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INFORMATION DOCUMENT TO SUPPORT MAIN MOTIONS PRESENTED IN DOCUMENT 'OM12-05 RULES PROPOSALS FOR THE INTERNATIONAL RULES FOR SEED TESTING 2014 EDITION'

Method Validation Reports on Rules Proposals for the International Rules for Seed Testing 2014 Edition

Contents

Revised method and ISTA method validation report for the germination of Lolium x boucheanum. L. multiflorum, L. perenne, Festuca filiformis, F. heterophylla, F. ovina, F. rubra, Poa nemoralis, P. palustris, P. pratensis.

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Summary

A validation study on the duration of the germination test for *Lolium perenne, Festuca rubra* and *Poa pratensis* was carried out. Eight ISTA-accredited laboratories in seven countries on three continents participated. Per species for samples were germinated at different temperature conditions, and seedlings were evaluated in different moments.

The results show that repeatability and reproducibility were similar for the last two counts in all species, and were at acceptable levels.

The variation introduced by shortening the duration is of the same magnitude as the variation caused by the different temperature regimes.

It is suggested to shorten the duration of the germination test of the indicated *Lolium* species to ten days, of the *Festuca* species to 14 days and of the *Poa* species to 21 days.

Introduction

Many years ago, when germination methods for grasses were established, little breeding had been done in these species. Now, after considerable breeding efforts, new varieties are in many aspects different from the older ones. One of these aspects is dormancy and speed of germination. Faster establishment is one of the breeding goals in grasses. Improvement in this area has resulted in faster germinating varieties, especially when germinated under optimal conditions (Oliveira et al, 2012).

Grasses are a group of species that is widely used in germination studies. Water and temperature are the most important factors affecting seed germination. However, often suboptimal conditions are applied, deviating substrates are used, or assessment of seedlings is done before the required stage of development has been reached (f.e. Naylor, 1981; Petroski et al, 1990; Ichihara et al, 2003; Larsen et al, 2004; Carbonnel et al, 2008, Karadavut, 2010; Sakanoue, 2010). These publications can not be used for estimating the duration of the germination test, but can be used for other qualitative aspects like effect of dormancy or seed age (Naylor, 2003).

Wiesner et al (1972) investigated dormancy in *Lolium perenne*. Although differences between varieties existed, when choosing optimal temperature conditions and applying a prechill and KNO_3, seeds germinated readily. Stanisavljevic et al (2011) found dormancy to be present in *Lolium mutiflorum* until 270 days after harvest. In *Festuca rubra* Stanisavljevic et al (2010) found dormancy effect on first counts until 240 days after harvest, on final counts until 120 days after harvest. However, it is not clear whether the tests were extended because of germinating seeds appearing during the final count.

Naylor (2003) showed that older seed lots of *Lolium multiflorum* germinated at a slower rate. Above 60% germination there was hardly an effect however, so this aspect will be of no importance for testing commercial seed lots. Pill et al (1997) showed a similar effect for *Poa pratensis* when wrongly primed.

In the present situation ISTA and AOSA durations for germination tests are the same for *Lolium perenne* and *Festuca rubra*, but differ for *Poa pratensis*:

Species		ISTA (days)	AOSA (days)
Lolium perenne	14	14	
Festuca rubra	21	21	
Poa pratensis	28	21	

Work done by Larsen et al (2004) confirms the relatively slow germination of *Poa pratensis* compared to *Festuca rubra* and *Lolium perenne*. It also suggests that *Festuca rubra* and *Lolium perenne* are differ little in speed of germination.

The present duration of the germination test for *Lolium, Festuca* and *Poa* is two, three and four weeks respectively. The prechill period, if applied, has to be added to these periods. Evidence presented during the germination workshop in Emmeloord in September 2009 suggested that hardly, if any, seeds germinate in the last week for the above mentioned species (Nijënstein, pers. comm.). The small differences present between 14 days and 21 days counts in *Festuca rubra* were not influenced by age of the seed lot nor by level of germination.

Shortening the duration will make the test more cost effective in the laboratory, and will result in seed lots being faster available for shipping.

The objective of this study was to shorten the duration of the germination test for *Lolium, Festuca* and *Poa* species.

Materials and methods

Participating laboratories

Eight laboratories from seven countries in three continents participated in this validation study:

- Agri Seed Testing, Inc., Salem, Oregon, USA.
- Landwirtschaftliches Technologie Zentrum Augustenberg, Karlsruhe, Germany.
- DLF-Trifolium Roskilde, Denmark.
- NAK, Emmeloord, the Netherlands.
- AsureQuality Limited, Palmerston North Seed Laboratory, Palmerston North, New Zealand.
- GEVES-SNES, Station Nationale d'Essais de Semences, Beaucouzé, France.
- Central Agricultural Office, Budapest, Hungary.
- Innoseeds BV, Kapelle, the Netherlands.

All participating labs are ISTA accredited for germination of the grasses in test. Samples were coded (Table 1, column 4), shipped July 11th 2011, and received by the labs within one week. Due to administrative difficulties, samples to USA and New Zealand were dispatched two weeks after the others. All tests were completed by September 19th 2011. In the report, labs are coded and in a order different from the above.

Materials

Seeds of *Lolium perenne, Festuca rubra and Poa pratensis* were used in this study. Per species four samples were germinated. Two different varieties per species, and per variety a high and a low germination percentage sample was chosen. Speed of germination was considered to be high when the difference between the first count and final count results were small. Table 1 shows the characteristics per sample at the moment of choosing it.

Table 1: Characteristics per sample.

Species	Variety	Sample id company	Sample id ISTA study	First count	Germination percentage	Speed	Level
Lolium perenne	Fornax	371011	Lp3	90	93	Fast	High
Lolium perenne	Fornax	372580	Lp1	78	80	Fast	Low
Lolium perenne	Elgon	335498	Lp2	55	97	Slow	High
Lolium perenne	Elgon	335493	Lp4	38	80	Slow	Low
Festuca rubra	Carousel	369085	Fr1	91	97	Fast	High
Festuca rubra	Carousel	372923	Fr3	74	78	Fast	Low
Festuca rubra	Casanova	372916	Fr4	59	83	Slow	High
Festuca rubra	Casanova	367305	Fr2	60	70	Slow	Low
Poa pratensis	Yvette	371935	Pp1	92	92	Fast	High
Poa pratensis	Yvette	371970	Pp2	67	68	Fast	Low
Poa pratensis	Oxford	372833	Pp3	64	81	Slow	High
Poa pratensis	Oxford	371938	Pp4	60	62	Slow	Low

All seed lots were older than six months. As a consequence hardly any dormancy if at all will have been present. Reasons for avoiding dormant samples were of practical and theoretical nature. Practical, because screening for dormancy in samples, and dispatching them within a few weeks would be a too heavy burden for a lab in such a busy part of the season. Theoretical, because from present day germination testing in grasses we already know that sometimes the duration of the test has to be extended because of dormant seeds. Shortening the duration would for sure result in the final count being different from the one before.

In order to avoid any influence of doing the purity in individual labs, seeds of all species was purified by blowing out all empty seeds by the organising lab before dispatch. Other seeds were not removed; labs discarded other seeds, whenever found at starting the germination tests.

Test method

If not indicated differently, labs adhered to the instructions as in the ISTA Rules (Anonymous, 2011).

Germination temperatures may affect the speed of germination. Therefore samples were germinated at all temperatures that are allowed according to the ISTA Rules (Anonymous, 2011):

- *Lolium* at 15/25, 20/30 and 20°C,
- *Festuca* at 15/25 and 20/30,
- *Poa* at 10/30, 15/25 and 20/30.

Lab no. 5 was not able to do the 15/25 temperature regime.

For dormancy breaking, both KNO_3 and a prechill of 2 days at 7°C were applied at the same time on all samples.

Counts were made at the following stages:

- *Lolium*: 7, 10, 14 days.
- *Festuca*: 7, 14, 21 days.
- *Poa*: 7, 14, 21, 28 days.

This comparative test thus involved a total of 3 species * 4 lots/species * 2 (*Festuca*)-3 (*Lolium* and *Poa*) temperatures/sample = 32 germination tests completed by each of the participants.

Before distributing the seeds to the participating labs, the samples were tested for homogeneity at temperature regimes of 20/30, 15/25 and 20°C. Of all 32 ‘treatments’, 8 samples were prepared and germinated. Results were heterogeneity was calculated according to ISTA Rules, chapter 2 (Anonymous, 2011). All results were within tolerance.

Labs reported normal seedlings, abnormal seedlings, fresh non germinated seeds and dead seeds. The data received from the participants were checked for completeness and plausibility, and found to be ok. In order to limit the workload, only normal seedlings will be dealt with in this report.

Statistical analysis of the test results were performed using methods derived from Generalized Linear modelling and ISTA Tolerance approach.

Results

Statistical analysis – calculation of repeatability

Repeatability is the closeness of the agreement between the results of successive measurement of the same measure and carried out in the same conditions of measurement.

For each temperature, let:

I be the total number of lots

J be the total number of labs

K be the number of reps of *m* seeds for a given lot in a given lab

p_{ijk} be the percentage of germinated seeds for lot *i*, lab *j* and rep *k* at a given number of days

The repeatability standard-deviation is computed as:

$$S_r = \sqrt{f_r^2 \frac{\overline{p}_{...}\left(100 - \overline{p}_{...}\right)}{m}}$$

where:

$\overline{p}_{...}$ is the overall average percentage of germinated seeds.

f_r^2 is an estimate of the dispersion parameter:

$$f_r^2 = \frac{1}{IJ}\sum_{i,j}\frac{var\_obs_{ij}}{var\_bin_{ij}}$$

where:

$var\_obs_{ij} = \frac{1}{K-1}\sum_k\left(p_{ijk} - \overline{p}_{ij.}\right)^2$ and $var\_bin_{ij} = \frac{\overline{p}_{ij.}\left(100 - \overline{p}_{ij.}\right)}{m}$ with $\overline{p}_{ij.}$ being the average percentage of germinated seeds in lot *i* and lab *j*

If f_r^2 > 1 one speaks of overdispersion because the data have larger variance than expected under the assumption of a binomial distribution.

Statistical analysis – calculation of reproducibility

Reproducibility is the precision under reproducibility conditions, i.e. conditions where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment.

For each temperature, the reproducibility standard-deviation is computed as:

$$S_R = \sqrt{\frac{1}{I}\sum_i\sum_j\frac{\left(p_{ij.} - \overline{p}_{i..}\right)^2}{J-1}}$$

where:

i = 1, 2, …, *I* *j* = 1, 2, …, *J*

$p_{ij.}$ is the percentage of germinated seeds out of *n* in lot *i* and lab *j* at a given number of days

$$\overline{p}_{i..} = \frac{\sum_j p_{ij.}}{J}$$

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

$$\mathrm{Var}\left(p_{ij.}\right) = \frac{p_{ij.}\left(100 - p_{ij.}\right)}{n}$$

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

$$f_R^2 = \frac{n S_R^2}{\overline{p}_{...}\left(100 - \overline{p}_{...}\right)} \text{ where } \overline{p}_{...} = \frac{\sum_{i,j} p_{ij.}}{IJ}$$

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

Data checking

Data checking has been performed according to ISTA rules by computing tolerances for germination test replicates. See Annex 2 for details. All results of *Lolium* are within tolerance.

For *Festuca* a number of results out of tolerance were found:

	Normal 7 days	Normal 10 days	Normal 14 days
Temp 15-25	6	1	1
Temp 20-30	1	0	0

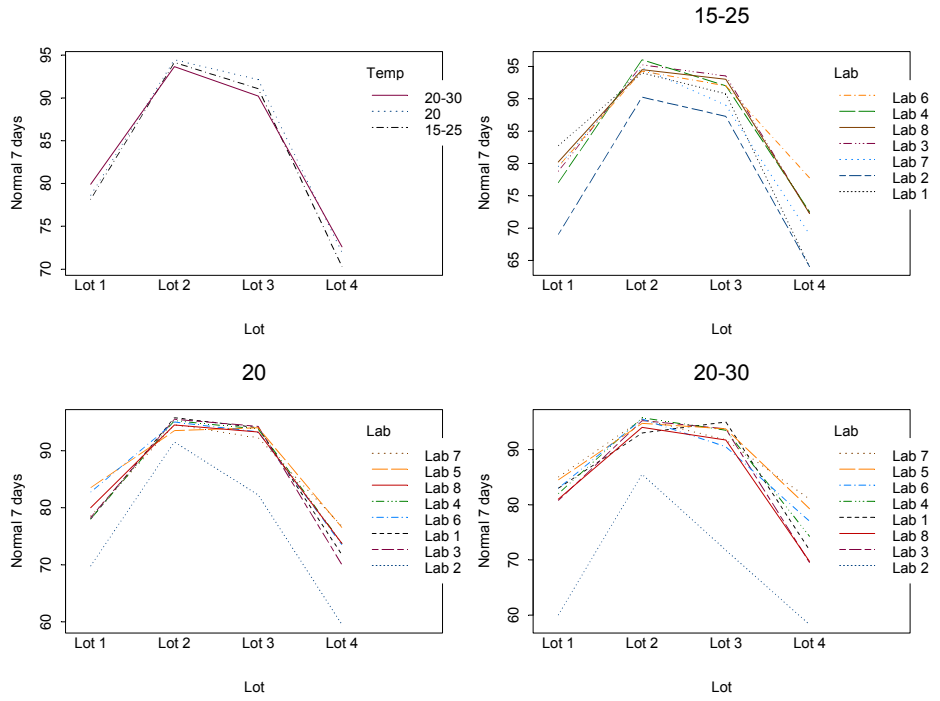
Number of results out of tolerance for *Poa:*

	Normal 7 days	Normal 14 days	Normal 21 days	Normal 28 days
Temp 10-30	1	1	1	1
Temp 15-25	2	0	0	1
Temp 20-30	0	1	2	0

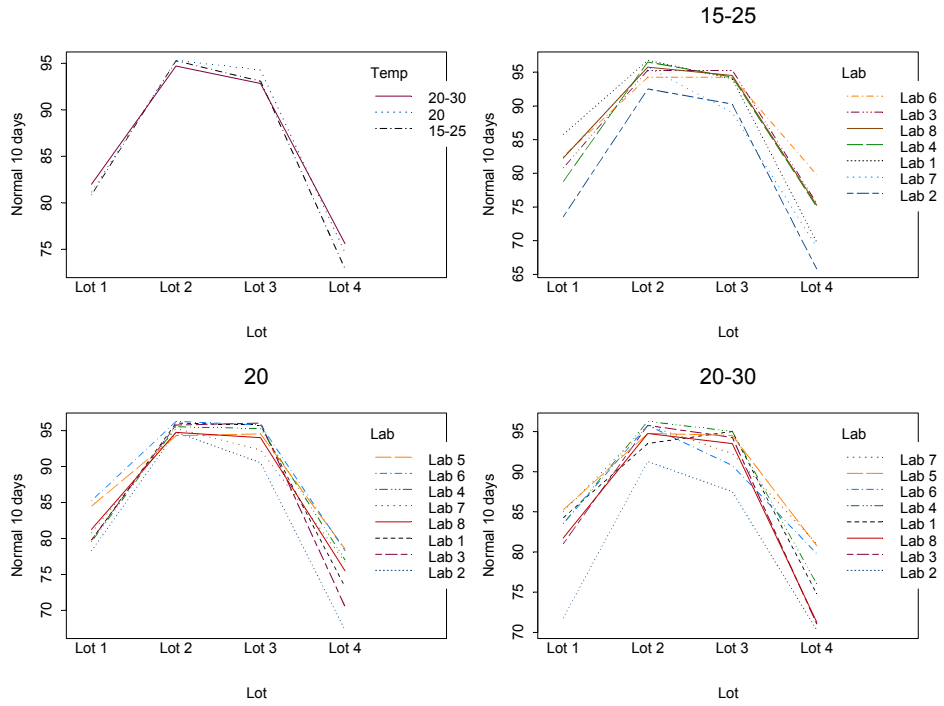
Data exploration with interaction plots - *Lolium*

Interaction plots are used in order to find deviating results. The first figure are results averaged over all labs. Next figures show individual results per lab and per temperature regime.

