of the methods varied according to the sample tested. Compared to *M. nivale* the levels of reproducibility for *F. roseum* were lower. Method 1 had the best levels of reproducibility for *F. roseum* whereas Method 3 was best for *M. nivale*. For *Fusarium* spp detection, greatest levels of reproducibility were obtained using Methods 1 and 2.

Repeatability studies showed a lack of repeatability for Method 2 compared to both Methods 1 and 3, with variability exceeding that expected for random sampling. Method 1 had the best repeatability over all three fungi.

Participating laboratories were requested to record the time taken to test the samples using the three methods; to score the methods for ease of identification and comment on their experience with the different methods.

There was a wide variation in times depending on a laboratories experience with a particular method (Table 12). The average time for completion of 12 sub-samples was less for Method 1.

For Method 1, two laboratories noted the lack of sporulation after incubation in the dark. However, most laboratories noted that the characteristic colour of *F. roseum* colonies (carmine red) was generally better in Method 1. Four laboratories commented that it was difficult to identify colonies in Method 2 after 5 days incubation. *M. nivale* colonies were sometimes small and it was sometimes difficult to separate *F. roseum* from *Epicocum* sp. For this reason laboratories incubated samples for 2-3 additional days before reporting the
results. One laboratory noted that a disadvantage of a five-day incubation time meant there were only three plating days per normal 5-day working week.

Although one laboratory noted that Method 3 was best for sporulation, four laboratories noted that *M. nivale* colonies were smaller and mycelium sparser than expected using this method making identification more difficult. It was also noted that in some cases prolonged incubation was required. This method was already disadvantaged by its longer incubation time compared to the other methods. The average score for ease of identification was best for Method 1 and lowest for Method 2. There was a wide range of scores for each method with laboratories favouring the method currently used in their laboratory.

One laboratory highlighted a problem associated with the use of distilled water rather than tap water in their laboratory and suggested that the optimum growth of *M. nivale* was not achieved particularly in method 3. The same laboratory showed that the use of distilled water rather that tap water could have an affect on the growth of some *F. roseum* colonies and their production of the characteristic carmine red pigmentation. This effect was not tested in the comparative test and all laboratories with the exception of one used distilled water. The laboratory using tap water had already been excluded from the analysis for not following the method. Tap water varies from laboratory to laboratory and therefore would be very difficult to standardise.

Conclusions and Recommendations

Method 1 has shown the best characteristics for detection of both *M. nivale* and *F. roseum*. Although Method 3 showed better reproducibility than Method 1 for *M. nivale*, detection levels were lower, the ease of *M. nivale* identification by this method was considered more difficult than Method 1 with additional incubation being required. Method 3 was not suitable for *F. roseum* testing. Method 2 was poorest overall for *M. nivale* testing.
From the results of this comparative test and the comments provided by the participants, it is recommended that Method 1 briefly described below, should be accepted as an ISTA Validated Method for detection of *M. nivale*.
Evidence from this comparative test shows that Method 1 had the highest level of repeatability and reproducibility for both '*F. roseum*' and *Fusarium* spp. when compared to Method 2 and Method 3. As the impact on wheat seed and plant health differs for each Fusarium spp within the group known as '*F. roseum*' it is important to ensure that any future procedures are capable of identifying to species level. It is therefore recommended that to accommodate future routine testing of individual *Fusarium* spp. previously considered under the species name of *F. roseum* that Method 1 should be considered for further validation studies.

Method Description.
Seeds are pre-treated in sodium hypochlorite (1% available chlorine) for ten minutes and drained before plating on to PDA or MA with streptomycin sulphate. Plates are incubated at 20ºC in the dark for 7 days.

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The authors wish to thank the participants (Jim Sheppard, Isabelle Serendat, Henrik Hansen, Guro Brodal, Jane Thomas, Toos Dekker-Nooren, Gabriele Schachermayr, Manfred Weinhappel, and Margaret Jacks) for their invaluable contribution to this report.

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Table 1. Incubation regimes for different methods.

Method	Incubation No. of days	Temperature	Light regime
1	7	20°C	Dark
2	5	22°C	Dark/NUV
3	7	5°C	Dark
	3	20°C	Dark/NUV

Table 2. Variance components for full logistic random effects model.

Factor	*M. nivale*	*F. roseum*	Fusarium spp
Sample	4.254	2.430	1.806
Sub-sample within sample[1]	0.040	0.096	0.118
Replicate within sub-sample	0.008	0.042	0.035
Laboratory	0.045	0.043	0.435
Method	0.073	0.159	0.062
Sample x Laboratory	0.059	0.165	0.255
Sample x Method	0.031	0.013	0.011
Laboratory x Method	0.048	0.289	0.250
Sample x Laboratory x Method	0.115	0.104	0.125
Dispersion factor for residual variance	1	1	1

[1] actually sub-sample nested in sample*laboratory*method

Table 3. Outliers at 1% level with deletion residuals.

Laboratory	Method	Sample	Sub-sample	Replicate	*M. nivale*	*F.roseum*	*Fusarium*. spp
2	1	6	278	1	3.5		
2	3	3	119	2	2.5		
3	1	6	284	1	2.4		
3	2	2	69	1	4.0		
3	2	3	123	2		2.6	2.6
3	2	4	177	1		2.8	
4	1	2	73	2		-4.0	2.9
4	1	2	74	1		6.8	
4	1	2	74	2	4.5		-4.5
4	1	3	128	1			2.6
4	1	4	181	2			4.3
4	1	5	236	1	-2.8	2.5	
4	2	3	130	2			3.7
4	2	4	184	2			3.8
4	2	6	292	1			2.5
4	3	2	77	1	-2.7	-2.6	2.4
4	3	4	185	1	3.6		-3.9
4	3	4	185	2	2.9	-3.6	-2.7
4	3	4	186	1	-2.8	3.4	3.1
4	3	4	186	2	-4.2		3.6
4	3	5	240	1			2.6
4	3	5	240	2	-2.6		2.3
5	1	4	188	2		2.4	2.2
5	1	6	295	2		2.5	
5	2	6	297	2			2.4
5	3	3	138	2	2.7		
6	1	2	85	2		2.5	2.5
6	2	2	88	2	2.4		
6	2	3	141	1		2.9	2.9
6	2	6	304	2		2.6	3.0
6	3	4	198	1		2.6	2.6
7	2	2	93	1		2.4	
7	3	3	150	1			2.5
8	1	3	152	1	2.5		
8	1	4	205	1		2.6	2.6
8	2	3	154	1	2.6		
8	2	4	207	1		2.5	2.5
8	2	4	207	2			2.7
8	2	6	315	2		3.7	2.9
8	3	4	209	2		3.0	3.1

Table 4. Dispersion factors for each laboratory, for linear logistic model, with factors sample, method and interaction.

	M. nivale		*F. roseum*		FUSARIUM *SPP*	
Lab	Dispersion	p-value	Dispersion	p-value	Dispersion	p-value
2	1.34	0.098	0.88	0.548	0.98	0.968
3	1.39	0.061	1.49	0.023	1.52	0.016
4	2.58	<0.001	2.54	<0.001	3.87	<0.001
5	1.21	0.286	1.37	0.135	1.32	0.111
6	1.31	0.129	1.70	0.002	1.63	0.005
7	1.12	0.510	0.86	0.470	1.32	0.111
8	1.28	0.163	1.59	0.008	2.07	<0.001
9	0.77	0.218	0.80	0.295	n/a	

Table 5. Mean percentages of *M. nivale* infected seeds over laboratories with standard errors of differences.

Sample	Method 1	2	3	Sample Mean	SED between methods by sample
1	4.9	2.8	3.1	3.6	0.54
2	70.9	56.3	73.4	66.9	1.35
3	32.5	19.7	21.0	24.4	1.24
4	20.4	10.8	17.1	16.1	1.06
5	3.6	2.5	2.2	2.8	0.48
6	1.1	0.2	0.3	0.5	0.21
Method Mean	22.2	15.3	19.5		

Average standard error differences between different methods =0.37%

Table 6. Mean percentages of *F. roseum* infected seeds over laboratories with standard errors of differences.

Sample	Method 1	2	3	Sample Mean	SED between methods by sample
1	0.7	0.5	0.4	0.5	0.23
2	6.8	7.0	3.3	5.7	0.74
3	0.1	1.7	0.1	0.6	0.23
4	22.8	20.3	15.2	19.4	1.22
5	7.7	6.4	2.5	5.5	0.72
6	3.7	3.6	1.0	2.8	0.51
Method Mean	7.0	6.6	3.7		

Average standard error differences between different methods =0.29%

Table 7. Mean percentages of *Fusarium* spp infected seeds over laboratories with standard errors of differences.

Sample	Method 1	2	3	Sample Mean	SED between methods by sample
1	1.0	0.8	0.5	0.8	0.31
2	8.3	8.0	4.9	7.1	0.90
3	0.3	1.9	0.6	0.9	0.32
4	26.9	27.6	20.4	25.0	1.50
5	8.9	8.0	4.0	7.0	0.88
6	6.4	4.8	2.1	4.4	0.71
Method Mean	8.6	8.5	5.4		

Average standard error differences between different methods =0.36%

Table 8. Dispersion factors for each method (repeatability) for linear logistic model with factors sample, laboratory and interaction: without laboratory 4.

	M. nivale		*F. roseum*		FUSARIUM *SPP*	
Method	Dispersion	p-value	Dispersion	p-value	Dispersion	p-value
1	1.18	0.186	1.11	0.380	1.17	0.219
2	1.30	0.025	1.55	<0.001	1.84	<0.001
3	1.13	0.316	1.09	0.444	1.41	0.006

Table 9. Reproducibility for each method and sample given by the sum of the three variance components from the linear logistic random effects model with factors laboratory, sub-sample and replicate (dispersion=1): without laboratory 4: *M. nivale*.

Sample	Mean proportion of infected seeds – *M. nivale*	Mean proportion of infected seeds – *Fusarium* spp	Reproducibility Method 1	Method 2	Method 3
1	3%	1%	0.168	0.286	0.397
2	65%	8%	0.200	0.264	0.043
3	24%	4%	0.039	0.411	0.112
4	17%	24%	0.298	0.129	0.267
5	3%	8%	1.208	0.859	0.392
6	1%	5%	0.862	1.382	0.451

Table 10. Reproducibility for each method and sample given by the sum of the three variance components from the linear logistic random effects model with factors laboratory, sub-sample and replicate (dispersion=1): without laboratory 4: *F. roseum*

Sample	Mean proportion of infected seeds – *F. roseum*	Mean proportion of infected seeds – *M. nivale*	Reproducibility Method 1	Method 2	Method 3
1	1%	3%	0.252	0.764	1.590
2	6%	65%	0.227	0.469	0.699
3	1%	24%	1.731	7.610	2.014
4	19%	17%	0.524	0.421	1.062
5	5%	3%	0.046	0.499	0.776
6	3%	1%	0.646	0.832	0.615

Table 11. Reproducibility for each method and sample given by the sum of the three variance components from the linear logistic random effects model with factors laboratory, sub-sample and replicate (dispersion=1): without laboratory 4: *Fusarium* spp

Sample	Mean proportion of infected seeds – *Fusarium* spp	Mean proportion of infected seeds – *M. nivale*	Reproducibility Method 1	Method 2	Method 3
1	1%	3%	0.247	0.699	1.543
2	8%	65%	0.221	0.123	0.986
3	4%	24%	1.571	3.362	1.988
4	24%	17%	0.126	0.150	0.836
5	8%	3%	0.085	0.329	0.639
6	5%	1%	0.419	0.554	1.000

Table 12. Range and average times for preparation and evaluation of 12 sub-samples of 200 seeds.

	Method 1	Method 2	Method 3
Range	5hrs 24mins – 16 hrs 20mins	6hrs 19mins – 18hrs	6hrs 8mins – 16hrs
Average	9hrs 59mins	10hrs 58mins	10hrs 36mins

Table 13. Range and average scores given by laboratories relating to ease of identification of *M. nivale* and *F. roseum*. 1= very straight forward, 5= very difficult.