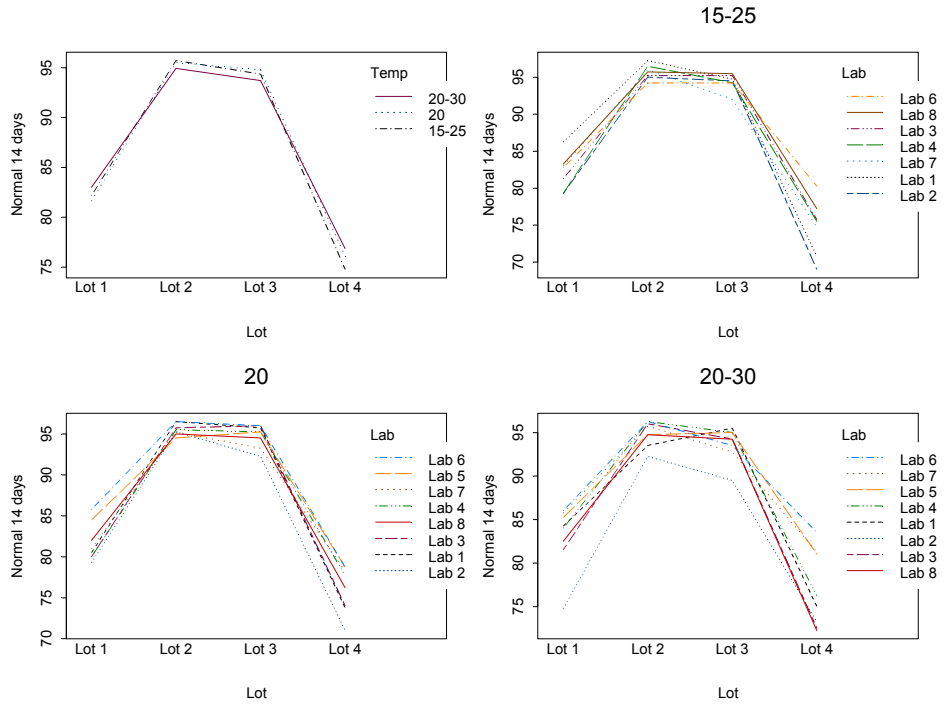
Normal 7 days: Lab 2 results very different from the others for temp 20 and 20-30, and to a lesser degree, for temp 15-25



Normal 10 days: Lab 2 results very different from the others for temp 20-30, and to a lesser degree, for temp 20 and 15-25

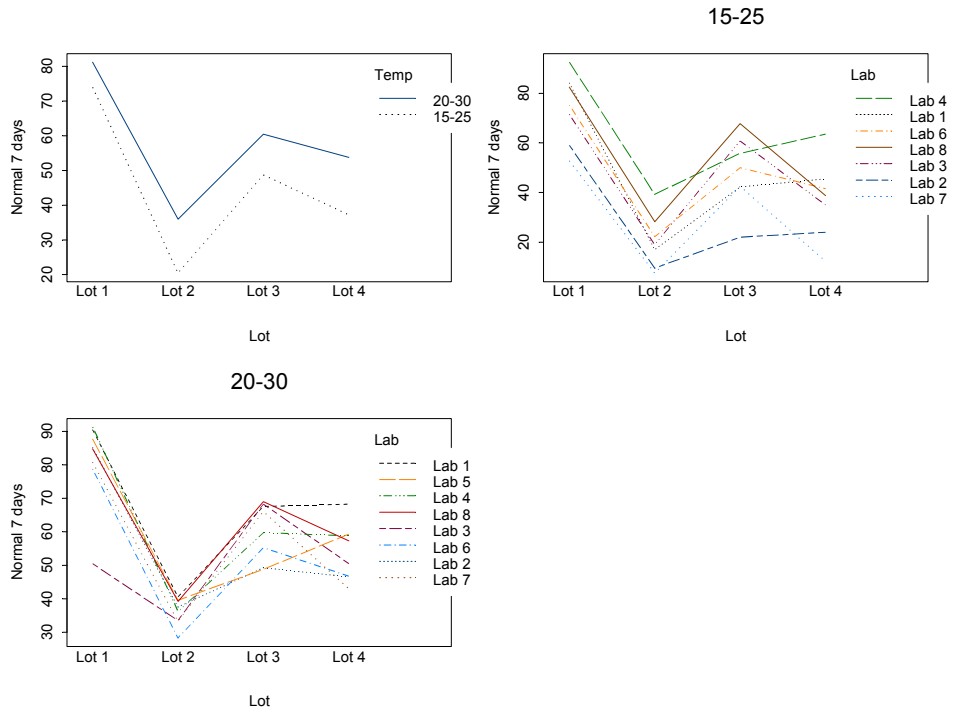


Normal 14 days: Lab 2 results different from the others for temp 20-30

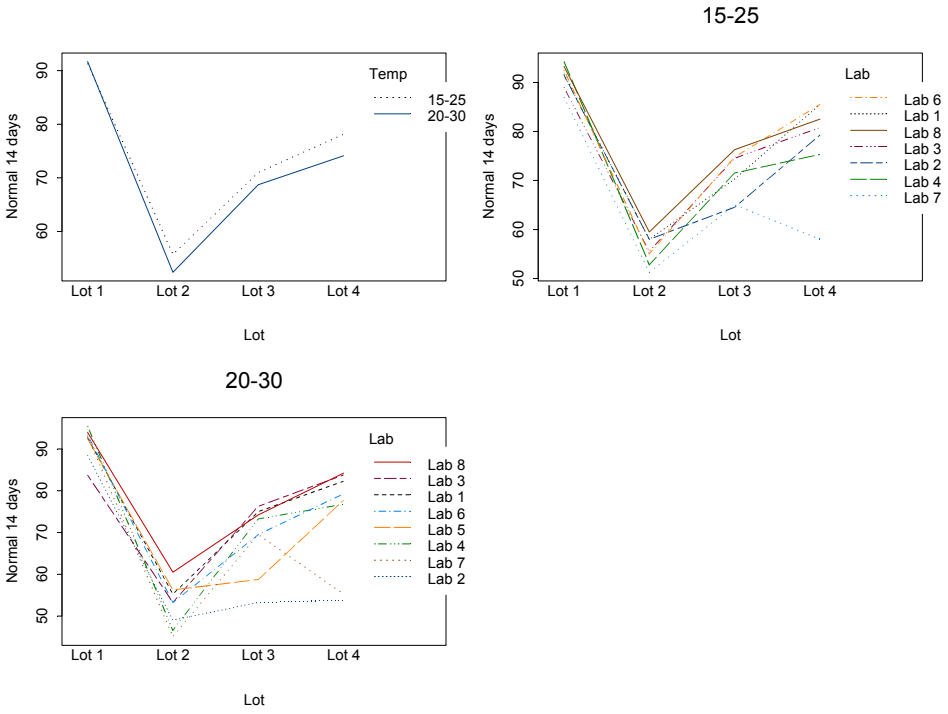


Data exploration with interaction plots - *Festuca*

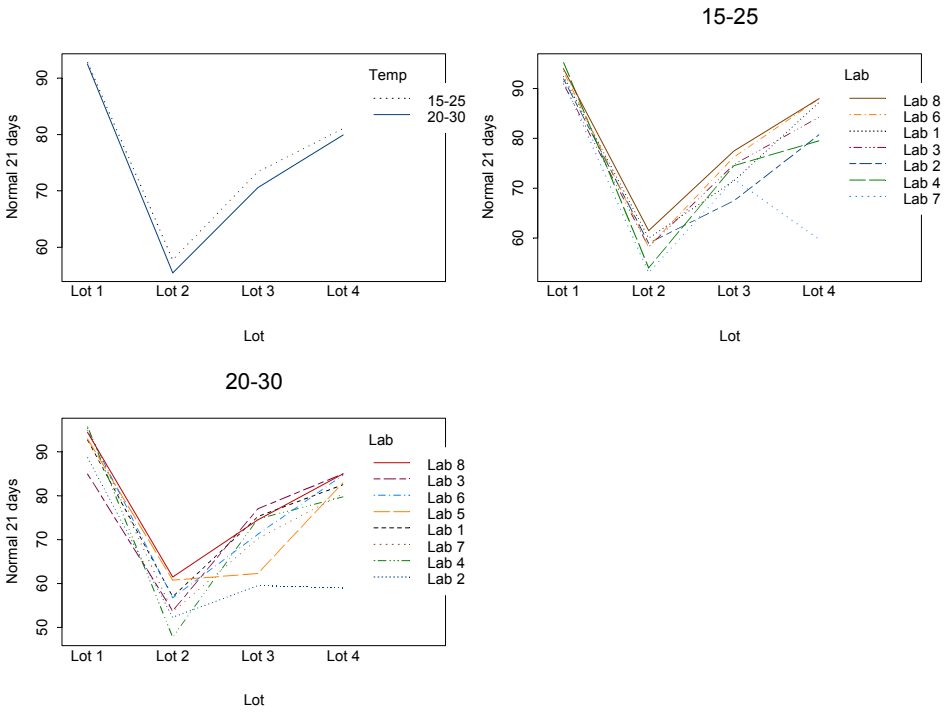
Normal 7 days:



Normal 14 days:



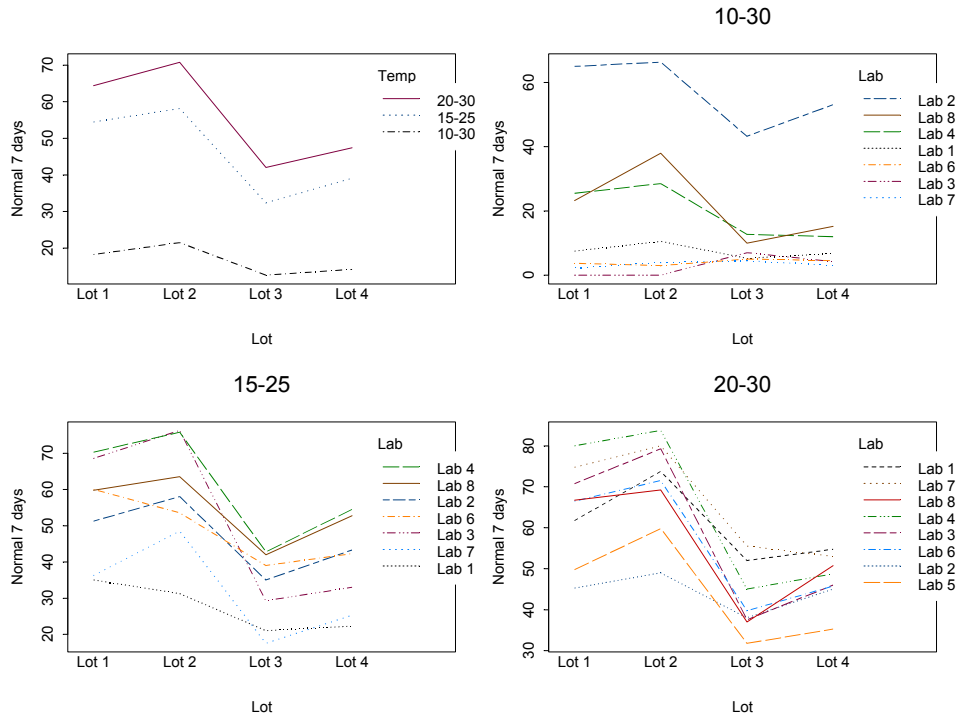
Normal 21 days:



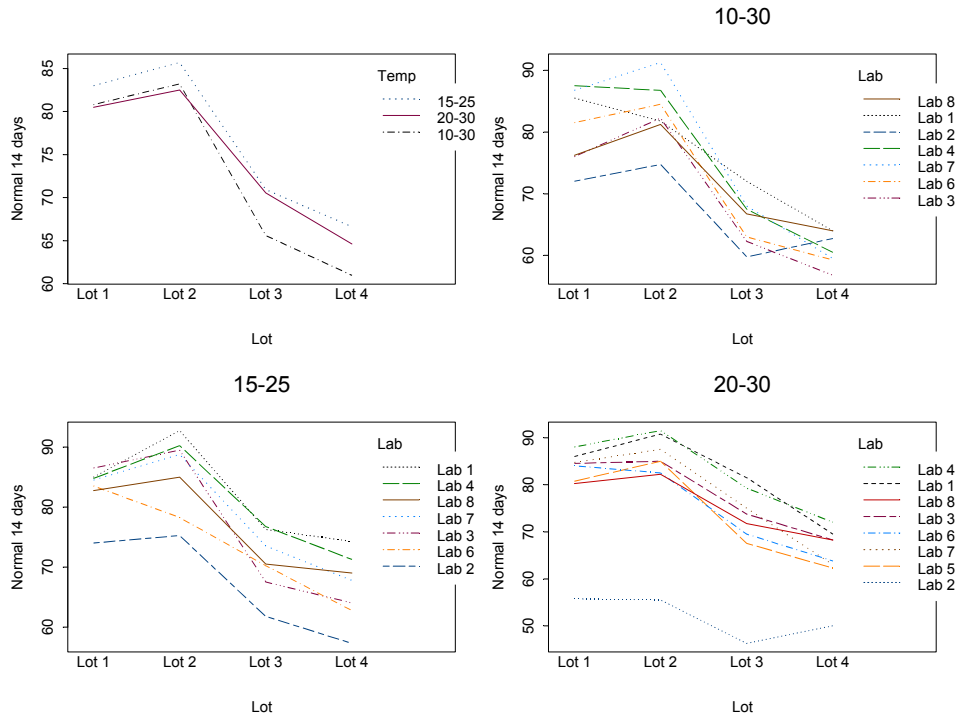
Labs 2 and 7 exhibit sometimes different results from the others for lot 4 in particular.

Data exploration with interaction plots - *Poa*

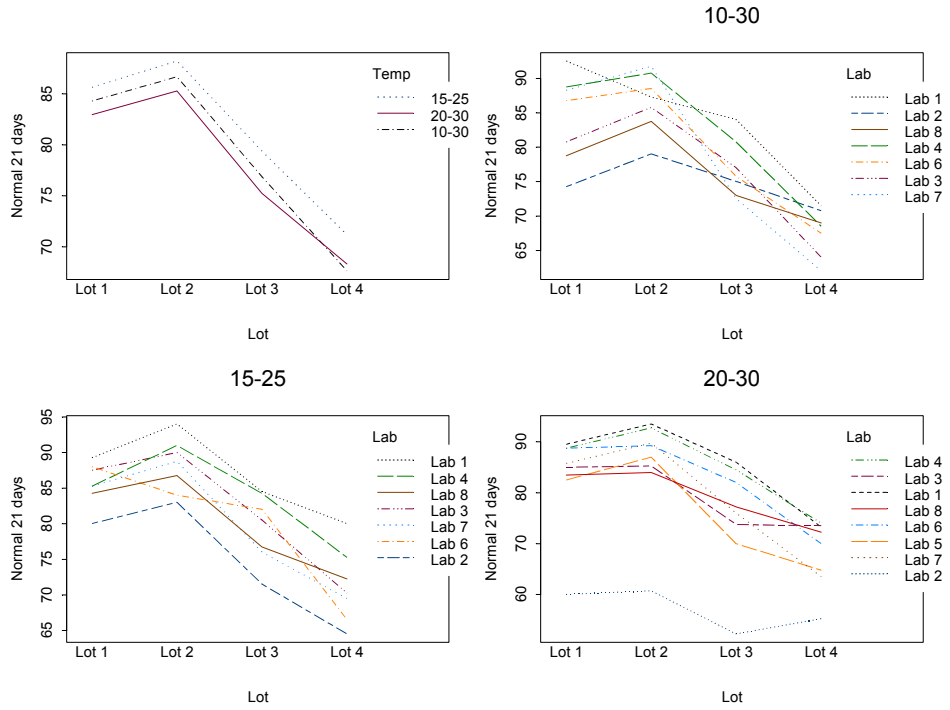
Normal 7 days: high between-labs variability.



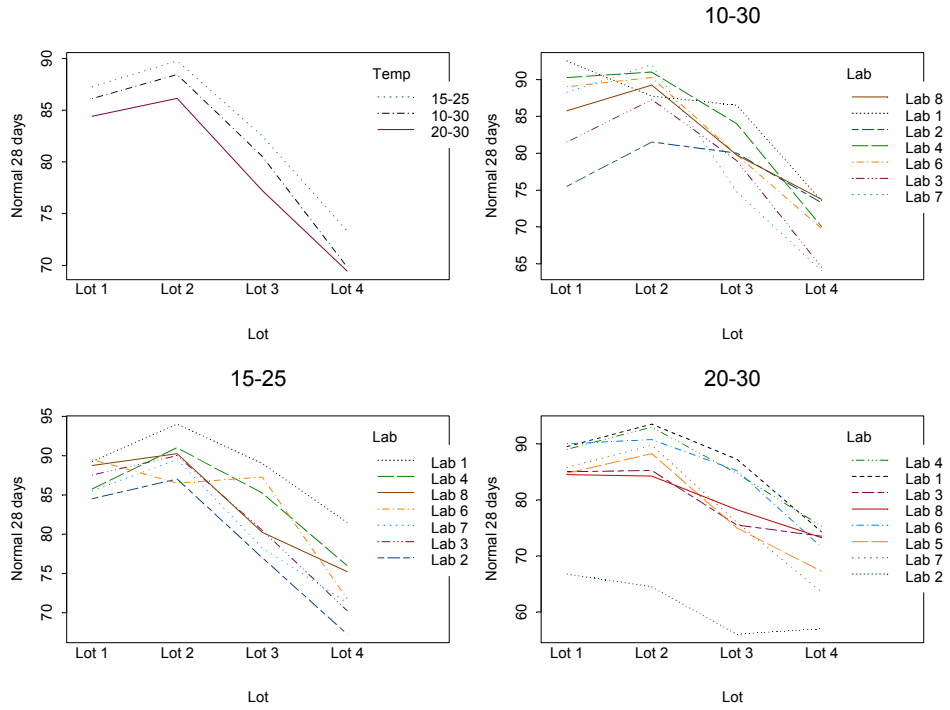
Normal 14 days: Lab 2 results very different from the others for temp 20-30, and to a lesser degree, for temp 10-30 and 15-25.



Normal 21 days: Lab 2 very different from the others for temp 20-30, and to a lesser degree, for temp 10-30 and 15-25.



Normal 28 days: Lab 2 very different from the others for temp 20-30, and to a lesser degree, for temp 10-30.



Repeatability and reproducibility:

$\overline{p}_{...}$ is the overall average percentage of germinated seeds.

S_r is the repeatability standard-deviation

S_R is the reproducibility standard-deviation

f_r^2 is an estimate of the dispersion parameter

If f_r^2 > 1 one speaks of overdispersion because the data have larger variance than expected under the assumption of a binomial distribution.