	Method 1	Method 2	Method 3
	M. nivale		
Range	1-5	1-5	2-5
Average	2.3	2.6	2.4
	F. roseum		
Range	1-5	1-5	2-5
Average	2.4	2.4	2.8

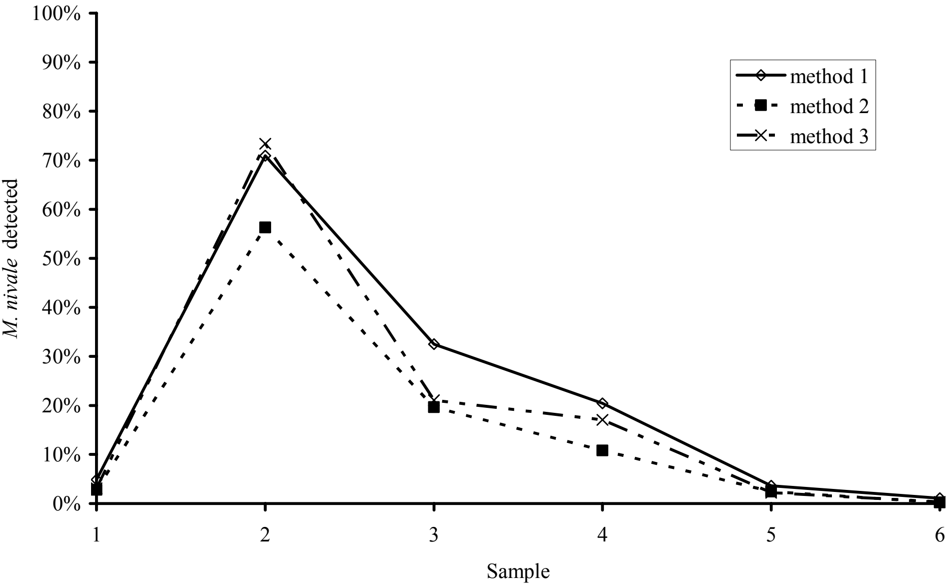

Figure 1. Mean proportions of *M. nivale* detected by method and sample.

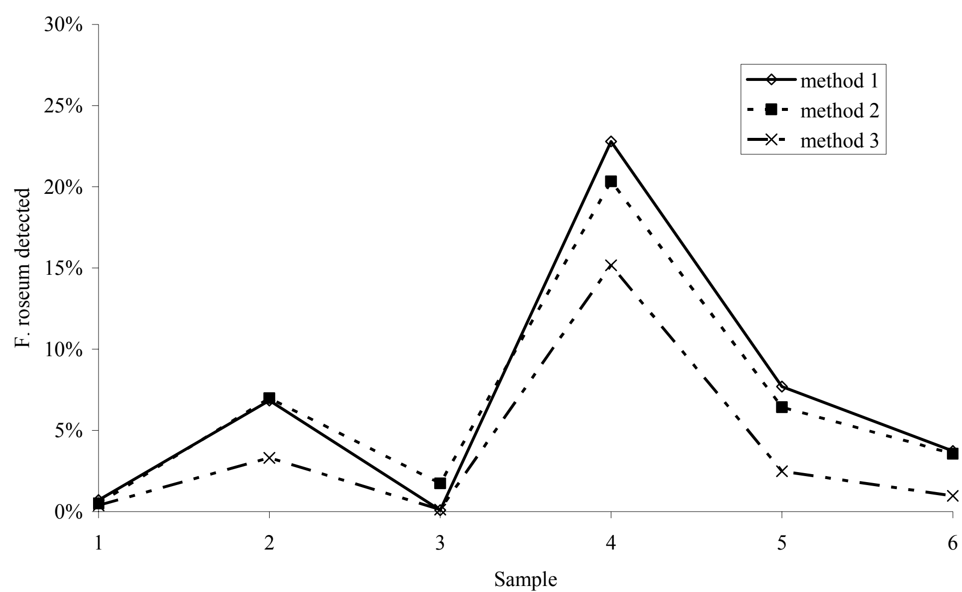

Figure 2. Mean proportions of *F. roseum* detected by method and sample.

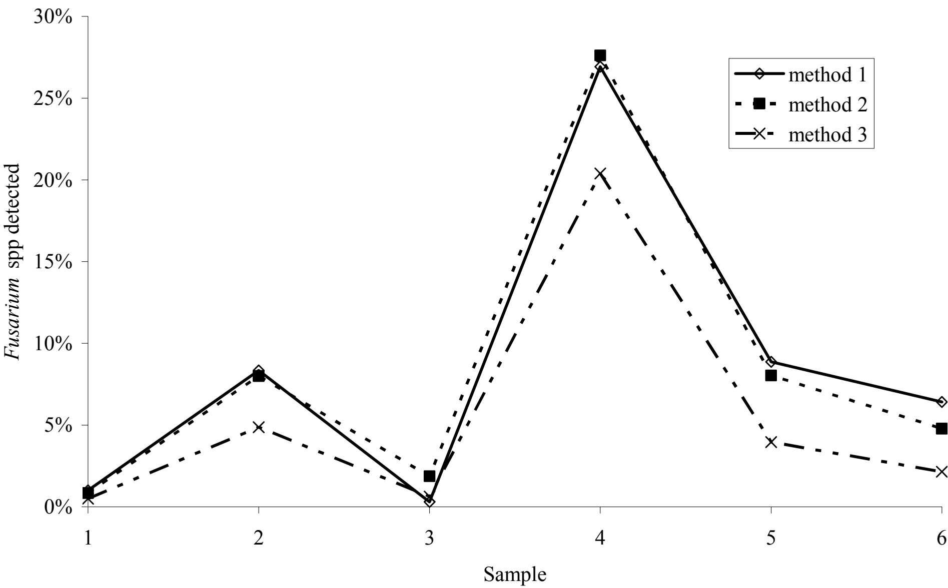

Figure 3. Mean proportions of *Fusarium* spp detected by method and sample.

Aranguren, B., Don, R., Duffy, G., Grégoire, S. and Martinelli, A. (2007) *ISTA Method Validation Reports* **4**, 53-62

Validation of Tetrazolium method for *Brassica* spp.

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Introduction

This work has been carried out at the request of the ISTA Tetrazolium Committee to validate tetrazolium testing of *Brassica* spp. tetrazolium testing in order that it may be included in the ISTA Rules.

Material and methods

A comparative test was organised in order to assess the repeatability and reproducibility of tetrazolium testing of *Brassica* spp. and to compare the results with the repeatability and reproducibility of germination testing of *Brassica* spp.

- Seed material

Three seed lots of canola seed (*Brassica napus* L.) were used in this study. From these lots samples 1G, 2G, 3G (for germination testing only) and 4T, 5T and 6T (for tetrazolium testing only) were obtained; Samples 1G and 6T from Lot 1, Samples 2G and 4T from lot 2 and samples 3G and 5T from lot 3.

- Germination method

The standard procedure prescribed for *Brassica napus* L. in the ISTA Rules was used. Four replicates of 100 pure seeds per sample were germinated on top of paper at 20°C or 20-30ºC (+/- 2ºC). The tests were illuminated for 8 hours in every 24 hours cycle (during the high temperature period where seed were germinated at 20-30ºC). Pre-chilling was not used and the first and final counts were completed 5 and 7 days (respectively) after planting. The number of normal seedlings was subsequently analysed.

- Tetrazolium method

The following procedure was used:

Number of seed tested: four replicates of 100 seeds.
Pre-soaking: 16 hours in tap water at 5-10ºC.
Preparation before staining: seed coats are removed.
Staining: 5 hours at 30ºC in a 1% tetrazolium chloride solution.
Evaluation: 8 categories of viable and 8 categories of non-viable seed as described below and illustrated in Figure 1 were recorded. The sum total of the 8 viable categories was subsequently analysed.

Viable seed

1. **VIABLE**: Completely stained seed of a normal red colour.
2. **VIABLE:** Upper 1/3 of the cotyledons unstained (if pervading).
3. **VIABLE:** Upper 1/2 of each cotyledon unstained (if superficial).
4. **VIABLE:** Minor unstained spots on lower ½ of each cotyledon in areas other than at junction of radicle-hypocotyl axis and cotyledons.
5. **VIABLE:** Not more than 1/3 of the extreme tip of radicle-hypocotyl axis unstained whether or not this extends into or through the conducting tissue.

6. **VIABLE:** Unstained area in the radicle-hypocotyl axis, not extending into the conducting tissue.
7. **VIABLE:** Extreme of the radicle stained dark red, not extended into the conducting tissue.
8. **VIABLE:** Other examples not described here as viable.

<u>**Non-viable seeds**</u>

9. **NON-VIABLE:** Unstained area of more than 1/3 of each cotyledon (if pervading).
10. **NON-VIABLE**: Unstained area of more than 1/2 of each cotyledon (if superficial).
11. **NON-VIABLE:** Unstained area of cotyledons extending into the region where radicle-hypocotyl axis and cotyledons are attached (whether or not this includes the shoot meristem).
12. **NON-VIABLE:** Unstained area of radicle-hypocotyl axis extending into or through the conducting tissue.
13. **NON-VIABLE:** Unstained area involving more than 1/3 of extreme tip of radicle and extending into or through the conducting tissue
14. **NON-VIABLE:** Completely green, brown or whitish yellow embryo.
15. **NON-VIABLE:** Seed completely unstained.
16. **NON-VIABLE:** Other examples not described here as non-viable.

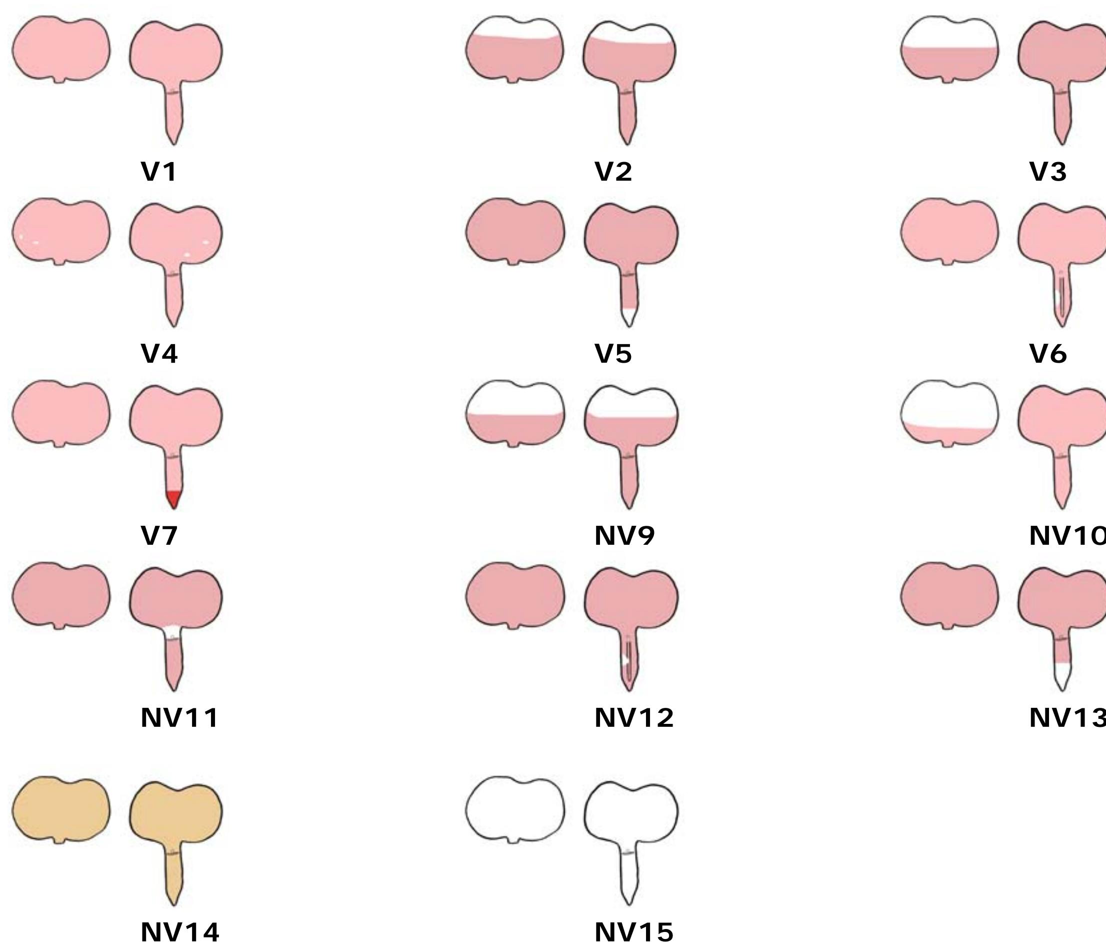

Figure 1. Evaluation guide for viable (V) and non-viable (NV) *Brassica* spp. seeds (illustrations include, on the left side, the separated outer cotyledon).