See page 4 and 5 for explanation of calculation of these parameters.

Repeatability *Lolium*:

Normal 7 days

15-25

$\overline{p}_{...}$	S_r	f_r
83.39	3.21	0.86

Normal 10 days

15-25

$\overline{p}_{...}$	S_r	f_r
85.5	2.89	0.82

Normal 14 days

15-25

$\overline{p}_{...}$	S_r	f_r
86.74	2.79	0.82

20

$\overline{p}_{...}$	S_r	f_r
84.27	4.03	1.11

20

$\overline{p}_{...}$	S_r	f_r
86.33	3.71	1.08

20

$\overline{p}_{...}$	S_r	f_r
87	3.49	1.04

20-30

$\overline{p}_{...}$	S_r	f_r
84.09	3.92	1.07

20-30

$\overline{p}_{...}$	S_r	f_r
86.29	3.56	1.04

20-30

$\overline{p}_{...}$	S_r	f_r
87.13	3.36	1.00

No overdispersion for temp 15-25 at all days and for temp 20-30 at 14 days. Largest overdispersion factors are observed for temp 20 at 7 and 10 days and for temp 20-30 at 7 days.

Results repeatability - *Festuca*:

Normal 7 days

15-25

$\overline{p}_{...}$	S_r	f_r
45.07	6.7	1.35

Normal 14 days

15-25

$\overline{p}_{...}$	S_r	f_r
74.03	4.59	1.05

Normal 21 days

15-25

$\overline{p}_{...}$	S_r	f_r
76.25	4.4	1.03

20-30

$\overline{p}_{...}$	S_r	f_r
57.86	5.24	1.06

20-30

$\overline{p}_{...}$	S_r	f_r
71.73	3.81	0.85

20-30

$\overline{p}_{...}$	S_r	f_r
74.62	3.75	0.86

There is no overdispersion for temp 20-30 at 14 and 21 days. Very high overdispersion (as confirmed by the number of results out of tolerances) for temp 15-25 at 7 days.

Results repeatability - *Poa*

Normal 7 days

10-30

$\overline{p}_{...}$	S_r	f_r

Normal 14 days

10-30

$\overline{p}_{...}$	S_r	f_r

Normal 21 days

10-30

$\overline{p}_{...}$	S_r	f_r

Normal 28 days

10-30

$\overline{p}_{...}$	S_r	f_r

16.57	3.7	1.00	72.64	4.91	1.10	78.86	4.67	1.14	81.22	4.1	1.05

15-25 15-25 15-25 15-25

$\overline{p}_{...}$	S_r	f_r	$\overline{p}_{...}$	S_r	f_r	$\overline{p}_{...}$	S_r	f_r	$\overline{p}_{...}$	S_r	f_r
45.98	4.84	0.97	76.55	4.18	0.99	81.1	4.07	1.04	83.21	3.93	1.05

20-30 20-30 20-30 20-30

$\overline{p}_{...}$	S_r	f_r	$\overline{p}_{...}$	S_r	f_r	$\overline{p}_{...}$	S_r	f_r	$\overline{p}_{...}$	S_r	f_r
56.17	5.2	1.05	74.55	4.56	1.05	77.95	4.76	1.15	79.31	4.1	1.01

The largest overdispersion factors are observed for temperature 10-30 at 14 days and 21 days and for temperature 20-30 at 21 days.

Results reproducibility – *Lolium*

Normal 7 days | Normal 10 days | Normal 14 days

15-25 | 15-25 | 15-25

$\overline{p}_{...}$	S_R	f_R	f	$\overline{p}_{...}$	S_R	f_R	f	$\overline{p}_{...}$	S_R	f_R	f
83.39	3.62	1.94	1.69	85.5	3.4	1.93	1.67	86.74	2.39	1.41	1.66

20 | 20 | 20

$\overline{p}_{...}$	S_R	f_R	f	$\overline{p}_{...}$	S_R	f_R	f	$\overline{p}_{...}$	S_R	f_R	f
84.27	4.06	2.23	1.68	86.33	2.58	1.50	1.66	87.00	2	1.19	1.66

20-30 | 20-30 | 20-30

$\overline{p}_{...}$	S_R	f_R	f	$\overline{p}_{...}$	S_R	f_R	f	$\overline{p}_{...}$	S_R	f_R	f
84.09	6.86	3.75	1.68	86.29	3.51	2.04	1.66	87.13	3.12	1.86	1.65

The f_R values are inferior to the f values for temp 15-25 at 14 days and for temp 20 at 10 and 14 days.

Let's see the reproducibility computations after excluding laboratory 2, for which different results from the other labs are observed.

Results – Lab 2 excluded:

Normal 7 days | Normal 10 days | Normal 14 days

15-25 | 15-25 | 15-25

$\overline{p}_{...}$	S_R	f_R	f
84.35	2.62	1.44	1.68

$\overline{p}_{...}$	S_R	f_R	f
86.33	2.62	1.53	1.66

$\overline{p}_{...}$	S_R	f_R	f
87.12	2.11	1.26	1.66

20

$\overline{p}_{...}$	S_R	f_R	f
85.49	1.76	1.00	1.67

20

$\overline{p}_{...}$	S_R	f_R	f
86.85	2.02	1.19	1.66

20

$\overline{p}_{...}$	S_R	f_R	f
87.37	1.69	1.02	1.65

20-30

$\overline{p}_{...}$	S_R	f_R	f
86.27	2.6	1.51	1.66

20-30

$\overline{p}_{...}$	S_R	f_R	f
87.16	2.46	1.47	1.65

20-30

$\overline{p}_{...}$	S_R	f_R	f
87.80	2.51	1.53	1.65

All the f_R values are now inferior to the f values.

Results reproducibility - *Festuca*:

Normal 7 days

15-25

$\overline{p}_{...}$	S_R	f_R	f
45.07	14.23	5.72	1.91

Normal 14 days

15-25

$\overline{p}_{...}$	S_R	f_R	f
74.03	5.68	2.59	1.76

Normal 21 days

15-25

$\overline{p}_{...}$	S_R	f_R	f
76.25	5.56	2.61	1.75

20-30

$\overline{p}_{...}$	S_R	f_R	f
57.86	9.14	3.70	1.90

20-30

$\overline{p}_{...}$	S_R	f_R	f
71.73	8.14	3.62	1.78

20-30

$\overline{p}_{...}$	S_R	f_R	f
74.62	6.16	2.83	1.76

All the f_R values are greater than the f values.

Let's see the reproducibility computations after excluding laboratories 2 and 7, for which different results from the other labs are observed, in particular for lot 4.

Results – Labs 2 and 7 excluded:

Normal 7 days

15-25

$\overline{p}_{...}$	S_R	f_R	f
51.62	9.57	3.83	1.95

Normal 14 days

15-25

$\overline{p}_{...}$	S_R	f_R	f
75.91	2.96	1.38	1.75

Normal 21 days

15-25

$\overline{p}_{...}$	S_R	f_R	f
77.99	2.68	1.30	1.73

20-30

$\overline{p}_{...}$	S_R	f_R	f
58.75	9.80	3.98	1.89

20-30

$\overline{p}_{...}$	S_R	f_R	f
74.50	4.77	2.19	1.76

20-30

$\overline{p}_{...}$	S_R	f_R	f
76.19	4.30	2.02	1.75

The f_R values are smaller than the f values for temp 15-25 at 14 and 21 days.

Results reproducibility - *Poa*:

Normal 7 days

10-30

$\overline{p}_{...}$	S_R	f_R	f
16.57	20.18	10.85	1.68

15-25

$\overline{p}_{...}$	S_R	f_R	f
45.98	13.39	5.37	1.92

20-30

$\overline{p}_{...}$	S_R	f_R	f
56.17	9.71	3.91	1.91

Normal 14 days

10-30

$\overline{p}_{...}$	S_R	f_R	f
72.64	4.7	2.11	1.78

15-25

$\overline{p}_{...}$	S_R	f_R	f
76.55	5.49	2.59	1.74

20-30

$\overline{p}_{...}$	S_R	f_R	f
74.55	10.03	4.60	1.76

Normal 21 days

10-30

$\overline{p}_{...}$	S_R	f_R	f
78.86	4.76	2.33	1.72

15-25

$\overline{p}_{...}$	S_R	f_R	f
81.10	4.34	2.22	1.71

20-30

$\overline{p}_{...}$	S_R	f_R	f
77.95	9.51	4.59	1.73

Normal 28 days

10-30

$\overline{p}_{...}$	S_R	f_R	f
81.22	4.41	2.26	1.7

15-25

$\overline{p}_{...}$	S_R	f_R	f
83.21	3.67	1.96	1.69

20-30

$\overline{p}_{...}$	S_R	f_R	f
79.31	8.4	4.15	1.72

All the f_R values are greater than the f values.

Let's see the reproducibility computations after excluding laboratory 2, for which different results from the other labs are observed at 14, 21 and 28 days:

Results – Lab 2 excluded:

Normal 7 days

10-30

$\overline{p}_{...}$	S_R	f_R	f

Normal 14 days

10-30

$\overline{p}_{...}$	S_R	f_R	f

9.85	10.03	6.73	1.62	73.53	3.95	1.79	1.77

15-25

$\overline{p}_{...}$	S_R	f_R	f
45.83	14.6	5.86	1.92

15-25

$\overline{p}_{...}$	S_R	f_R	f
78.14	3.89	1.88	1.73

20-30

$\overline{p}_{...}$	S_R	f_R	f
57.87	8.3	3.36	1.9

20-30

$\overline{p}_{...}$	S_R	f_R	f
77.79	3.89	1.87	1.73

Normal 21 days

10-30

$\overline{p}_{...}$	S_R	f_R	f
79.54	4.14	2.05	1.72

Normal 28 days

10-30

$\overline{p}_{...}$	S_R	f_R	f
81.83	3.64	1.89	1.7

15-25

$\overline{p}_{...}$	S_R	f_R	f
82.16	3.6	1.88	1.7

15-25

$\overline{p}_{...}$	S_R	f_R	f
83.92	3.37	1.83	1.68

20-30

$\overline{p}_{...}$	S_R	f_R	f
80.93	4.3	2.19	1.71

20-30

$\overline{p}_{...}$	S_R	f_R	f
81.92	4.03	2.09	1.7

At 14, 21 and 28 days, all the f_R values are closer to the f values but still greater.

Statistics – conclusion

In *Lolium* the 15-25 temperature regime is the temperature for which the repeatability and reproducibility criteria are all met after excluding lab 2. These criteria are almost met for temp 20-30 at 10 and 14 days.

For *Festuca* there is no temperature for which repeatability and reproducibility criteria are all met. These criteria are almost met at 14 and 21 days for the two temperatures.

Repeatability and reproducibility are similar for the last two counts in all species.

Also in *Poa* there is no temperature for which repeatability and reproducibility criteria are all met. Temp 15-25 is however a temperature for which these criteria are almost met at 14, 21 and 28 days.

This means that there is more variation within lab (repeatability variance) and across labs (reproducibility variance than expected), also after excluding lab 2 for *Poa* and labs 2 and 7 for *Festuca*. This accounts for all counting dates, including the ones present in the ISTA Rules at this moment. Therefore there is no reason for not accepting the shorter durations because of not fully meeting the present statistical requirements for repeatability and reproducibility.

At this moment no statistical tests exist for comparing observed repeatabilities/reproducibilities with expected repeatabilities/reproducibilities. However, as the observed results are only slightly different from the expected ones, it can be concluded that the results are acceptable.

Validation study comparison of species, temperature and duration of the test

Lab 2 had some deviating results. Therefore averages were calculated including and excluding lab 2. The results in Annex 3 demonstrate that influence on the results of lab 2 is limited. The pattern is the same for both sets. This section is therefor based on all results, including lab 2.

Table 1: Average results per treatment, for all labs. See for explanation of first two columns Materials-section. In top row: species, temperature regime. In second row counting dates.

Lp		**15-25**			**20**			**20-30**		
		7	**10**	**14**	**7**	**10**	**14**	**7**	**10**	**14**
Lp3	**fast high**	91,1	93,1	94,4	92,1	94,3	94,8	90,2	92,8	93,7
Lp1	**fast low**	78,1	80,8	82,1	78,6	81,1	81,6	79,9	82,0	83,0
Lp2	**slow high**	94,1	95,3	95,7	94,4	95,3	95,5	93,7	94,7	94,9
Lp4	**slow low**	70,3	72,9	74,8	71,9	74,6	76,1	72,6	75,6	76,9
avg		80,8	83,0	84,2	81,6	83,7	84,4	82,1	84,1	84,9

Fr		**15-25**			**20-30**		
		7	**14**	**21**	**7**	**14**	**21**
Fr1	**fast high**	74,0	91,3	92,8	81,2	91,7	92,5
Fr3	**fast low**	48,7	71,0	73,4	60,5	68,7	70,6
Fr4	**slow high**	37,2	78,1	81,1	53,8	74,1	79,9
Fr2	**slow low**	20,4	55,7	57,7	36,0	52,4	55,4
avg		45,1	74,0	76,3	57,9	71,7	74,6

Pp		**10-30**				**15-25**				**20-30**			
		7	**14**	**21**	**28**	**7**	**14**	**21**	**28**	**7**	**14**	**21**	**28**
Pp1	**fast high**	**18,2**	80,8	84,3	86,1	54,4	83,0	85,6	87,3	64,4	80,5	83,0	84,4
Pp2	**fast low**	**21,5**	83,2	86,7	88,4	58,1	85,7	88,2	89,8	70,8	82,5	85,3	86,2
Pp3	**slow high**	**12,5**	65,6	76,9	80,5	32,4	70,9	79,4	82,5	42,1	70,6	75,2	77,3
Pp4	**slow low**	**14,1**	61,0	67,6	69,8	39,0	66,6	71,2	73,3	47,4	64,6	68,3	69,4
avg		16,6	72,7	78,9	81,2	46,0	76,6	81,1	83,2	56,2	74,6	78,0	79,3

Final germination percentages per species are very similar for all temperature treatments (table 1).

Of the seven labs, only one lab reported fresh non-germinated seeds (6 seeds out of 12.800 tested by this lab). This confirms that hardly any to no dormancy was present in the samples.

The effect of temperature on speed of germination is clearly visible in all samples of *Festuca* and *Poa*: a higher temperature results in highest first count. Second counts in *Festuca* and second and third counts are slightly higher at 15-25 compared with 20-30^{o}C however.

This temperature effect is not visible in *Lolium*.

All sample type combinations of fast/slow and high/low respond in the same pattern.

Differences between last counts and the ones before are 0.7-1.2% for *Lolium*, 2.2-2.9% for *Festuca* and 1.4-2.3% in *Poa* (table 2).

In *Lolium* and *Poa* the effect of sample type is not apparent. In *Lolium* these differences range from 0.2 – 1.9% and in *Poa* from 0.9-3.6%. In *Festuca* this ranger is wider (0.8-5.8%), and there seems to be more of a variety effect: fast ranging 7.5-8.7% overall, and slow 14.2-17.5%.

In *Poa* there is clear tendency towards smaller differences between 28 and 21 days counts when temperatures increase (2.3 → 2.1 → 1.4%). In *Lolium* and *Festuca* this effect is not visible.

Table 2: Differences between counting dates. See for explanation of first two columns Materials-section. In top row: species, temperature regime. In second row counting dates.

Lp		15-25		20		20-30	
		10-7	14-10	10-7	14-10	10-7	14-10
Lp3	fast high	2,0	1,3	2,2	0,5	2,6	0,9
Lp1	fast low	2,7	1,3	2,5	0,5	2,1	1,0
Lp2	slow high	1,2	0,4	0,9	0,2	1,0	0,2
Lp4	slow low	2,6	1,9	2,7	1,5	3,0	1,3
avg		2,2	1,2	2,0	0,7	2,0	0,8

Fr		15-25		20-30	
		14-7	21-14	14-7	21-14
Fr1	fast high	17,3	1,5	10,5	0,8
Fr3	fast low	22,3	2,4	8,2	1,9
Fr4	slow high	40,9	3,0	20,3	5,8
Fr2	slow low	35,3	2,0	16,4	3,0
avg		29,0	2,2	13,9	2,9

Pp		10-30			15-25			20-30		
		14-7	21-14	28-21	14-7	21-14	28-21	14-7	21-14	28-21
Pp1	fast high	62,6	3,5	1,8	28,6	2,6	1,7	16,1	2,5	1,4
Pp2	fast low	61,7	3,5	1,7	27,6	2,5	1,6	11,7	2,8	0,9
Pp3	slow high	53,1	11,3	3,6	38,5	8,5	3,1	28,5	4,6	2,1
Pp4	slow low	46,9	6,6	2,2	27,6	4,6	2,1	17,2	3,7	1,1
avg		56,1	6,2	2,3	30,6	4,6	2,1	18,4	3,4	1,4

The differences between the last count and the count before were statistically analyzed by means of multiple comparisons using the global model with a focus on comparing the two last counts globally and for each lot. See annex 1 for details.

Table 3: differences between counting dates and temperatures compared. See for explanation of first two columns Materials-section. In top row: what is compared; in second species and temperature regime. In third row: counting days that are compared or temperature regimes that are compared.