- Participants

There were 22 participants:

Belén Aranguren (ARGENTINA)
Rita Amengual (ARGENTINA)
Silvia Benavídez (ARGENTINA)
David Bitzel (USA)
Claudia Carracedo (ARGENTINA)
María A. Chazarreta (ARGENTINA)
Roque Craviotto (ARGENTINA)
Daniel Curry (USA)
Patricia Del Fueyo (ARGENTINA)
Sylvie Ducournau (FRANCE)
Teresita Farrás (URUGUAY)
Eduardo Firpo (ARGENTINA)
Marilú Makuch (ARGENTINA)
Darío Moreno (ARGENTINA)
Alejandra Petinari (ARGENTINA)
Victoria F. de Picardi (ARGENTINA)
María E. R. de Sagripanti (ARGENTINA)
Mauricia Sala (ARGENTINA)
Graeme Smith (AUSTRALIA)
Adolph Steiner (GERMANY)
Silvia Tascón (ARGENTINA)
Ha Ung (USA)

- Statistical analysis

The results were analysed in a number of ways:

1. Z-scores were calculated and results assessed as in ISTA Proficiency tests. 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.

2. Laboratories were assigned ratings for the germination and tetrazolium tests they carried out in this validation study. The z-score for an item indicates how far and in what direction it differs from the mean. It is the test used by ISTA for evaluation of proficiency tests and a z-score of ± 1.96 indicates a significant difference from the mean value ($p=0.05$). 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

3. Repeatability and reproducibility have been 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

4. Effect of the different factors (laboratory, sample, test) have been analysed by variance analysis.

Results

Three of the participants failed to provide complete sets of verified results and the analysis were completed on 19 sets of results.

Z- scores and ISTA Rating/Score

The results of tetrazolium tests obtained by the participant laboratories on the three seed lots are illustrated in Figure 2.

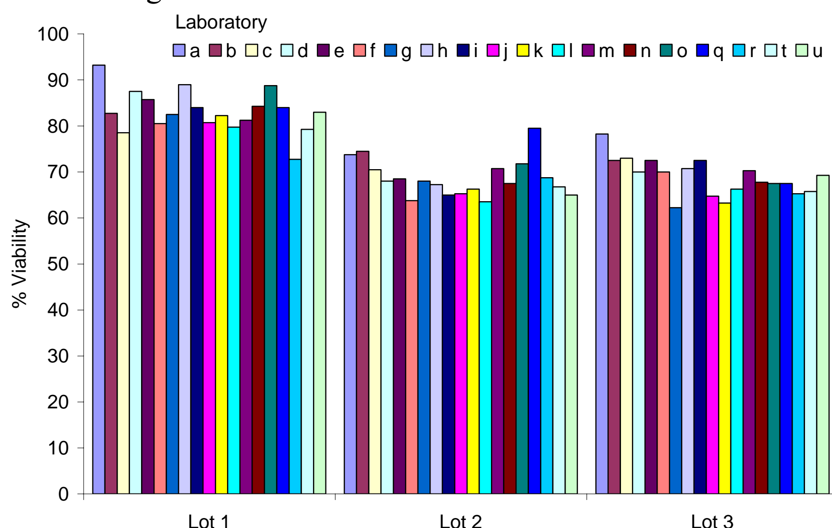

Figure 2. The results of tetrazolium tests on the 3 seed lots used in the *Brassica* spp. tetrazolium validation study

From the z-scores (table 1) we see that laboratory a obtains significantly higher tetrazolium results for lots 1 and 3, as does laboratory q for lot 2. Laboratory r obtains significantly lower results for lot 1. In terms of an overall score or rating all participating laboratories obtained an A score in this validation study with the exception of laboratory a, which has a C score.

Table 1 Z-Scores obtained by different laboratories when conducting tetrazolium tests on the 3 seed lots used in the *Brassica* spp. tetrazolium validation study

	z-score			Absolute	ISTA
Laboratory	Lot 1	Lot 2	Lot 3	Total	Score
a	2.23	1.26	2.37	5.86	C
b	-0.09	1.44	0.91	2.44	A
c	-1.02	0.46	1.04	2.52	A
d	0.96	-0.16	0.28	1.40	A
e	0.57	-0.04	0.91	1.52	A
f	-0.58	-1.2	0.28	2.06	A
g	-0.14	-0.16	-1.69	1.99	A
h	1.29	-0.34	0.47	2.10	A
i	0.19	-0.9	0.91	2.00	A
j	-0.53	-0.83	-1.05	2.41	A
k	-0.2	-0.59	-1.44	2.23	A
l	-0.75	-1.27	-0.67	2.69	A
m	-0.42	0.52	0.34	1.28	A
n	0.24	-0.28	-0.29	0.81	A
o	1.24	0.76	-0.36	2.36	A
q	0.19	2.67	-0.36	3.22	A
r	-2.29	0.03	-0.93	3.25	A
t	-0.86	-0.47	-0.8	2.13	A
u	-0.03	-0.9	0.09	1.02	A

The results of germination tests obtained by the participant laboratories on the three seed lots are illustrated in Figure 3.

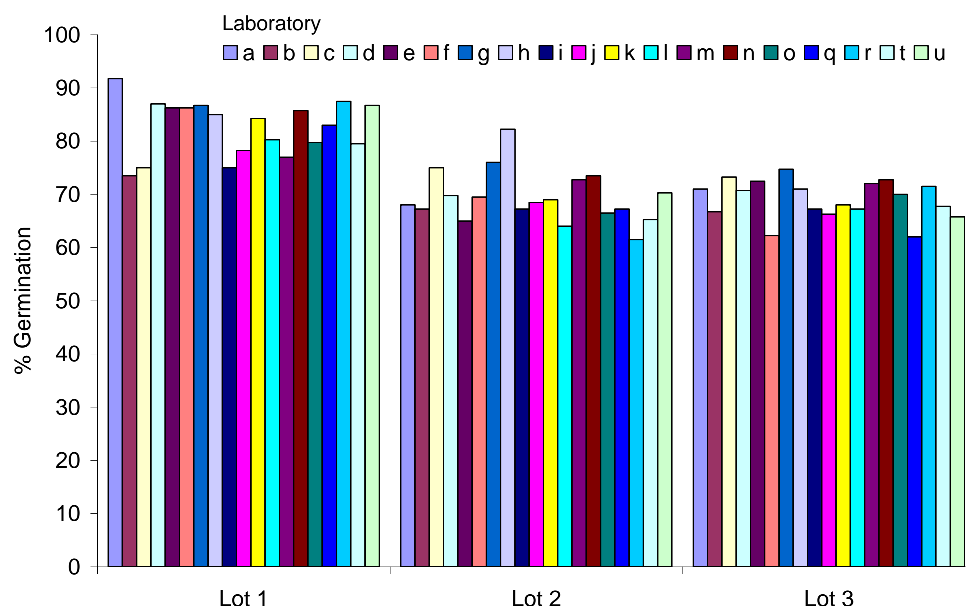

Figure 3 The results of germination tests on the 3 seed lots used in the *Brassica* spp. tetrazolium validation study

From the tetrazolium z-scores (table 2) we see that laboratory h obtains a significantly higher germination results for lot 2. In terms of an overall score or rating laboratories c, g and h obtained a B score whilst all other laboratories obtained an A rating.