All labs		between last two counts					differences between temperatures			
Lp		15-25	20	20-30						
		14-10	14-10	14-10	AVG		15/25-20	15/25-20/30	20-20/30	AVG
Lp3	fast high	1,3	0,5	0,9	**0,9**		0,4	0,7	1,1	**0,7**
Lp1	fast low	1,3	0,5	1,0	**0,9**		0,5	0,9	1,4	**0,9**
Lp2	slow high	0,4	0,2	0,2	**0,3**		0,2	0,8	0,6	**0,5**
Lp4	slow low	1,9	1,5	1,3	**1,6**		1,3	2,1	0,8	**1,4**
avg		**1,2**	**0,7**	**0,8**	**0,9**		**0,2**	**0,7**	**0,5**	**0,5**
Fr		15-25	20-30							
		21-14	21-14				15/25-20	15/25-20/30	20-20/30	
Fr1	fast high	1,5	0,8		**1,2**			0,3		**0,3**
Fr3	fast low	2,4	1,9		**2,2**			2,8		**2,8**
Fr4	slow high	3,0	5,8		**4,4**			1,2		**1,2**
Fr2	slow low	2,0	3,0		**2,5**			2,3		**2,3**
avg		**2,2**	**2,9**		**2,6**			**1,7**		**1,7**
Pp		10-30	15-25	20-30						
		28-21	28-21	28-21			15/25-10/30	15/25-20/30	10/30-20/30	
Pp1	fast high	1,8	1,7	1,4	**1,6**		1,2	2,9	1,7	**1,9**
Pp2	fast low	1,7	1,6	0,9	**1,4**		1,4	3,6	2,2	**2,4**
Pp3	slow high	3,6	3,1	2,1	**2,9**		2,0	5,2	3,2	**3,5**
Pp4	slow low	2,2	2,1	1,1	**1,8**		3,5	3,9	0,4	**2,6**
avg		**2,3**	**2,1**	**1,4**	**1,9**		**2,0**	**3,9**	**1,9**	**2,6**

The differences between final counts of different temperatures are similar in magnitude compared to differences between the two last counts (Table 3). In *Lolium* and *Festuca* differences between the last two counts are slightly bigger than the differences between temperature regimes. In *Poa* the differences between temperature regimes are slightly bigger.

Table 4: Results and statistical evaluations of last two counts per species. Averages and P-value. A P-value in read indicates: statistically significant differences between the two values at p<0.05.

Festuca	14 days	21 days	P-value
Overall mean	78.74%	80.66%	0.0002
Lot 1	92.48%	93.36%	0.1385
Lot 2	54.97%	56.91%	0.0828
Lot 3	73.61%	74.77%	0.2360
Lot 4	81.80%	84.61%	0.0009

Lolium	10 days	14 days	P-value
Overall mean	89.30%	89.89%	0.0442
Lot 1	82.18%	82.83%	0.3023
Lot 2	95.59%	95.75%	0.6423
Lot 3	94.15%	94.65%	0.1981
Lot 4	75.12%	76.48%	0.0590

Poa	21 days	28 days	P-value
Overall mean	82.53%	84.07%	<.0001
Lot 1	86.79%	87.86%	0.0578
Lot 2	89.13%	90.03%	0.0821
Lot 3	79.59%	82.16%	0.0001
Lot 4	70.34%	72.03%	0.0269

The difference between two counts for the overall test result is always significant as the error df is high for this experiment (table 4).

There are some significant differences for some lots (highlighted in red). However, when having a closer look at the interaction plots in Annex 1, it is cleat that this has no consequence on the lot rankings (no 'cross overs' in the figures in Annex 1 between last and last but one count).

Additional remarks made by labs

One lab (no. 7) reported mould on germination blotters of the *Festuca* samples. This mould was not confined to one sample, but was present on the entire plate at one specific temperature condition.

Lab 2 reported 'much more root abnormalities' in *Festuca* seedlings at 20/30°C.

None of these appear to have had an effect on the final results.

The AOSA Rules already have a germination test duration of 21 days for *Poa* species.

Additional results from routine tests

Particpants were asked to submit data from own testing, when available. An overview of these data can be found in Annex 4. Averages per species are summarized in Table 5. These additional data are in line with the findings obtained in the comparative study, as can be seen in the last two columns.

Table 5: Routine data received from participating laboratories.

			Average per count (days)						Differences		
Lab	Species	# samples	6	7	10	14	21	28	2nd-1st	3rd-2nd	4rd-3rd
4	Lolium perenne	33		80,6	87,3	89,0			6,6	1,7	
5	Lolium perenne		81,0			90,5			9,5		
5	Lolium multiflorum	4	86,3			89,5			3,3		
5	Loliumxboucheanu	4	89,0			92,3			3,3		
4	Festuca rubra	29		54,0		83,6	85,1		29,7	1,4	
4	Poa pratensis	34		70,7		87,3	88,7		16,7	1,3	0,7

Discussion

This validation study confirm the findings that the difference between the final count and the one before is small. Only very limited numbers of seeds germinate in the last weeks in all three species.

As all seed lots were older than six months and hardly any fresh non-germinated seeds were found, limited dormancy levels will have been present in the samples (Stanisavljevic et al, 2010 and 2011). The study by Wiesner et al (1972) is already more than 40 years old. In the mean time dormancy levels in modern varieties will have been lowered through breeding.

The objective of this validation study was to be critical at all counting dates, and really count all normal seedlings present at all counts. However, as it was not indicated very precisely in the protocol, some of the labs in case of doubt may have left un-assessed questionable seedlings on the blotter until the next count. When forced to take a decision before the final count, part of these would have ended up in the normal seedlings categories.

When asked, 4 out of the 6 responding labs confirmed that this might have happened in their lab.

Therefore the difference in germination percentage between the last two counts is at least in part caused by this bias.

The fact that the AOSA Rules already have a 21 days test for *Poa* species supports the present proposal.

The ISTA Rules allow labs to terminate germination tests and issue a final Orange ISTA Certificate before the final day, provided all seedlings are at a stage of development where all essential structures can be accurately assessed, and also the remaining seeds can be assessed correctly, without any doubt (ISTA Rules, par 5.6.5). Laboratories could probably increase the use of the option, although introducing this approach in a lab would make the job of an analyst more difficult.

On the other hand, when having problems with correct assessment, the ISTA Rules in 5.6.4 allows the germination test to be extended beyond the final count. Analysts could use this option to extend the test for any doubtful seedlings at the end of the new (shortened) count time.
Shortening the duration of the germination test for these grass species improves the logistics and economies of the seed trade, but further improvements can be made. Meeting minimum legal germination levels is the critical parameter for shipping of many seed lots. Considerable numbers of samples reach this minimum level at the first (*Lolium* and *Festuca*) or second (*Poa*) count already. This is confirmed in the present study.

Conclusions

For most samples differences between the last two counts are not statistically significant.

Part of the bias is probably caused by postponing the final evaluation of questionable seedlings.

The variation introduced by shortening the duration of the germination test for the tested species is similar to the variation caused by the different temperature regimes allowed according to the ISTA Rules.

Therefore it is suggested to change the duration of the germination test as follows:

Species	Old (days)	New (days)
Lolium spp	14	10
Festuca spp	21	14

Poa sp	28	21

Although this validation study covered only one species per genus, the results will be valid for the following species.

- Lolium x boucheanum. L. multiflorum, L. perenne
- Festuca filiformis (syn. F. tenuifolia), F. heterophylla (syn. F. rubra heterophylla), F. ovina, F. rubra
- Poa nemoralis, P. palustris, P. pratensis

Reason : in the present ISTA Rules, the species in one group have the same germination method already.

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References

Anonymous (2012) AOSA Rules for testing seeds. Volume 1. Principles and procedures. Association of Official Seed Analysts.

Anonymous (2007) ISTA Method Validation for Seed Testing, International Seed Testing Association, Bassersdorf, Switzerland (http://www.seedtest.org/upload/cms/user/ISTAMethodValidationforSeedTesting-V1.01.pdf).

Anonymous (2011). ISTA Rules 2011, International Seed Testing Association, Bassersdorf, Switzerland.

Carbonnel, M.V., Martinez, E., Flórez, M., Maqueda, R., Pintor-López, A. & Amaya, J.M. (2008). Magnetic field treatments improve germination and seedling growth in *Festuca arundiancea* Schreb. and *Lolium perenne* L. *Seed Sci. & Technol.,* **36,** 31-37.

Ichihara, M., Yamashita, M., Sawada, H., Kida, Y. & Asai, M. (2009). Influence fo after-ripening environments and seed fate of Italian ryegrass (*Lolium multiflorum)*. *Weed Biology and Management,* **9,** 217-224.

Karadavut, U. (2010). Comparison of some statistical models for describing seedling growth of *Lolium perenne* plants in early period. *Journal of Animal and Veterinary Advances,* **9(1),** 190-194.

Larsen S.U. (2004). A study of factors affecting germination, establishment, and competition of the turfgrass species red fescue (*Festuca rubra L. spp. litoralis Vasey*), perennial ryegrass (*Lolium perenne L.*), and Kentucky bluegrass (*Poa pratensis L.*) – Forest & Landscape Research, No. 34-2004. Danish Centre for Forest, Landscape and Planning, Hørsholm. 141 pp.

Miles, S.R. (1963), Handbook of tolerances and measures of precision for seed testing. *Proc. Int. Seed Test. Ass.* **28 (3).**

Naylor, R.E.L. (1981). An evaluation of various germination indices for predicting differences in seed vigour in Italian ryegrass. *Seed Sci. & Technol.,* **9,** 593-600.

Naylor, R.E.L. (2003). Germination of seed lots of Italian ryegrass (*Lolium multiflorum Lam.)* after extended natural ageing in cool storage. *Seed Sci. & Technol.,* **31,** 177-185.

Oliveira, G., Nunes, A., Clemente, A. & Correira, O. (2012). Testing germination of species for hydroseeding degraded mediterranean áreas. *Restoration Ecology*, **20(5),** 623-630.

Petroski, R.J., Dornbos, D.L. & Powell, R.G. (1990). Germination and growth inhibition of annual ryegrass (*Lolium multiflorum L.*) and alfalfa (*Medicago sativa L.*) by loline alkaloids and synthethic N-acylloline derivatives. *J. Agric. Food Chem.* **1990**, 1716-1718.

Pill, W.G. & Korengel, T.K. (1997). Seed priming advances the germination of Kentucky bluegrass *(Poa pratensis* L.). *Journal of Turf Management*, **2(1),** 27-42.

Sakanoue, S. (2010). Use of a simple distribution function to estimate germination rates and thermal time requirements for seed germination in cool-season herbage species. *Seed Sci. & Technol.***, 38,** 612-623.

Stanisavljevic, R., Dragicevic, V., Milenkovic, J., Djukanovic, L., Djokic, D., Terzic, D., Dodig, D. (2010). Effects of the duration of after-ripening period on seed germination and seedling size in three fescue species. *Spanish Journal of Agricultural Research*, **8(2),** 454-459.

Stanisavljevic, R., Djokic, D., Milenkovic, J., Dukanovic, L., Stevovic, V., Simic, A. & Dodig, D. (2011). Seed germination and seedling vigour of Italian ryegrass, cocksfoot and timothy following harvest and storage. *Cienc. Agrotec, Lavras,* **35(6),** 1141-1148.

Wiesner, L.E. & Grabe. D.F. (1961). Effect of temperature preconditioning and cultivar on ryegrass seed dormancy. *Crop Sci.* **12(6),** 760-764.

Annex 1: Statistical analysis of: "Duration of the germination test of some grass species"

Complements

(Jean-Louis Laffont – ISTA Statistics Committee)

1. Model for each species: Generalized Linear Model

Normal_ seedlings_counts$_{ijklm}$ ~ Binomial(100, π_{ijklm})

$$\text{logit}\left(\pi_{ijklm}\right) = \log\left(\frac{\pi_{ijklm}}{1-\pi_{ijklm}}\right)$$
$$= \mu + \alpha_i + \beta_j + \delta_k + \left(\alpha\beta\right)_{ij} + \left(\alpha\delta\right)_{ik} + \left(\beta\delta\right)_{jk} + \left(\alpha\beta\delta\right)_{ijk} + c_l + \left(\alpha c\right)_{il} + \left(\beta c\right)_{jl} + \left(\delta c\right)_{kl}$$

where:

μ is the general effect.

α_i is the fixed effect of lot *i*.

β_j is the fixed effect of temperature *j*.

δ_k is the fixed effect of number of days *k*.

$(\alpha\beta)_{ij}$ is the fixed interaction effect between lot *i* and temperature *j*.

$(\alpha\delta)_{ik}$ is the fixed interaction effect between lot *i* and number of days *k*.

$(\beta\delta)_{jk}$ is the fixed interaction effect between temperature *j* and number of days *k*.

$(\alpha\beta\delta)_{ijk}$ is the fixed interaction effect between lot *i*, temperature *j* and number of days *k*.

c_l is the fixed effect of lab *l*.

$(\alpha c)_{il}$ is the fixed interaction effect between lot *i* and lab *l*.

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

$(\delta c)_{kl}$ is the fixed interaction effect between number of days *k* and lab *l*.

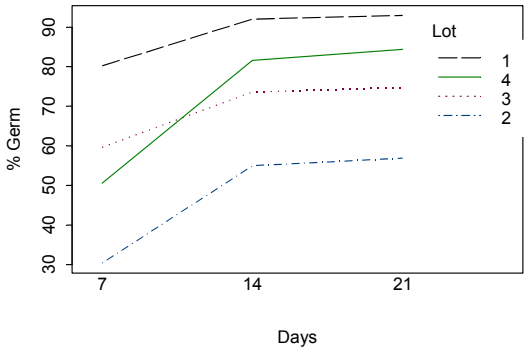
This model has been fitted using the SAS GLIMMIX procedure.

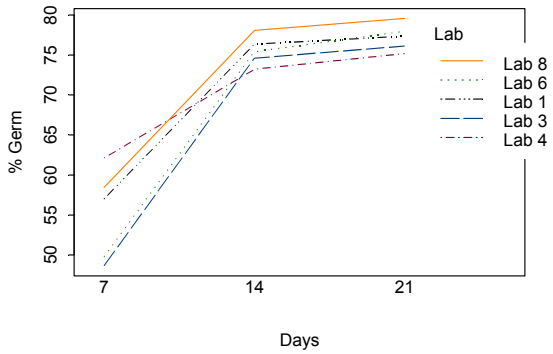
2. Results

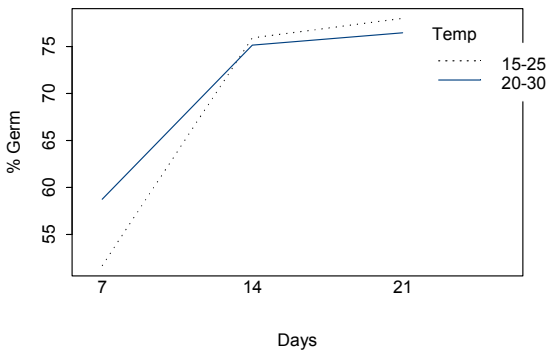
In the following analyses, Lab 5 has been suppressed as Temp 15-25 is missing for the three species and as Temp 20-30 is also missing for *Poa* in this laboratory.

Festuca:

	Num DF	Den DF	F Value	Pr > F
LOT	3	360	1426.67	<.0001
TEMP	1	360	9.75	0.0019
Number of DAYS	2	360	1008.88	<.0001
LAB	4	360	28.86	<.0001
LOT*TEMP	3	360	0.76	0.5155
LOT*DAYS	6	360	38.89	<.0001
TEMP*DAYS	2	360	31.47	<.0001
LAB*LOT	12	360	18.09	<.0001
LAB*TEMP	4	360	5.78	0.0002
LAB*DAYS	8	360	14.14	<.0001
LOT*TEMP*DAYS	6	360	3.94	0.0008
LAB*LOT*DAYS	24	360	4.28	<.0001
LAB*TEMP*DAYS	8	360	5.75	<.0001
LAB*LOT*TEMP	12	360	5.36	<.0001
LAB*LOT*TEMP*DAYS	24	360	0.71	0.8388



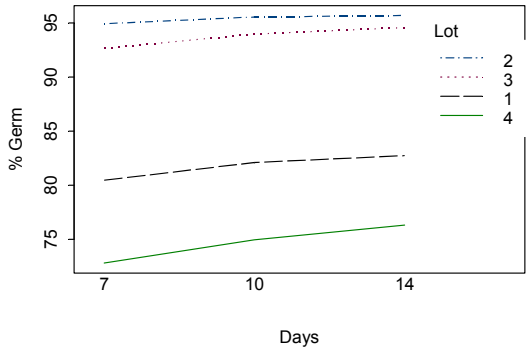


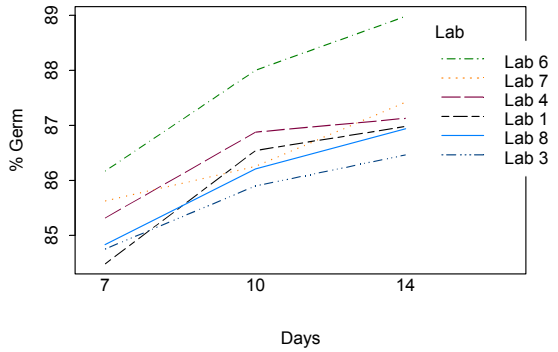


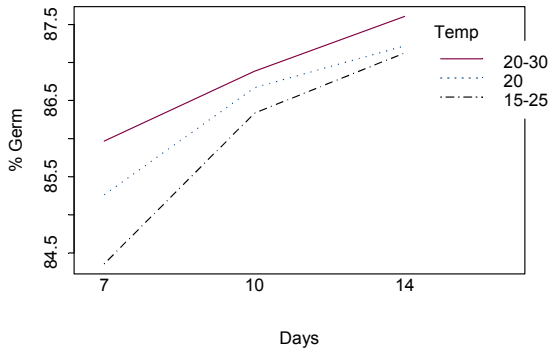
No crossover interactions with DAYS from 14 days.

Lolium:

	Num DF	Den DF	F Value	Pr > F
LOT	3	648	1502.18	<.0001
TEMP	2	648	1.76	0.1724
Number of DAYS	2	648	26.19	<.0001
LAB	5	648	2.46	0.0323
LOT*TEMP	6	648	3.96	0.0007
LOT*DAYS	6	648	0.86	0.5216
TEMP*DAYS	4	648	0.83	0.5078
LAB*LOT	15	648	6.57	<.0001
LAB*TEMP	10	648	2.57	0.0047
LAB*DAYS	10	648	0.41	0.9407
LOT*TEMP*DAYS	12	648	0.08	1.0000
LAB*LOT*DAYS	30	648	0.12	1.0000
LAB*TEMP*DAYS	20	648	0.43	0.9861
LAB*LOT*TEMP	30	648	2.48	<.0001
LAB*LOT*TEMP*DAYS	60	648	0.11	1.0000



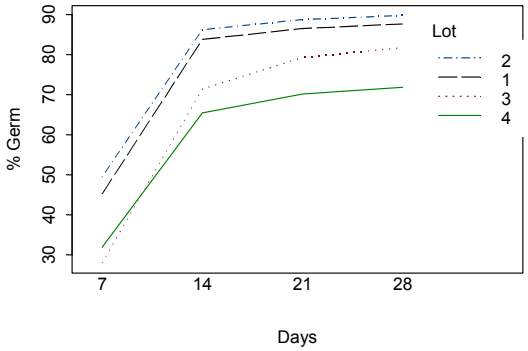


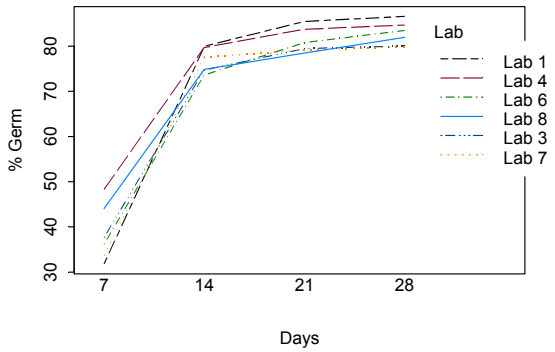


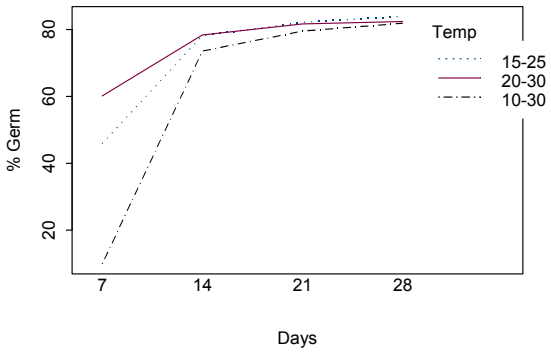
No crossover interactions with DAYS except for LAB x DAYS.

Poa:

	Num DF	Den DF	F Value	Pr > F
LOT	3	864	69.6	<.0001
TEMP	2	864	25.3	<.0001
Number of DAYS	3	864	104.88	<.0001
LAB	5	864	62.51	<.0001
LOT*TEMP	6	864	1.57	0.1529
LOT*DAYS	9	864	17.37	<.0001
TEMP*DAYS	6	864	53.18	<.0001
LAB*LOT	15	864	7.61	<.0001
LAB*TEMP	10	864	12.06	<.0001
LAB*DAYS	15	864	37.12	<.0001
LOT*TEMP*DAYS	18	864	1.17	0.2806
LAB*LOT*DAYS	45	864	2.12	<.0001
LAB*TEMP*DAYS	30	864	15.05	<.0001
LAB*LOT*TEMP	30	864	4.28	<.0001
LAB*LOT*TEMP*DAYS	90	864	1.24	0.0741







No crossover interactions between LOT and DAYS from 14 days; limited interactions between TEMP and DAYS from 14 days.

Annex 2: Data check for identifying deviating results (outliers).

Lolium

Normal 7 days

15-25

Lot 1	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	15	18	16	17	16	16	16
Obs range	6	13	7	3	11	4	4
Mean	82,75	69,00	78,75	77,00	79,50	79,50	80,25

Lot 2	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	10	12	9	8	10	9	10
Obs range	8	10	4	0	7	3	1
Mean	94,00	90,25	95,25	96,00	94,25	94,75	94,50

Lot 3	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	11	13	10	11	11	12	10
Obs range	5	1	8	7	3	3	4
Mean	90,75	87,25	93,50	92,00	92,00	89,00	93,00

Lot 4	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	19	19	18	18	16	18	18
Obs range	10	8	8	8	5	5	2
Mean	64,00	64,00	72,25	72,50	77,75	69,00	72,25

20

Lot 1	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	16	18	16	16	14	15	16	16
Obs range	10	9	9	12	7	2	7	2
Mean	78,00	69,75	78,25	78,00	83,50	82,75	78,50	80,00

Lot 2	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	8	11	8	9	10	9	10	10
Obs range	5	4	3	5	10	8	5	1
Mean	95,75	91,50	95,50	95,25	93,50	95,00	94,50	94,50

Lot 3	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	10	15	10	10	10	10	11	10
Obs range	10	12	8	5	10	3	8	3
Mean	94,00	82,25	94,25	93,75	94,00	93,25	92,25	93,25

Lot 4	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	18	19	18	17	17	17	17	17
Obs range	8	9	13	11	9	12	7	4
Mean	71,75	59,50	70,00	73,75	76,50	73,50	76,75	73,75

20-30

Lot 1	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	15	19	15	15	14	15	14	15
Obs range	4	18	6	6	6	8	6	2
Mean	83,00	60,00	80,75	82,00	84,50	83,00	85,00	81,00

Lot 2	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	10	14	9	8	9	8	8	10
Obs range	4	13	8	4	7	4	4	3
Mean	93,00	85,50	95,25	95,75	94,75	95,50	95,50	94,00

Lot 3	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	9	18	10	10	10	12	11	11
Obs range	7	13	3	3	9	6	5	2
Mean	95,00	71,75	93,75	93,50	93,75	90,50	91,75	91,75

Lot 4	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	18	19	18	17	16	17	15	18
Obs range	18	10	9	9	12	14	9	5
Mean	71,75	58,25	69,50	74,25	79,25	77,00	81,00	69,75

Normal 10 days

15-25

Lot 1	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	14	17	15	16	15	15	15
Obs range	3	11	7	2	10	7	3
Mean	85,75	73,50	80,75	78,75	82,50	82,25	82,25

Lot 2	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	7	11	9	8	10	8	8
Obs range	6	10	4	2	7	3	1
Mean	96,75	92,50	95,25	96,50	94,25	95,75	95,75

Lot 3	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	10	12	9	10	10	12	10
Obs range	5	2	5	6	1	3	2
Mean	94,00	90,25	95,25	94,25	94,25	89,00	94,50

Lot 4	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	18	19	17	17	16	18	17
Obs range	10	7	9	4	6	5	3
Mean	69,75	65,75	75,50	75,00	79,75	69,00	75,25

20

Lot 1	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	16	16	16	16	14	14	15	15
Obs range	12	3	7	11	9	3	4	1
Mean	79,75	78,25	79,75	79,50	84,50	85,25	80,75	81,25

Lot 2	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	8	9	8	8	10	8	9	9
Obs range	4	3	3	5	8	6	6	1
Mean	96,00	94,75	95,75	95,50	94,25	96,25	95,25	94,75

Lot 3	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	8	12	8	9	10	8	11	10
Obs range	6	8	8	4	9	3	8	2
Mean	95,75	90,50	96,00	95,25	94,50	95,75	92,25	94,00

Lot 4	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	17	18	18	17	16	16	17	17
Obs range	11	7	13	11	7	15	7	4
Mean	73,25	67,25	70,50	77,00	78,50	78,25	76,75	75,50

20-30

Lot 1	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	14	18	15	14	14	14	14	15
Obs range	4	14	7	5	6	7	6	3
Mean	84,25	71,75	81,00	83,50	85,25	83,50	85,00	81,75

Lot 2	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	10	11	8	8	9	8	8	9
Obs range	5	11	7	4	7	5	5	2
Mean	93,50	91,25	95,75	96,25	94,75	95,75	95,75	94,75

Lot 3	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	9	13	10	9	10	11	11	10
Obs range	7	10	2	4	9	5	5	1
Mean	95,00	87,50	94,25	95,00	94,50	90,75	92,25	93,50

Lot 4	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	17	18	18	17	15	16	15	18
Obs range	14	5	12	8	7	11	9	4
Mean	74,75	70,25	71,00	76,00	80,75	79,75	81,00	71,25

Normal 14 days

15-25

Lot 1	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	14	16	15	16	15	15	15
Obs range	4	12	7	2	9	7	3
Mean	86,25	79,25	81,25	79,25	82,75	83,00	83,25

Lot 2	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	7	9	9	8	10	8	8
Obs range	5	8	4	2	7	2	1
Mean	97,25	95,00	95,25	96,50	94,25	96,00	95,75

Lot 3	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	9	10	9	10	10	11	8
Obs range	3	4	5	6	1	3	2
Mean	94,75	94,50	95,25	94,25	94,25	92,00	95,50

Lot 4	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	18	18	17	17	16	17	17
Obs range	8	6	9	5	6	1	3
Mean	70,75	69,00	75,75	75,50	80,25	74,75	77,25

20

Lot 1	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	16	16	16	16	14	14	15	15
Obs range	12	4	6	11	9	3	4	2
Mean	80,50	79,25	80,00	80,00	84,50	85,75	81,00	82,00

Lot 2	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	8	9	8	8	10	8	9	9
Obs range	4	4	3	5	7	6	6	0
Mean	96,50	95,25	95,75	95,50	94,50	96,50	95,25	95,00

Lot 3	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	8	11	8	9	9	8	10	10
Obs range	6	6	8	4	6	2	6	2
Mean	95,75	92,25	96,00	95,25	95,25	96,00	93,25	94,50

Lot 4	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	17	18	17	16	16	16	16	17
Obs range	9	9	13	9	6	13	9	3
Mean	73,75	71,00	74,00	77,75	78,75	78,75	78,25	76,25

20-30

Lot 1	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	14	17	15	14	14	14	14	15
Obs range	4	13	7	6	6	7	6	3
Mean	84,25	74,75	81,50	84,00	85,25	86,00	85,75	82,50

Lot 2	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	10	11	8	8	9	8	8	9
Obs range	5	9	7	4	7	4	5	2
Mean	93,50	92,25	96,00	96,25	94,75	96,25	95,75	94,75

Lot 3	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	8	12	10	9	9	10	10	10
Obs range	6	8	2	4	8	1	5	2
Mean	95,50	89,50	94,25	95,00	95,00	93,50	92,75	94,25

Lot 4	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	17	17	18	17	15	14	15	18
Obs range	13	6	11	7	7	10	8	6
Mean	75,00	73,25	72,50	76,25	81,00	83,50	81,25	72,25

Festuca

Normal 7 days
15-25

Lot 1	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	14	19	18	11	17	20	15
Obs range	7	21	13	6	22	15	6
Mean	84,25	59,00	71,50	92,50	75,25	52,75	82,50

Lot 2	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	14	11	15	19	16	10	17
Obs range	7	9	4	9	21	1	6
Mean	17,00	9,50	19,00	39,25	22,25	7,50	28,25

Lot 3	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	19	16	19	19	20	19	18
Obs range	20	16	15	12	11	17	6
Mean	42,25	22,00	60,75	55,75	50,00	42,50	67,75

Lot 4	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	20	17	19	19	19	12	19
Obs range	6	6	6	20	33	5	11
Mean	45,50	24,00	35,00	63,50	41,50	12,25	38,75

20-30

Lot 1	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	12	14	20	11	13	16	15	14
Obs range	1	10	14	7	4	5	6	2
Mean	90,50	85,25	50,50	91,25	87,75	78,75	80,75	84,75

Lot 2	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	19	19	18	19	19	17	18	19
Obs range	5	9	9	5	9	11	9	2
Mean	40,50	37,50	33,50	36,25	39,50	28,25	33,25	39,25

Lot 3	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	18	20	18	19	20	20	19	18
Obs range	10	11	13	30	9	15	11	7
Mean	67,50	49,25	68,25	59,75	48,75	55,25	66,00	69,00

Lot 4	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	18	20	20	19	19	20	19	19
Obs range	14	11	14	16	15	11	12	4
Mean	68,25	46,50	50,50	58,75	59,25	46,75	43,00	57,25

Normal 14 days
15-25

Lot 1	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	11	11	12	10	11	13	10
Obs range	6	4	7	3	14	6	2
Mean	91,75	91,50	89,00	94,25	92,50	87,00	93,25

Lot 2	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	19	19	19	20	20	20	19
Obs range	7	14	8	15	14	7	1
Mean	58,00	58,00	55,50	52,75	55,00	51,25	59,50

Lot 3	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	18	19	17	18	17	19	17
Obs range	12	7	9	11	12	6	4
Mean	70,25	64,50	74,50	71,50	74,75	65,00	76,25

Lot 4	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	14	16	15	17	14	19	15
Obs range	1	12	4	17	10	19	3
Mean	85,50	79,25	80,75	75,25	85,50	58,00	82,50

20-30

Lot 1	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	10	13	14	8	11	10	10	10
Obs range	1	7	7	3	3	8	5	0
Mean	92,75	88,50	83,75	95,50	92,50	93,50	93,00	94,00

Lot 2	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	20	20	20	20	19	20	19	19
Obs range	10	14	6	10	7	3	13	2
Mean	55,25	49,00	53,25	46,50	56,25	53,25	45,00	60,50

Lot 3	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	17	20	17	17	19	18	18	17
Obs range	7	10	9	4	3	13	11	6
Mean	75,00	53,25	76,25	73,25	58,75	69,50	69,50	74,25

Lot 4	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	15	20	14	17	16	16	20	14
Obs range	9	6	7	11	11	3	10	5
Mean	82,25	53,75	83,75	76,75	77,75	79,25	55,25	84,25

Normal 21 days
15-25

Lot 1	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	11	11	11	9	10	11	10
Obs range	5	5	5	3	11	4	2
Mean	92,50	92,00	91,00	95,25	93,50	91,50	94,00

Lot 2	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	19	19	19	20	19	20	19
Obs range	5	16	5	18	13	7	2
Mean	60,00	59,00	58,25	54,00	58,25	53,00	61,50

Lot 3	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	18	18	17	17	17	18	16
Obs range	10	8	8	10	12	5	4
Mean	71,50	67,50	74,75	74,50	76,25	71,75	77,50

Lot 4	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	13	15	14	16	13	19	13
Obs range	6	13	7	16	11	18	4
Mean	87,25	80,75	84,25	79,50	88,00	59,75	88,00

20-30

Lot 1	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	10	12	14	8	10	9	8	10
Obs range	1	7	7	3	3	6	3	1
Mean	92,75	88,75	85,00	95,75	93,00	95,00	95,50	94,50

Lot 2	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	19	20	20	20	19	19	20	19
Obs range	10	4	7	8	9	7	13	3
Mean	57,00	52,25	53,75	47,75	60,75	56,75	53,50	61,50

Lot 3	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	17	19	17	17	19	18	18	17
Obs range	7	9	9	5	6	15	11	6
Mean	75,25	59,50	77,00	74,75	62,25	71,25	70,25	74,50

Lot 4	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	15	19	14	16	15	14	16	14
Obs range	9	8	7	12	13	8	5	4
Mean	82,50	59,00	85,00	79,75	83,00	84,75	80,50	85,00

Poa

Normal 7 days

10-30

Lot 1	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	10	19		17	7	5	16
Obs range	4	8	0	13	5	2	10
Mean	7,50	65,00	0,00	25,50	3,75	2,25	23,25

Lot 2	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	11	19		17	6	7	19
Obs range	5	8	0	**23**	2	5	7
Mean	10,50	66,25	0,00	28,50	3,00	4,00	38,00

Lot 3	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	8	19	10	13	8	7	11
Obs range	7	11	10	7	4	5	6
Mean	5,25	43,25	7,00	12,75	5,00	4,50	10,00

Lot 4	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	10	20	7	12	7	6	14
Obs range	6	7	6	5	1	3	2
Mean	6,75	53,00	4,25	12,00	4,50	3,00	15,25

15-25

Lot 1	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	19	20	18	18	19	19	19
Obs range	10	15	14	7	9	**20**	5
Mean	35,00	51,25	68,50	70,25	60,00	36,25	59,75

Lot 2	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	18	19	17	17	20	20	19
Obs range	10	12	7	9	5	8	3
Mean	31,25	58,00	76,25	75,75	53,50	48,50	63,50

Lot 3	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	16	19	18	19	19	15	19
Obs range	**18**	5	12	6	7	6	5
Mean	21,00	35,00	29,25	42,75	39,00	17,50	42,00

Lot 4	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	16	19	18	20	19	17	20
Obs range	8	8	15	12	11	6	8
Mean	22,25	43,25	33,00	54,50	42,25	25,25	52,75