Table 2 Z-Scores obtained by different laboratories when conducting tetrazolium tests on the 3 seed lots used in the *Brassica* spp. tetrazolium validation study

		z-score		Absolute	ISTA Proficiency
Laboratory	Lot 1	Lot 2	Lot 3	Total	Score
a	1.78	-0.29	0.53	2.60	A
b	-1.76	-0.44	-0.65	2.85	A
c	-1.46	1.16	1.16	3.78	B
d	0.86	0.07	0.46	1.40	A
e	0.72	-0.91	0.95	2.58	A
f	0.72	0.02	-1.91	2.65	A
g	0.81	1.36	1.58	3.76	B
h	0.47	2.65	0.53	3.66	B
i	-1.46	-0.44	-0.51	2.42	A
j	-0.83	-0.18	-0.79	1.81	A
k	0.33	-0.08	-0.30	0.71	A
l	-0.45	-1.11	-0.51	2.07	A
m	-1.08	0.69	0.81	2.58	A
n	0.62	0.85	1.02	2.49	A
o	-0.54	-0.60	0.25	1.39	A
q	0.09	-0.44	-1.98	2.51	A
r	0.96	-1.63	0.67	3.26	A
t	-0.59	-0.86	-0.37	1.82	A
u	0.81	0.18	-0.93	1.92	A

- Reproducibility and Repeatability of the results

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.

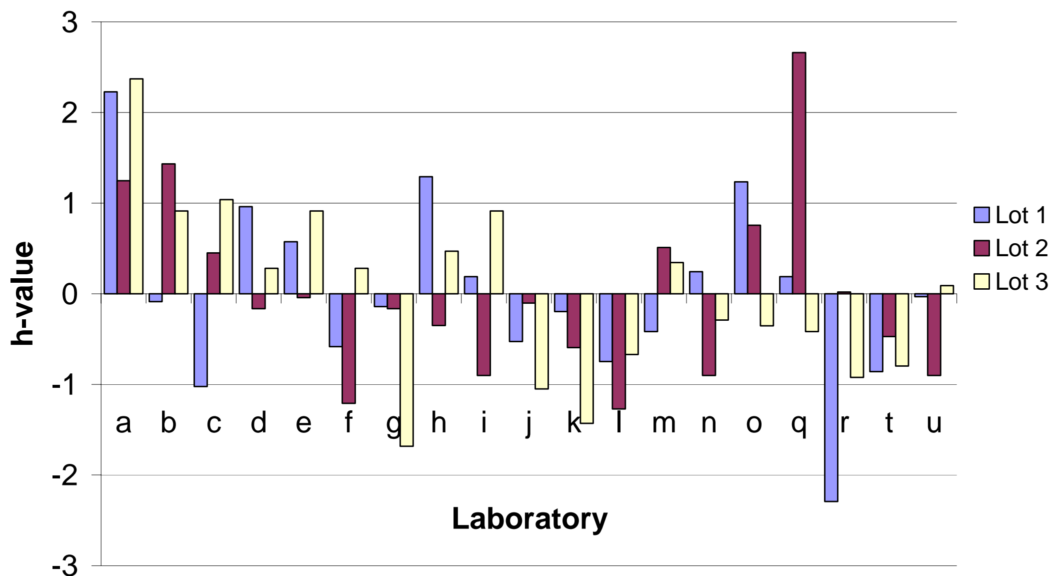

Figure 4 The h-values obtained by participant laboratories carrying out tetrazolium tests on the 3 *Brassica* spp. seed lots used in this validation study

For the tetrazolium test, h-value results (figure 4) are similar to those obtained using z scores. Laboratory a obtains significantly higher tetrazolium results for lots 1 ($p>0.01<0.05$) and 3 ($p<0.01$) as does laboratory q for lot 2 ($p<0.01$). Laboratory r obtains a significantly lower result for lot 1 ($p<0.01$).

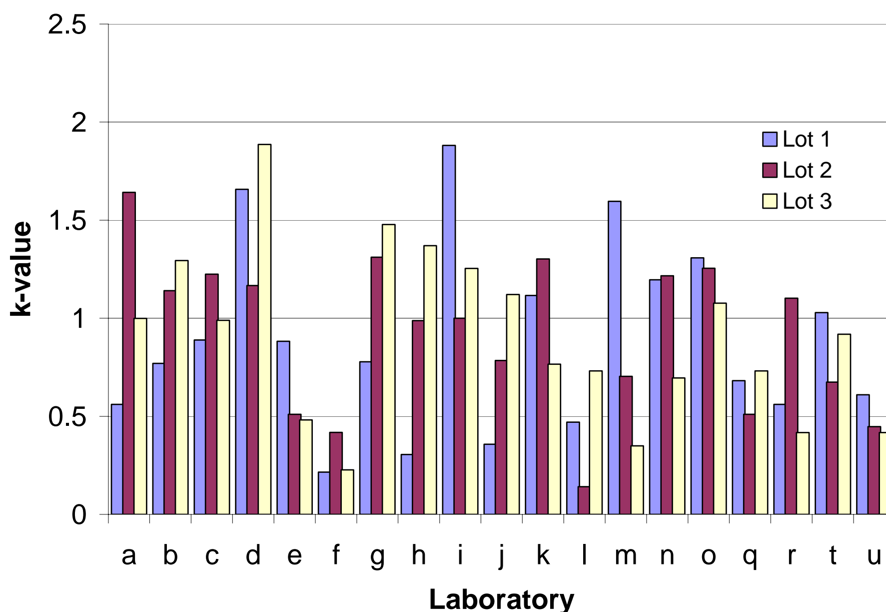

Figure 5 The k-values obtained by participant laboratories carrying out tetrazolium tests on the 3 *Brassica* spp. seed lots used in this validation study

An examination of tetrazolium k-values indicates significant variation between replicate results for: Lot 1 tested at labs d, i and m; for lot 2 tested at lab a; and for lot 3 tested at lab d (all at $p<0.05>0.01$).

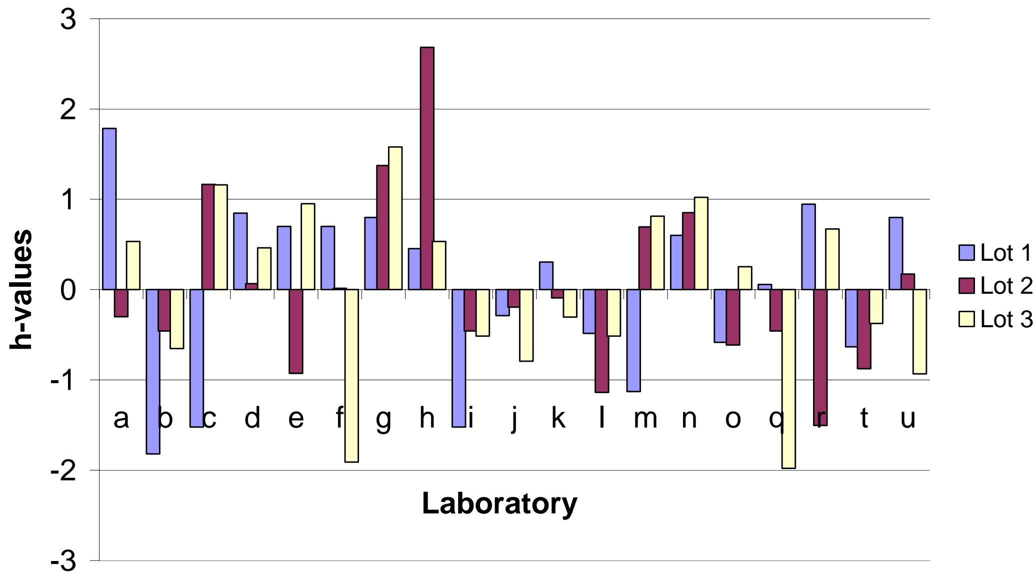

Figure 6 The h-values obtained by participant laboratories carrying out germination tests on the 3 *Brassica* spp. seed lots used in this validation study

Evaluation of germination test h-value results (figure 6) indicates that lab h obtains a significantly higher germination result for lot 2 ($p<0.01$) and labs f and h obtain significantly lower results on lot 3 ($p>0.01<0.05$). The significance of this deviation in the results for labs f and h, when testing lot 3, was not identified in the analysis of z-scores.

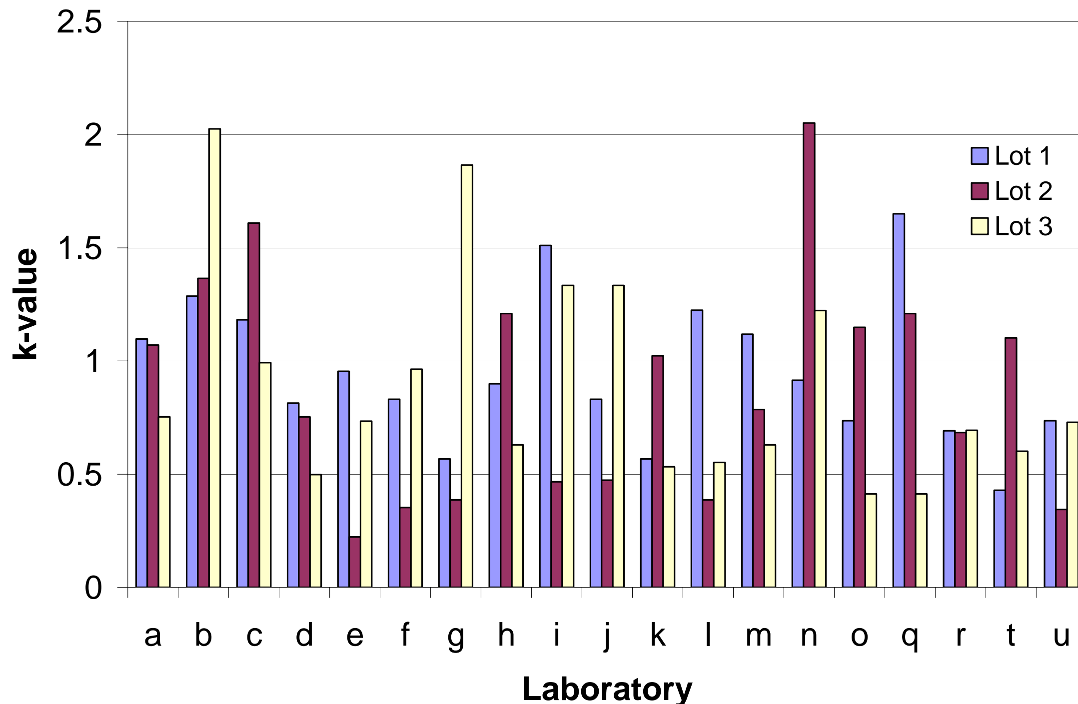

Figure 7 The k-values obtained by participant laboratories carrying out germination tests on the 3 *Brassica* spp. seed lots used in this validation study

An examination of germination k-values (figure 7) indicates significant variation between replicate results for: Lot 1 tested at lab q ($p<0.05>0.01$); lot 2 tested at labs c ($p<0.05>0.01$) and n ($p<0.01$); and for lot 3 tested at labs b and n (both at $p<0.01$).

In terms of overall repeatability and reproducibility the tetrazolium and germination test are relatively similar (table 3)

Table 3 Overall Repeatability and Reproducibility values for tetrazolium and germination tests on the three seed lots used in this validation trial

Test	Seed Lot	Mean Result	Repeatability (sr^2)	Reproducibility (sR^2)
Tetrazolium	1	83.15	2.6762	5.0935
	2	68.67	4.0820	5.3913
	3	68.89	3.5958	5.0282
Germination	1	82.71	3.0102	5.6941
	2	69.43	3.6581	5.7330
	3	69.09	3.4291	4.6534

- Variance Analysis

Variance analysis reveals that for all 3 lots there is no significant difference between tetrazolium results and germination results and that lot 1 has significantly higher results ($p<0.001$) than lots 2 and 3 which are not significantly different (figure 8).