20-30

Lot 1	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	19	19	18	16	20	19	17	18
Obs range	3	10	17	8	14	19	6	8
Mean	61,75	45,25	70,75	80,00	49,75	66,50	74,75	66,75

Lot 2	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	17	20	16	14	19	18	16	18
Obs range	14	9	5	13	5	12	5	4
Mean	73,75	49,00	79,25	83,75	59,75	71,50	80,00	69,25

Lot 3	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	20	19	19	19	18	19	19	19
Obs range	19	18	2	13	10	4	13	0
Mean	52,00	38,00	37,50	45,00	31,75	39,75	55,50	37,00

Lot 4	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	20	19	20	20	19	20	20	20
Obs range	9	18	8	8	10	8	10	4
Mean	54,75	45,00	46,00	48,75	35,25	45,75	53,00	50,75

Normal 14 days

10-30

Lot 1	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	14	18	17	13	15	13	17
Obs range	7	9	9	6	8	10	6
Mean	85,50	72,00	76,00	87,50	81,50	86,75	76,25

Lot 2	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	15	17	15	13	14	11	15
Obs range	7	9	3	9	3	10	3
Mean	81,75	74,75	82,25	86,75	84,50	91,25	81,25

Lot 3	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	18	19	19	18	19	18	18
Obs range	13	16	12	**20**	14	18	12
Mean	72,00	59,75	62,25	67,50	63,00	68,00	66,75

Lot 4	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	19	19	19	19	19	19	19
Obs range	11	9	10	17	12	5	6
Mean	64,00	62,75	56,75	60,50	59,25	59,50	64,00

15-25

Lot 1	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	14	17	14	14	14	14	15
Obs range	13	9	9	8	11	5	4
Mean	85,00	74,00	86,50	84,75	83,50	84,50	82,75

Lot 2	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	10	17	12	12	16	12	14
Obs range	4	8	3	9	2	9	4
Mean	92,75	75,25	89,50	90,25	78,25	88,75	85,00

Lot 3	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	17	19	18	17	18	17	18
Obs range	15	10	6	14	10	4	9
Mean	76,25	61,75	67,50	76,75	70,25	73,50	70,50

Lot 4	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	17	19	19	18	19	18	18
Obs range	5	9	17	6	14	7	8
Mean	74,25	57,25	64,00	71,25	62,75	67,75	69,00

20-30

Lot 1	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	14	19	14	13	15	14	14	16
Obs range	8	13	13	8	6	11	6	5
Mean	86,00	55,75	84,50	88,00	80,75	84,00	84,75	80,25

Lot 2	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	11	19	14	11	14	15	13	15
Obs range	5	19	2	3	8	9	2	1
Mean	90,75	55,50	85,00	91,50	85,00	82,50	87,50	82,25

Lot 3	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	15	20	17	16	18	18	17	18
Obs range	13	10	9	5	6	9	11	5
Mean	81,50	46,25	73,75	79,25	67,50	69,50	75,00	71,75

Lot 4	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	18	20	18	18	19	19	19	18
Obs range	6	**23**	12	8	10	11	19	5
Mean	69,50	50,00	68,25	72,00	62,25	63,75	63,00	68,25

Normal 21 days

10-30

Lot 1	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	11	17	15	12	13	13	16
Obs range	3	10	6	4	3	10	5
Mean	92,50	74,25	80,75	88,75	86,75	88,25	78,75

Lot 2	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	13	16	14	11	13	11	14
Obs range	6	9	5	5	1	11	4
Mean	87,25	79,00	85,75	90,75	88,50	91,75	83,75

Lot 3	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	14	17	17	15	17	18	17
Obs range	15	17	13	14	**22**	16	9
Mean	84,00	75,00	77,00	80,75	75,75	72,50	73,00

Lot 4	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	18	18	19	18	18	19	18
Obs range	11	15	14	9	11	7	3
Mean	71,50	70,75	64,00	68,50	67,50	62,00	69,00

15-25

Lot 1	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	12	16	13	14	13	14	14
Obs range	12	12	10	6	11	6	3
Mean	89,25	80,00	87,50	85,25	88,00	85,25	84,25

Lot 2	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	10	15	12	11	14	12	13
Obs range	4	6	2	9	6	9	4
Mean	94,00	83,00	90,00	91,00	84,00	88,75	86,75

Lot 3	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	14	18	16	14	15	17	17
Obs range	13	10	9	9	8	3	7
Mean	84,50	71,50	80,50	84,25	82,00	76,00	76,75

Lot 4	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 7	Lab 8
Max Tol range	16	19	18	17	19	18	18
Obs range	11	4	11	8	14	10	7
Mean	80,00	64,50	70,25	75,25	66,50	69,50	72,25

20-30

Lot 1	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	12	19	14	12	15	12	14	14
Obs range	7	16	12	8	6	10	7	5
Mean	89,50	60,00	85,00	88,75	82,50	88,75	85,75	83,50

Lot 2	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	10	19	14	10	13	12	12	14
Obs range	6	**22**	2	4	8	5	6	2
Mean	93,50	60,75	85,25	92,75	87,00	89,25	89,75	84,00

Lot 3	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	14	20	17	14	18	15	17	17
Obs range	12	12	9	4	7	12	13	6
Mean	86,00	52,25	73,75	84,50	70,00	82,00	76,00	77,25

Lot 4	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8
Max Tol range	17	20	17	17	19	18	19	18
Obs range	9	**22**	10	7	12	11	19	5
Mean	73,25	55,25	73,50	74,00	64,75	70,00	63,50	72,25

Annex 3: Averages per sample type, temperature regime and differences (analysed in tables 1 and 2 in the report).

All labs

Lp		15-25					20					20-30				
		7	10	14	10-7	14-10	7	10	14	10-7	14-10	7	10	14	10-7	14-10
Lp3	fast high	91,1	93,1	94,4	2,0	1,3	92,1	94,3	94,8	2,2	0,5	90,2	92,8	93,7	2,6	0,9
Lp1	fast low	78,1	80,8	82,1	2,7	1,3	78,6	81,1	81,6	2,5	0,5	79,9	82,0	83,0	2,1	1,0
Lp2	slow high	94,1	95,3	95,7	1,2	0,4	94,4	95,3	95,5	0,9	0,2	93,7	94,7	94,9	1,0	0,2
Lp4	slow low	70,3	72,9	74,8	2,6	1,9	71,9	74,6	76,1	2,7	1,5	72,6	75,6	76,9	3,0	1,3
avg		80,8	83,0	84,2	2,2	1,2	81,6	83,7	84,4	2,0	0,7	82,1	84,1	84,9	2,0	0,8

Fr		15-25										20-30				
		7	14	21	14-7	21-14						7	14	21	14-7	21-14
Fr1	fast high	74,0	91,3	92,8	17,3	1,5						81,2	91,7	92,5	10,5	0,8
Fr3	fast low	48,7	71,0	73,4	22,3	2,4						60,5	68,7	70,6	8,2	1,9
Fr4	slow high	37,2	78,1	81,1	40,9	3,0						53,8	74,1	79,9	20,3	5,8
Fr2	slow low	20,4	55,7	57,7	35,3	2,0						36,0	52,4	55,4	16,4	3,0
avg		45,1	74,0	76,3	29,0	2,2						57,9	71,7	74,6	13,9	2,9

Pp		10-30							15-25							20-30						
		7	14	21	28	14-7	21-14	28-21	7	14	21	28	14-7	21-14	28-21	7	14	21	28	14-7	21-14	28-21
Pp1	fast high	**18,2**	80,8	84,3	86,1	62,6	3,5	1,8	54,4	83,0	85,6	87,3	28,6	2,6	1,7	64,4	80,5	83,0	84,4	16,1	2,5	1,4
Pp2	fast low	**21,5**	83,2	86,7	88,4	61,7	3,5	1,7	58,1	85,7	88,2	89,8	27,6	2,5	1,6	70,8	82,5	85,3	86,2	11,7	2,8	0,9
Pp3	slow high	**12,5**	65,6	76,9	80,5	53,1	11,3	3,6	32,4	70,9	79,4	82,5	38,5	8,5	3,1	42,1	70,6	75,2	77,3	28,5	4,6	2,1
Pp4	slow low	**14,1**	61,0	67,6	69,8	46,9	6,6	2,2	39,0	66,6	71,2	73,3	27,6	4,6	2,1	47,4	64,6	68,3	69,4	17,2	3,7	1,1
avg		16,6	72,7	78,9	81,2	56,1	6,2	2,3	46,0	76,6	81,1	83,2	30,6	4,6	2,1	56,2	74,6	78,0	79,3	18,4	3,4	1,4

Excl lab 2

Lp		15-25					20					20-30				
		7	10	14	10-7	14-10	7	10	14	10-7	14-10	7	10	14	10-7	14-10
Lp3	fast high	91,7	93,5	94,3	1,8	0,8	93,5	94,8	95,1	1,3	0,3	92,9	93,6	94,3	0,7	0,7
Lp1	fast low	79,6	82,0	82,6	2,4	0,6	79,9	81,5	82,0	1,6	0,5	82,8	83,5	84,2	0,7	0,7
Lp2	slow high	94,8	95,7	95,8	0,9	0,1	94,9	95,4	95,6	0,5	0,2	94,8	95,2	95,3	0,4	0,1
Lp4	slow low	71,3	74,0	75,7	2,7	1,7	73,7	75,7	76,8	2,0	1,1	74,6	76,4	77,4	1,8	1,0
avg		81,9	83,9	84,7	2,0	0,8	82,8	84,2	84,8	1,4	0,6	84,1	85,0	85,6	1,0	0,6

Fr		15-25										20-30				
		7	14	21	14-7	21-14						7	14	21	14-7	21-14
Fr1	fast high	76,5	91,3	93,0	14,8	1,7						80,6	92,1	93,1	11,5	1,0
Fr3	fast low	53,2	72,0	74,4	18,8	2,4						62,1	70,9	72,2	8,8	1,3
Fr4	slow high	39,4	77,9	81,1	38,5	3,2						54,8	77,0	82,9	22,2	5,9
Fr2	slow low	22,2	55,3	57,5	33,1	2,2						35,8	52,9	55,9	17,1	3,0
avg		47,8	74,1	76,5	26,3	2,4						58,3	73,2	76,0	14,9	2,8

Pp		10-30							15-25							20-30						
		7	14	21	28	14-7	21-14	28-21	7	14	21	28	14-7	21-14	28-21	7	14	21	28	14-7	21-14	28-21
Pp1	fast high	**10,4**	82,3	86,0	87,9	71,9	3,7	1,9	55,0	84,5	86,6	87,7	29,5	2,1	1,1	67,2	84,0	86,3	86,9	16,8	2,3	0,6
Pp2	fast low	**14,0**	84,6	88,0	89,6	70,6	3,4	1,6	58,1	87,4	89,1	90,2	29,3	1,7	1,1	73,9	86,4	88,8	89,3	12,5	2,4	0,5
Pp3	slow high	**7,4**	66,6	77,2	80,6	59,2	10,6	3,4	31,9	72,5	80,7	83,4	40,6	8,2	2,7	42,6	74,0	78,5	80,3	31,4	4,5	1,8
Pp4	slow low	**7,6**	60,7	67,1	69,3	53,1	6,4	2,2	38,3	68,2	72,3	74,3	29,9	4,1	2,0	47,8	66,7	70,2	71,2	18,9	3,5	1,0
avg		9,9	73,6	79,6	81,9	63,7	6,0	2,3	45,8	78,2	82,2	83,9	32,3	4,0	1,7	57,9	77,8	81,0	81,9	19,9	3,2	1,0

Annex 4: results from the past as submitted by participating labs (supportive evidence, analysed in table 5 of the report).

	Species	Prechill	Substrate	Temperature	7	10	14	10-7	14-10
Lab 4	Lolium perenne	No	TP, KNO3	15-25	62,0	70,8	74,0	8,8	3,2
					66,8	77,5	80,3	10,7	2,8
					66,3	77,3	82,0	11,0	4,7
					73,3	79,5	82,8	6,2	3,3
					74,0	82,8	83,5	8,8	0,7
					70,3	79,5	84,0	9,2	4,5
					78,0	83,8	85,3	5,8	1,5
					74,3	82,8	85,5	8,5	2,7
					78,8	84,5	85,5	5,7	1,0
					79,3	85,5	86,3	6,2	0,8
					82,3	82,8	86,5	0,5	3,7
					79,0	84,3	87,5	5,3	3,2
					83,3	86,3	87,8	3,0	1,5
					82,3	88,0	88,5	5,7	0,5
					59,5	87,0	88,8	27,5	1,8
					83,8	88,3	90,3	4,5	2,0
					86,8	88,5	90,5	1,7	2,0
					85,0	88,3	90,5	3,3	2,2
					84,3	85,8	90,8	1,5	5,0
					85,8	88,8	91,3	3,0	2,5
					72,8	90,3	91,3	17,5	1,0
					86,8	91,8	92,3	5,0	0,5
					88,5	91,5	92,5	3,0	1,0
					84,3	90,8	92,5	6,5	1,7
					85,0	92,0	92,8	7,0	0,8
					84,3	92,3	92,8	8,0	0,5
					88,0	93,3	93,3	5,3	0,0
					89,3	91,5	93,3	2,2	1,8
					87,5	93,3	93,3	5,8	0,0
					92,0	94,3	94,5	2,3	0,2
					84,5	95,0	95,0	10,5	0,0
					86,8	95,5	95,8	8,7	0,3
					95,8	96,5	96,5	0,7	0,0
				avg	80,6	87,3	89,0	6,6	1,7

	Species	Prechill	Substrate	Temperature	7	14	21	14-7	21-14
Lab 4	Festuca rubra	No	TP, KNO3	15-25	5,0	11,0	12,5	6,0	1,5
					13,0	30,0	32,8	17,0	2,8
					39,0	70,8	73,0	31,8	2,2
					66,3	72,5	73,8	6,2	1,3
					32,0	80,5	83,0	48,5	2,5
					26,0	82,0	84,0	56,0	2,0
					71,5	85,3	85,8	13,8	0,5
					60,0	87,5	87,8	27,5	0,3
					83,3	88,0	88,3	4,7	0,3
					65,3	87,0	89,8	21,8	2,8
					21,8	86,0	90,0	64,2	4,0
					53,0	88,0	90,0	35,0	2,0
					66,0	86,3	90,3	20,3	4,0
					33,8	84,5	90,3	50,7	5,8
					64,5	90,0	90,5	25,5	0,5
					51,0	89,8	90,5	38,8	0,7
					73,5	91,0	91,3	17,5	0,3
					63,8	88,8	91,5	25,0	2,7
					36,8	89,3	91,8	52,5	2,5
					60,8	92,5	92,5	31,7	0,0
					54,8	92,8	92,8	38,0	0,0
					87,3	91,8	93,0	4,5	1,2
					66,3	93,3	93,3	27,0	0,0
					64,5	93,5	93,5	29,0	0,0
					61,8	94,5	94,8	32,7	0,3
					77,3	94,5	94,8	17,2	0,3
					44,5	94,8	94,8	50,3	0,0
					36,8	94,5	95,0	57,7	0,5
					85,5	95,0	95,8	9,5	0,8
				avg	54,0	83,6	85,1	29,7	1,4

	Species	Prechill	Substrate	Temperature	7	14	21	28	14-7	21-14	28-21
Lab 4	Poa pratensis	7d10oC	TP, KNO3	15-25	59,5	79,5	80,8	81,5	20,0	1,3	0,7
					75,8	82,8	83,0	83,0	7,0	0,2	0,0
					70,3	80,5	82,8	83,3	10,2	2,3	0,5
					60,8	81,5	82,5	84,5	20,7	1,0	2,0
					71,8	83,0	84,3	84,8	11,2	1,3	0,5
					73,0	83,8	84,8	84,8	10,8	1,0	0,0
					52,8	80,0	83,8	85,3	27,2	3,8	1,5
					59,8	82,3	85,5	86,5	22,5	3,2	1,0
					76,5	87,0	87,8	87,8	10,5	0,8	0,0
					60,0	82,3	86,3	88,0	22,3	4,0	1,7
					80,8	86,8	87,3	88,5	6,0	0,5	1,2
					66,0	88,3	88,5	89,3	22,3	0,2	0,8
					67,0	88,0	88,8	89,3	21,0	0,8	0,5
					49,3	88,0	88,5	89,8	38,7	0,5	1,3
					77,5	87,3	88,5	90,0	9,8	1,2	1,5
					82,5	90,0	90,3	90,3	7,5	0,3	0,0
					66,5	87,5	88,8	90,3	21,0	1,3	1,5
					80,5	89,5	90,5	90,5	9,0	1,0	0,0
					81,0	89,0	90,5	90,5	8,0	1,5	0,0
					76,0	88,8	89,8	90,5	12,8	1,0	0,7
					75,0	88,8	89,8	90,8	13,8	1,0	1,0
					72,3	90,0	91,0	91,3	17,7	1,0	0,3
					71,5	89,3	90,3	91,5	17,8	1,0	1,2
					78,3	90,0	90,0	91,5	11,7	0,0	1,5
					76,8	89,8	90,5	91,5	13,0	0,8	1,0
					67,3	88,8	91,3	91,5	21,5	2,5	0,2
					82,3	89,0	90,5	91,8	6,7	1,5	1,3
					68,0	92,0	92,0	92,0	24,0	0,0	0,0
					63,5	88,5	90,0	92,3	25,0	1,5	2,3
					84,5	90,3	92,3	92,3	5,8	2,0	0,0
					86,3	92,8	92,8	92,8	6,5	0,0	0,0
					49,5	90,3	93,3	93,3	40,8	3,0	0,0
					86,0	93,5	93,5	93,5	7,5	0,0	0,0
					55,0	90,8	94,8	94,8	35,8	4,0	0,0
				avg	70,7	87,3	88,7	89,4	16,7	1,3	0,7

	Species	Prechill	Substrate	Temperature	**6**	**14**			**14-6**		
Lab 5	Lolium multiflorum	7d-5oC	TP	20-30	88,0	89,0			1,0		
					90,0	95,0			5,0		
					79,0	85,0			6,0		
					88,0	89,0			1,0		
				avg	86,3	89,5			3,3		
	Species	Prechill	Substrate	Temperature	**6**	**14**	**18**		**14-6**	**18-14**	
Lab 5	Loliumxboucheanu	7d-5oC	TP	20-30	87,0	89,0	97,0		2,0	8,0	
					92,0	94,0			2,0		
					85,0	92,0			7,0		
					92,0	94,0			2,0		
				avg	89,0	92,3			3,3		
					6	**14**			**14-6**		
Lab 5	Lolium perenne	7d-5oC	TP	20-30	87,0	93,0			6,0		
					93,0	93,0			0,0		
					88,0	90,0			2,0		
					56,0	86,0			30,0		
				avg	81,0	90,5			9,5		

Proposal for a new method to detect Xanthomonas campestris pv. campestris in disinfected Brassica spp. seed lots

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Summary

The ISTA Rule 7-019 was modified to extract cells of *Xanthomonas campestris* pv. *campestris* (Xcc) located inside the seed coat of vegetable Brassica crops that may have survived a hot water or similar seed treatment. This new method includes wet seed grinding, ten-fold concentration of extracts, buffered saline (PBS) in a larger ratio to the seed, longer incubation time, changes to the semi-selective media and the pathogenicity test. Wet grinding of seeds was compared to simply soaking seed (as in the ISTA Rule 7-019) while the ten-fold concentration of seed extracts was compared to the undiluted and ten-fold diluted extracts in a comparative test organized by ISHI-Veg in which eight laboratories participated. Two naturally contaminated hot water treated cabbage seed lots with medium and high pathogen load, and a pathogen-free lot were utilized. The recovery of Xcc (cfu/ml) and the number of positive seed subsamples (from the 20 tested) obtained using the two extraction methods 'soaking' and 'wet grinding' were compared. Wet grinding strongly enhanced the extraction of viable Xcc cells compared to seed soaking. This same result was repeated and reproduced in three seed lots. In seed extracts with low Xcc and saprophytic load, the ten-fold concentration of the undiluted seed extract after grinding was shown to improve the detection of Xcc cells. The revised protocol was found to be a reliable method for detecting Xcc in disinfected Brassica spp. seed lots.

Introduction

Xanthomonas campestris pv. *campestris* (Xcc) is an important seed-borne bacterial pathogen and the causal organism of black rot in vegetable *Brassica* crops, such as broccoli, cabbage, calabrese, canola, cauliflower and oilseed rape. The use of healthy seed is a critical aspect of a disease management strategy.

The ISTA Rule 7-019 is the current reference method for the detection of Xcc in untreated seed lots (1). In this method bacteria are extracted by soaking whole seeds in saline buffer plus Tween, the extract is then diluted and plated on mCS20ABN and FS semi-selective media and suspect Xcc colonies are transferred to YDC. Finally, suspect Xcc colonies on YDC (Yeast Dextrose Chalk) are confirmed in a pathogenicity test. The development of typical black rot symptoms in inoculated plants of the pathogenicity test confirm that the seed lot is contaminated with Xcc. The ISTA Rule 7-019 is based on the results of a comparative test organised by the International Seed Health Initiative-Vegetables, ISF (ISHI-Veg) in 1995 (2) in which 13 laboratories participated.

Hot water and other similar proprietary treatments are a common practice for treating *Brassica* spp. seed lots found to be Xcc positive. The efficacy of such treatments in disinfecting is seed-lot dependent and therefore treated seed lots require to be retested thoroughly for the presence of viable bacteria that may have survived the treatment. ISTA Rule 7-019 involves just soaking the seed for extracting the pathogen. This relatively mild extraction does not allow for the detection of Xcc cells located in the seed that might have survived the treatment. Therefore, an adapted protocol has been developed for detecting Xcc in disinfected *Brassica* spp. seed lots.

Wet grinding of seeds after soaking was found to be crucial for detecting Xcc in disinfected seed lots (M. Asma; H. Koenraadt, personal communication). This practice facilitates the release of bacteria located in the seed. Moreover, the use of buffered saline (PBS) rather than saline, in a larger ratio of buffer to seed has been shown to prevent a reduction in Xcc recovery caused by a suboptimal pH of the extract associated with certain proprietary treatments (3). Concentration of the seed extract by centrifugation and a longer incubation time of the plated extracts on the mCS20ABN and FS semi-selective media have been introduced into this protocol to increase the sensitivity of the detection assay. Suspect bacterial colonies were confirmed in a pathogenicity test slightly different to the one described in ISTA Rule 7-019.

The composition of the semi-selective media used was according to the ISHI comparative test in 2005 with the sole exception being the addition of extra potassium dihydrogen phosphate (KH_2PO_4) and agar in the mCS20ABN medium. Agar enhanced the absorption of the seed wash extract (H. Koenraadt, personal communication). Suspect Xcc colonies obtained using the two extraction methods 'soaking' and 'wet grinding' were recorded on both media confirmed in a pathogenicity test, which is slightly different to the one described in ISTA Rule 7-019.

A comparative test was organised by the International Seed Health Initiative-Vegetables, ISF (ISHI-Veg) in which eight seed health testing laboratories from The Netherlands, France, U.S.A. and Japan participated.

Aim and objective of the comparative test

The aim of this comparative test was to demonstrate the beneficial effect of:

i) wet grinding over soaking as in the ISTA Rule 7-019, by comparing the findings "after grinding" to the findings "after soaking".

ii) ii) the ten-fold concentration of the seed extract over the undiluted and ten-fold diluted extracts by comparing the number of suspect Xcc (cfu/ml) in each extract

on the detection of surviving Xcc cells in disinfected *Brassica* spp. seed subsamples.

The ultimate objective was to develop an internationally accepted seed health testing method for detecting Xcc in disinfected *Brassica* spp. seed lots.

Sensitivity of the proposed method

This test method is suitable for seed that has been treated using physical (hot water) or chemical (chlorine) or proprietary processes with the aim of disinfection provided that any residue, if present, does not influence the reliability of the assay.

In the current ISTA Rule 7-019 for the detection of Xcc, 100 µl of the undiluted extract is plated on the media. The theoretical sensitivity for Xcc in untreated seed is 15 cfu/ml with a probability of 95%. In the proposed method, the additional ten-fold concentration of the bacterial extract increases the theoretical sensitivity to 1.5 cfu/ml. In practice, this sensitivity is not always reached due to the presence of saprophytes that might disguise the presence of Xcc. However, after a disinfection treatment the amount of saprophytes is strongly reduced and therefore the practical sensitivity is not far from the theoretical sensitivity.

Materials and Methods

Seed lots and seed subsamples

Three cabbage (*Brassica oleracea*), hot-water treated (25 min at 50°C) seed lots with variable levels of remaining (natural) contamination and saprophytes were selected by the laboratory of Bejo Zaden B.V. in The Netherlands. The seed lots were characterised prior to the comparative test based on the number of detected positive seed subsamples before and after grinding. Each subsample comprised 10 000 seeds.

One out of five subsamples tested from the seed lot P10.077 was positive after soaking and all five subsamples were positive after grinding. Of the 5 subsamples from seed lot P10.067, zero and three subsamples tested positive after soaking and after grinding, respectively. Thus, the seed lots P10.077 and P10.067 were characterised as "high" and "medium", respectively. The eight subsamples from lot P48.077 tested negative for Xcc after soaking and after grinding, and were characterised as being "healthy".

Each participating laboratory received three, ten and seven subsamples of 10 000 seeds from the high, medium and healthy seed lots, respectively. These subsamples were prepared by GEVES-SNES in France with the use of the rotary sample divider based on the thousand seed weight of each seed lot. The subsamples were coded randomly to ensure a blind comparative test.

Reference culture

All participating laboratories used the MATREF 2.3.2 (CFBP 7143) reference culture of Xcc grown on YDC medium in two petri-dishes provided by GEVES-SNES.

Media

All participating laboratories used the premixed medium ingredients from Duchefa (4) to prepare the mCS20ABN and FS semi-selective media. So as to avoid any differences in the batches premixed powder ingredients were included in the test package by the test organizer.

Each laboratory prepared the mCS20ABN (Duchefa Cat. No. C-5122) and FS (Fieldhouse-Sasser) (Duchefa Cat. No. C-5123) media using the premixed powders. For the mCS20ABN medium the final concentration of the compounds per liter of distilled water was: 2.8 g/l KH_2PO_4, 0.8 g/l $(NH_4)_2HPO_4$, 0.4 g/l $MgSO_4.7H_2O$, 2.0 g/l bacto tryptone, 2.0 g/l soya peptone, 6.0 g/l L-glutamine, 1.0 g/l L-histidine, 1.0 g/l dextrose, 25.0 g/l soluble starch (Merck 1252) and 18.0 g/l agar. The pH was adjusted to 6.5 and the medium was autoclaved at 121°C for 15 min. When the mixture cooled to 50°C the following sterile antibiotics were added: 35 mg/l nystatin, 40 mg/l neomycin sulphate and 100 mg/l bacitracine.

For the FS medium the final concentration of the compounds per liter of distilled water was: 0.8 g/l K_2HPO_4, 0.8 g/l KH_2PO_4, 0.5 g/l KNO_3, 0.1 g/l $MgSO_4.7H_2O$, 0.1 g/l yeast extract, 25.0 g/l soluble starch (Merck 1252), 15.0 g/l agar along with 1.5 ml methyl green (1% aq.) which the laboratories added. The pH was adjusted to 6.5 and the medium was autoclaved at 121°C for 15 min. When the mixture cooled to 50°C the following sterile antibiotics were added: 35 mg/l nystatin, 3 mg/l D-methionine, 1 mg/l pyridoxine HCl, 50 mg/l cephalexine, 30 mg/l trimethoprim. The medium plates with FS and mCS20ABN were stored for at least 4 days at 4°C prior to use so they became opaque due to the retrogradation of the starch. This facilitated the recognition of starch hydrolysis by Xcc colonies.

The YDC medium was prepared by each laboratory with the following composition: 10.0 g/l yeast extract, 20.0 g/l dextrose, 20.0 g/l $CaCO_3$ and 15.0 g/l agar per liter of distilled water. The medium was autoclaved at 121°C for 15 min and cooled down to 50°C.

Bacteria extraction from seed subsamples

All 10 000-seed subsamples were suspended in 250 mL sterile PBS (0.05M phosphate) (8.0g/l NaCl, 5.75 g/l Na_2HPO_4, 1.0 g/l KH_2PO_4, pH 7.2-7.4) plus Tween 20 (0.02% v/v) and then soaked for 2.5 h at room temperature (20°-25°C) under agitation by an orbital shaker at 100-125 rpm. Two (2) ml of the agitated seed wash (undiluted seed wash after soaking) of each subsample was collected in a sterile container. The seeds from all subsamples were then ground in the remaining 248ml PBS (0.05M phosphate) plus Tween 20 (0.02% v/v) with a suitable grinder such as Ultra Turrax T25 with S25N-25G dispersion tool until all seeds are ground - this point is reached in at most 2 minutes of grinding. The grinder was cleaned thoroughly by running it two times in hot water, one time in 70% ethanol and two times in sterile water. To prevent any cross contamination between subsamples the S25N-25G dispersion tool was autoclaved or disassembled and its parts were immersed in 70% ethanol and rinsed with sterile water to remover ethanol residues after assembling Coarse particles were filtered from the extracts using a bag filter model P 400 ml (InterScience, France) or universal extraction bag model with synthetic intermediate layer (Bioreba, Switzerland) or filter extraction bags (Neogen Europe, Scotland) and 3.5 ml of each filtrate (undiluted seed extract after grinding) were transferred to a centrifuge tube and kept on ice.

Dilution, concentration and plating

Undiluted seed wash after soaking (or 10^0) – From each seed wash a ten-fold (10^{-1}) and a hundred-fold (10^{-2}) dilution was prepared. The (10^{-1}) dilution was prepared by pipetting 0.5ml of the undiluted seed wash into 4.5 ml of sterile PBS (0.05M phosphate) plus Tween 20 (0.02% v/v) and was vortexed to mix. The (10^{-2}) dilution was prepared by pippetting 0.5 ml of the 10^{-1} dilution into 4.5 ml of sterile PBS (0.05M phosphate) plus Tween 20 (0.02% v/v) and was vortexed to mix. 100 µl each of the seed wash (10^0) and the two dilutions (10^{-1} and 10^{-2}) were plated in duplicate on the mCS20ABN and FS media.

Undiluted seed extract after grinding (10^0) – From the 3.5 ml of each undiluted seed extract 0.5 ml were used to prepare a ten-fold dilution (10^{-1}) and 2.0 ml were used to prepare a ten-fold concentration (10^{+1}).The remaining 1ml was used to plate the (10^0) undiluted seed extract. The tenfold dilution was prepared by pippetting 0.5 ml of the undiluted filtered seed extract into 4.5 ml of sterile PBS (0.05M phosphate) plus Tween 20 (0.02% v/v) and vortexed to mix. The ten-fold concentration was prepared by centrifuging 2 ml of the extract for 5 minutes at 5000 *g*, discarding the supernatant and re-suspending the pellet in 200 µl sterile PBS (0.05M phosphate) plus Tween 20 (0.02% v/v). 100 µl each of the seed extract (10^0) and the ten-fold dilution (10^{-1}) was plated in duplicate on the mCS20ABN and FS media while 100 µl of the ten-fold concentrate (10^{+1}) was plated once on the two media.

Positive control plates were prepared by plating 100 µl of serial ten-fold dilutions of the reference culture suspensions on each medium. Serial ten-fold dilutions of the PBS plus Tween 20 were plated on each medium and served as sterility check. All plates were incubated at 28°-30°C.

On the FS medium the Xcc colonies after 4-6 days of incubation appeared small, pale green, mucoid and were surrounded by a zone of starch hydrolysis. On the mCS20ABN medium the Xcc colonies after 4-6 days of incubation appeared pale yellow, mucoid and are surrounded by a zone of starch hydrolysis. On the last day of incubation, colonies from seed subsamples were visually compared to colonies of the reference culture on the same medium and were considered suspect if they appeared similar to the latter. The number of suspect Xcc colonies on each plate was counted. If present, up to six suspect colonies from each semi-selective medium per seed sub-sample and colonies from the reference culture were sub-cultured to sectored plates of YDC. The plates were incubated at 28°C-30°C for 3-4 days. The Xcc isolates on YDC medium appear yellow and mucoid. If present, at least two YDC suspect colonies from each semi-selective medium were confirmed in a pathogenicity test.

Pathogenicity test

Seedlings of a known susceptible cabbage variety Wirosa (5) were grown at 20°C-30°C until the 2-3 true leaf stage. The secondary veins of the two first true leaves of two seedlings were stab-inoculated with a YDC suspect bacterial isolate using a sterile toothpick. The same procedure was followed with the reference isolate which served as positive control. The inoculated plants were incubated at 20°-30°C. Ten and 14 days post-inoculation symptoms were compared to symptoms of positive control plants and the positive colonies were recorded. Typical Xcc symptoms are the local yellow necrotic lesions, followed by systemic blackening of veins.

Data analysis

For each combination (laboratory x contamination level x seed subsample x after soaking/after grinding x semi-selective medium x undiluted/diluted/concentrated extract x plate) the number of Xcc suspects in cfu/ml was recorded.

The number of positive colonies in the pathogenicity test was used to estimate the number of confirmed Xcc colonies obtained per subsample, semi selective medium and lot. This was calculated by multiplying the average number of Xcc recorded suspects with the proportion of Xcc positive colonies of the total tested in the pathogenicity test (6, 7) as shown in the formula:

Confirmed Xcc colonies (no.) = (number of suspects) $\times$ (number of positives ÷ total number tested)

For instance, if 100 suspect colonies were recorded and five were tested for pathogenicity, four positives in the confirmation test would give 100*4/5 or 80 confirmed colonies.

Laboratory 5 reported testing suspect and non-suspect Xcc colonies for pathogenicity. Although this was a deviation from the protocol, Laboratory 5's experience and skill in detecting and identifying Xcc colonies on both semi-selective media led the authors to include its results in the final analysis. The rest of laboratories confirmed all tested Xcc suspect colonies in the pathogenicity test.

The average number of Xcc cfu/ml from the results of the undiluted, diluted and concentrated extracts of the (laboratory x contamination level x seed subsample x after soaking/after grinding x semi-selective medium) combination was analysed in ANOVA with Statistica programme. Prior to the analysis values were transformed with $\log_{10}$ (v+1), where v equals to cfu/ml.