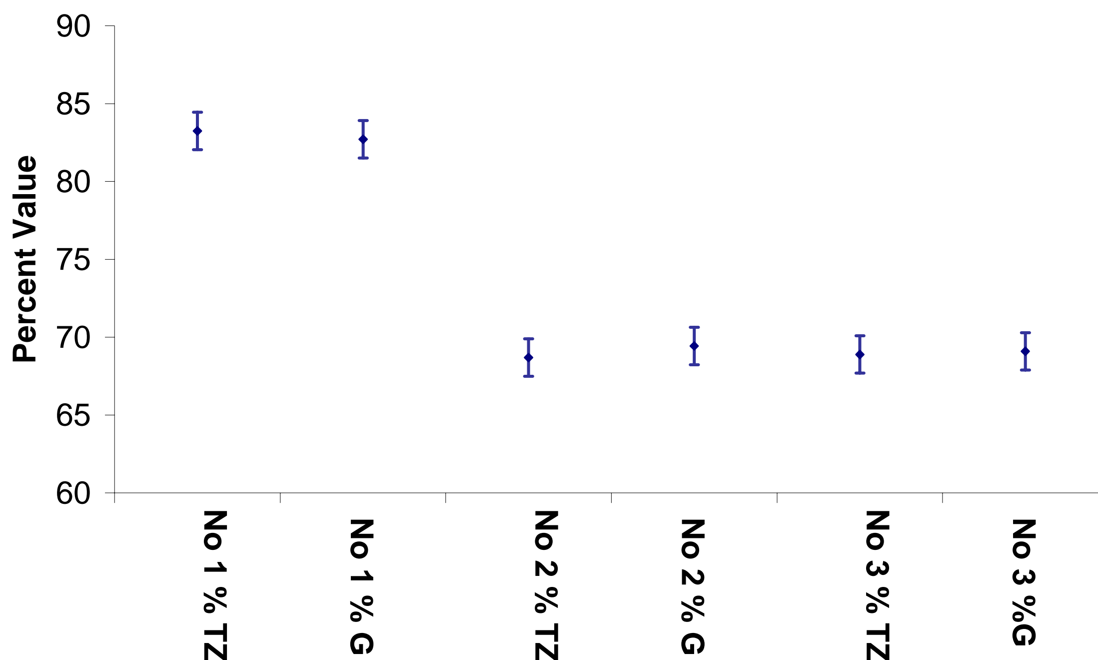

Figure 8 The mean germination and tetrazolium results* obtained by participating laboratories on the three seed lots used in this validation study.

There is a laboratory effect with a significant interaction between laboratory and test for all samples (see figure 9). Some laboratories obtain higher tetrazolium than germination results on the lots whilst others obtained higher germination than tetrazolium results.

* Error bars indicate LSD at P=0.05

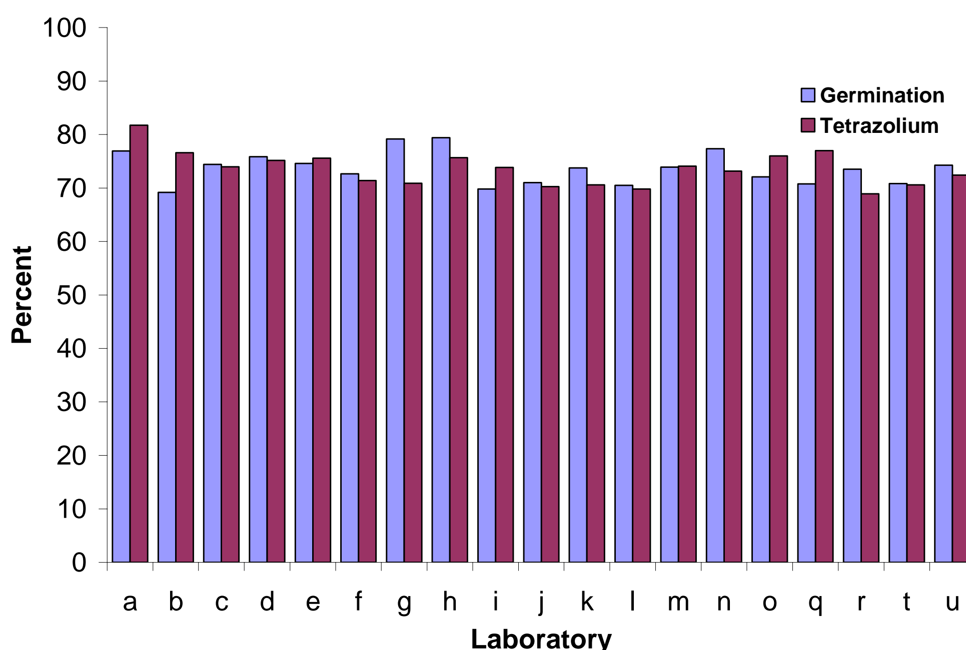

Figure 9 The overall mean germination and tetrazolium results obtained by individual participating laboratories in this validation study.

Discussion and Conclusion

The results obtained in this validation study support the inclusion of *Brassica* spp. testing in the ISTA Rules:

- There is no significant difference between germination and tetrazolium results obtained and the levels of repeatability and reproducibility for germination and tetrazolium tests are similar.
- When one compares the results obtained in this trial with the results obtained in ISTA proficiency tests we find lower levels of variation and a higher overall laboratory rating with little difference between tetrazolium and germination results in terms of variation and rating (table 3).

Table 3 Ratings obtained in various ISTA Germination and Tetrazolium Proficiency tests compared to ratings obtained in this validation trial

	ISTA Score/Rating			
Test	A	B	C	BMP
Brassica napus Germination Proficiency test (2001)	73%	14%	5%	8%
Mean of seven germination proficiency test rounds	72%	16%	5%	7%
Triticum aestivum Tetrazolium Proficiency test (2004)	77%	8%	6%	8%
This study (germination)	85%	15%	0%	0%
This study (tetrazolium)	95%	0%	5%	0%

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.

The significant interaction between test and lab has little practical meaning and is due in part to the scale of the trial (19 laboratories, 3 samples and 4 replicates) giving levels of precision that would not be expected in an applied seed testing situation. For example, least significant differences between mean results obtained by laboratories are less than 3 whereas tolerances are as much as 10.