Following the characterisation of seed lots, the SeedCalc Version 8 (8) spreadsheet application was used to calculate the contamination rate of Xcc in the "medium" lot after grinding. The number of expected positive subsamples from this lot in the comparative test at a probability higher than 5% was then calculated with the spreadsheet application developed by J. L. Laffont (ISTA Statistics Committee Chair). The 3 positive 10 000-seed subsamples of the 5 tested revealed a contamination rate of 0.01% and 4-9 expected positive subsamples of the 10 distributed to laboratories with a probability higher than 5%. In the "high" lot, as all 3 subsamples were positive after grinding during the characterization, it was expected that the laboratories would also find them positive after grinding.

For every subsample of the "high" and "medium" Xcc levels of contamination each laboratory also used the results of the pathogenicity test to record the number of positive seed subsamples detected after soaking and after grinding per level lot and laboratory. A subsample was considered positive if at least 1 YDC suspect colony isolated *either* from FS *or* mCS20ABN medium was confirmed in the pathogenicity test. A

subsample was considered negative if there were no Xcc suspect colonies recorded in *any* of the two semi-selective media. If there was no result from the YDC medium (laboratory 2, for instance, reported fungal contamination in YDC medium plates as a result of which confirmation of suspect Xcc in one "high" subsample was not feasible) it was considered to be a missing value.

The number of positive and negative subsamples using data from the (laboratory x level x subsample x after soaking /after grinding) combination was analysed in a Generalised Linear Model on the binary results (positive or negative result) with a logit link function in a Type I test implemented in the R version 2.13.1 (9) statistical program.

Concentration – dilutions comparison

The ten-fold concentrated (10^{+1}) seed extract was compared to the undiluted (10^{0}) and ten-fold (10^{-1}) diluted seed extracts for the suspect Xcc cfu/ml after grinding of both "medium" and "high" levels in their 208 total (laboratory x subsample x media) combinations.

Results

No laboratory recorded any Xcc suspects in the sterility check plates and in the "healthy" subsamples plated in both semi-selective media after grinding and after soaking.

A higher number of Xcc cfu/ml was recorded by all laboratories after grinding when compared with after soaking in both "medium" and "high" levels (Figures 1, 2). In effect, in each seed "medium" and "high" subsample that was found positive after grinding and after soaking in the mCS20ABN medium, all the laboratories recorded a higher number of Xcc cfu/ml after grinding than after soaking (data not shown). All the laboratories obtained a similar result for the FS medium also with the exception of laboratory 4 for one "high" subsample (data not shown).

The Analysis of Variances (ANOVA) on the average of Xcc cfu/ml of "medium" and "high" levels of both semi-selective media after soaking and after grinding showed a contamination level and a grinding effect (Table 1). However, the interaction between these factors was also significant (Table 1) demonstrating that the grinding effect was higher in the "high" than in the "medium" level (interaction graph not shown).

In both "medium" and "high" levels, subsamples that were negative after grinding were also negative after soaking (data not shown). Moreover, subsamples that were found positive after soaking were also found positive after grinding (data not shown).

The number of detected and expected positive subsamples out of the total tested after grinding and after soaking is presented in Tables 2 and 3 by contamination level and laboratory. The expected number of positive subsamples after grinding of the "medium" level was detected in all but Laboratories 2 and 3. The expected number of positive seed subsamples was detected by all laboratories in the "high" level after grinding (Table 2).

One subsample of the ten tested from the "medium" level was found positive after soaking in 3 laboratories. Likewise, only one of the three "high" subsamples tested after soaking was found positive in 5 laboratories (Table 3).

In Tables 4 and 5 the number of positive subsamples detected by the laboratories after soaking and after grinding, by semi-selective medium and contamination level is presented.

The analysis of deviances for the number of positive and negative subsamples after soaking and after grinding from "medium" and "high" levels using the Generalised Linear Model on the binary results showed a contamination level and a grinding effect (Table 6). No significant interaction was found between laboratories, contamination level and grinding (Table 6).

Concentration – dilutions comparison

Table 7 presents the comparison of suspect Xcc (cfu/ml) found in the concentrated and diluted extracts of the 208 total (laboratory x subsample x media) combinations of both "medium" and "high" levels. In 51 combinations, the number of Xcc suspects in the ten-fold concentrated seed extract was higher or equal to those in the 10^{0} and 10^{-1} seed extracts. A positive result in the ten-fold concentrated extract and a negative result in the undiluted extract and the ten-fold dilution were recorded in 25 combinations. A negative result in the ten-fold concentrated extract and a positive result in the undiluted extract and the ten-fold dilution were recorded in 8 combinations.

Discussion

No false positive results were recorded. There was no cross contamination as the sterility control plates and all the “healthy” subsamples were found Xcc-negative after soaking and after grinding.

The Xcc cfu/ml values were higher after grinding than after soaking on both media and in both “high” and “medium” levels, as shown in the ANOVA analysis. This result, in addition to the grinding effect shown in the Generalised Linear Model on the binary results analysis, confirmed the initial hypothesis that wet grinding has a beneficial effect over seed soaking as prescribed in the ISTA Rule 7-019 when detecting Xcc in seeds that have already undergone a disinfection treatment.

The difference in the level of contamination in the seed lots was distinguished by all the laboratories after soaking and after grinding. This was confirmed by both analyses. Moreover, the detection of a higher Xcc load (cfu/ml) in the “high” subsamples as compared with the “medium” after grinding and after soaking confirmed the initial characterization of the seed lots by their contamination level. The higher grinding effect in the “high” than in the “medium” level that was shown by the grinding x level interaction is explained by the lower than the expected number of positives – and subsequently of Xcc cfu/ml- that Laboratories 2 and 3 detected in the “medium” lot. Given the experience of these two laboratories with the method this result is attributed to a probable mishandling of the subsamples during test performance. However, the other six laboratories not only detected the expected number of positives in the range estimated by the statistical tool developed by J.L. Laffont but they also arrived at a similar number. These findings in conjunction with the absence of interactions in the Binomial model analysis show that the detection of Xcc after wet grinding of seed was repeatable and reproducible for all three contamination levels.

The ten-fold concentration of the seed extract after grinding improved the detection of the Xcc colonies in 76 (laboratory x subsample x media) combinations of both “medium” and “high” lots. This improvement was even more profound in the 25 combinations where the ten-fold concentration showed a higher than zero result and the undiluted and the ten-fold diluted extracts showed a zero result. The low saprophytic load that was recorded in the latter verified that the ten-fold concentration enhanced the detection of Xcc suspects in disinfected seed lots. However, in the eight combinations that showed the opposite result, the presence of saprophytic in various load was recorded which might serve as an explanation.

Conclusions and recommendations

Wet grinding *Brassica* spp. seed was shown to enable the extraction of internally located Xcc cells that had survived a disinfection treatment.

In seed extracts that contained low Xcc and saprophytic load, a ten-fold concentration of the undiluted seed extract after grinding was shown to improve detection of Xcc cells.

Wet grinding and a ten-fold concentration of the undiluted seed extract of disinfected *Brassica* spp. seed were essential for the detection of *X. c*. pv. *campestris* positive seed lots.

The protocol described in this report is a reliable method for detecting Xcc in disinfected *Brassica* spp. seed lots and is highly recommended for routine testing by seed health laboratories.

Acknowledgements

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Bibliography

1. International Rules for Seed Testing - Annexe to Chapter 7: Seed Health Testing Methods ISTA 7-019: Detection of *Xanthomonas campestris* pv. *campestris* on *Brassica* spp. ISTA 2005

2. Koenraadt, H., van Bilsen, J.G.P.M. and Roberts, S.J. (2005). Comparative test of four semi-selective media for the detection of *Xanthomonas campestris* pv. *campestris* in Brassica seed. *Seed Science and Technology* **33**, 115-125

3. Koenraadt, H., Borst, R. and van Vliet, A. (2007). Detection of *Xanthomonas campestris* pv. *campestris* in cabbage seeds after hot water treatment. *Phytopathology* **97**:S59.

4. Duchefa Biochemie (http://www.duchefa.com/)

5. Vicente, J.G., Conway, J., Roberts, S.J. and Taylor, J.D. (2001). Identification and origin of *Xanthomonas campestris* pv. *campestris* races and related pathovars. *Phytopathology* **91**, 492-499.

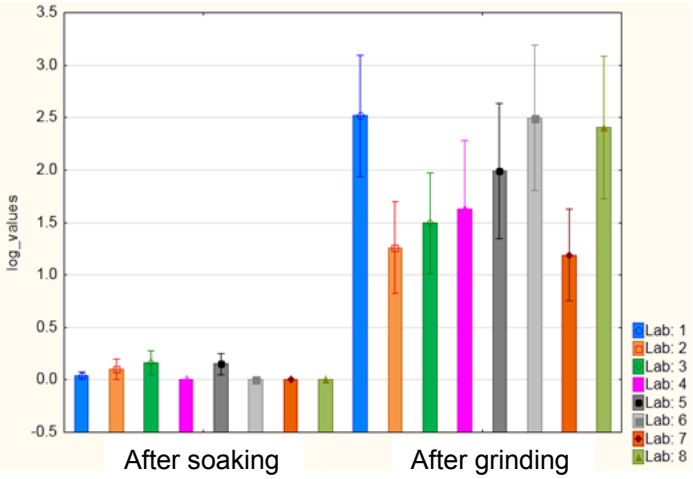
6. Asma, M. (2005). Proposal for a new method for detecting *Xanthomonas hortorum* pv.*carotae* on carrot seeds. ISTA Method Validation Reports 2, 1-17.

7. Kurowski, C. and Remeeus, P.M. (2007). Proposal for a new method for detecting *Pseudomonas savastanoi* pv. *phaseolicola* on bean seeds. *ISTA Method Validation reports 4*, 1–22.

8. Seedcalc version 8 (http://www.seedtest.org/en/statistical-tools-for-seed-testing-_content---1--1143--279.html)

9. R version 2.13.1. (2011-07-08), Copyright © 2011, The R Foundation for Statistical Computing, ISBN 3-900051-07-0, Platform: i386-pc-mingw32/i386 (32-bit).

Figure 1. Adjusted means and standard errors of the $\log_{10}$(Xcc cfu/ml+1) after soaking and after grinding in both contamination levels and both semi-selective media per laboratory.



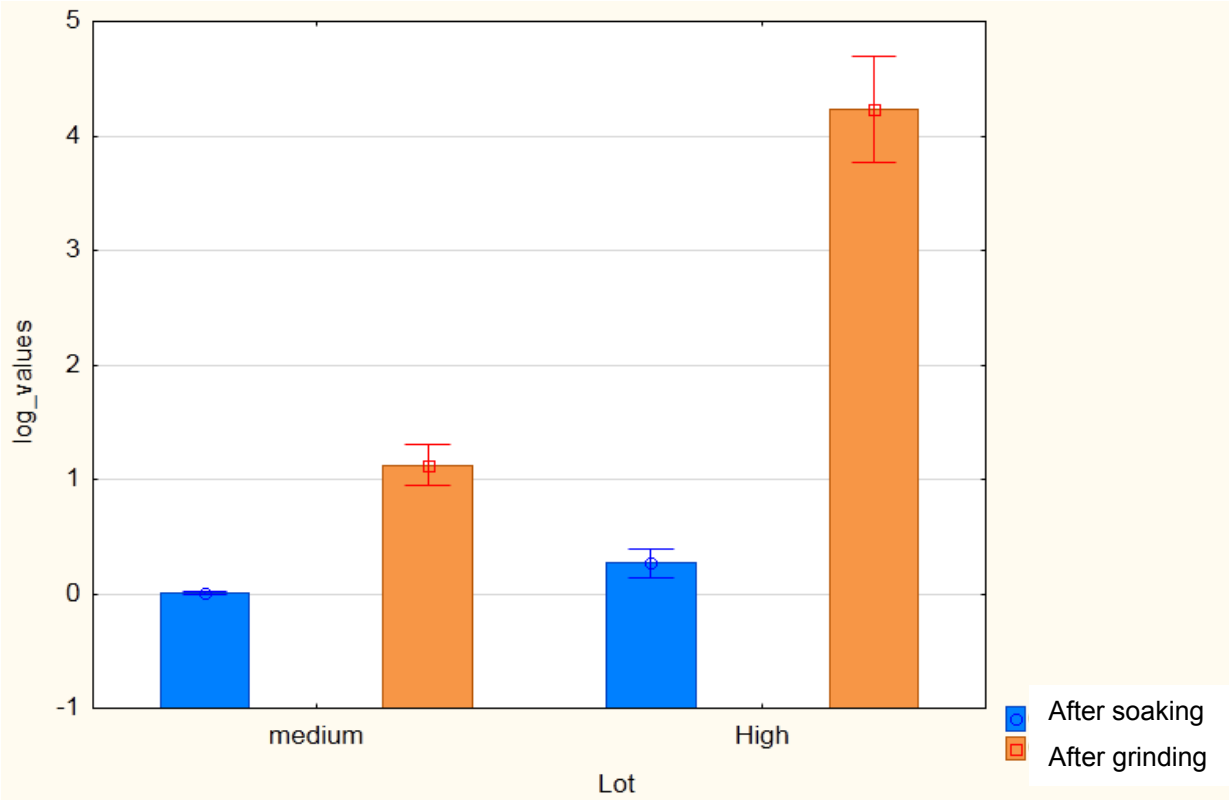


Figure 2. Adjusted means and standard errors of the log_{10}(Xcc cfu/ml +1) after soaking and after grinding in both semi-selective media and all laboratories per "medium" and "high" levels.

Table 1. Statistical output for the Xcc cfu/ml of both "medium" and "high" levels, both semi-selective media of all laboratories. Data prior use was transformed with the function: log_{10} (v+1), where v=Xcc cfu/ml.

	SS	Df	MS	F	P
Ord.Orig.	260.7207	1	260.7207	122.7072	0.000000
Lab	18.2726	7	2.6104	1.2286	0.287364
Cont.Level	143.5109	1	143.5109	67.5428	0.000000
Grinding	224.8676	1	224.8676	105.8331	0.000000
Lab*Cont.Level	12.7993	7	1.8285	0.8606	0.538355
Lab*Grinding	22.2685	7	3.1812	1.4972	1.68527
Cont.Level*Grinding	103.0693	1	103.0693	48.5091	0.000000
Lab*Cont.Level*Grinding	18.3711	7	2.6244	1.2352	0.283794
Error	539.6837	254	2.1247		

Table 2. Number of detected and expected positive subsamples out of the total tested after grinding in the "medium" and "high" levels per laboratory.

After grinding				
	No. of positive detected subsamples/total tested	No. of expected positive subsamples with a probability ≥ 5%	No. of positive detected subsamples/total tested	No. of expected positive subsamples with a probability ≥ 5%
Laboratories	Medium lot	Medium lot	High lot	High lot
1	6+/10	4-9+/10	3+/3	3+/3
2	2+/10	4-9+/10	3+/3	3+/3
3	2+/10	4-9+/10	3+/3	3+/3
4	6+/10	4-9+/10	3+/3	3+/3
5	6+/10	4-9+/10	3+/3	3+/3
6	7+/10	4-9+/10	3+/3	3+/3
7	6+/10	4-9+/10	3+/3	3+/3
8	7+/10	4-9+/10	3+/3	3+/3

Table 3. Number of positive detected subsamples out of the total tested after soaking per laboratory and contamination level.

After soaking		
Laboratories	Medium lot	High lot
1	0+/10	1+/3
2	0+/10	0+/2*
3	0+/10	1+/3
4	1+/10	1+/3
5	1+/10	1+/3
6	0+/10	0+/3
7	1+/10	1+/3
8	0+/10	0+/3

* One seed subsample was recorded as missing value.

Table 4. Number of positive detected subsamples after grinding per laboratory, contamination level and semi-selective medium.

Positive detected subsamples/total tested after grinding				
	Medium lot		High lot	
Laboratories	FS	mCS20ABN	FS	mCS20ABN
1	6+/10	5+/10	3+/3	3+/3
2	2+/10	2+/10	2+/3	3+/3
3	2+/10	2+/10	3+/3	2+/3
4	5+/10*	5+/10*	2+/3	3+/3
5	5+/10	6+/10	1+/3	3+/3
6	7+/10	5+/10	3+/3	3+/3
7	6+/10	5+/10	3+/3	3+/3
8	7+/10	6+/10	3+/3	3+/3

*Four seed subsamples were confirmed in both media. The fifth was confirmed in FS only and the sixth in mCS20ABN only.

Table 5. Number of positive detected subsamples after soaking per laboratory, contamination level and semi-selective medium.

Positive detected subsamples/total tested after soaking				
	Medium lot		High lot	
Laboratories	FS	mCS20ABN	FS	mCS20ABN
1	0+/10	0+/10	1+/3	0+/3
2	0+/10	0+/10	0+/3	0+/2*
3	0+/10	0+/10	1+/3	1+/3
4	1+/10	0+/10	1+/3	0+/3
5	0+/10	1+/10	1+/3	1+/3
6	0+/10	0+/10	0+/3	0+/3
7	1+/10	0+/10	1+/3	0+/3
8	0+/10	0+/10	0+/3	0+/3

** One seed subsample was recorded as missing value.

Table 6. Statistical output for the Generalised Linear model data analysis of the total number of Xcc positive and negative seed subsamples of medium and high contamination levels of all laboratories, after soaking and after grinding.

Response: Final Score

	Df	Deviance Res.	Df	Res.Deviance	Pr(>Chisq)
NULL			206	269.91	
Lab	7	6.583	199	263.33	0.4735.
Lot	1	17.465	198	245.86	2.927e-05***
Grinding	1	93.620	197	152.24	□2.2e-16***
Lab:Cont. level	7	4.867	190	147.38	0.6762
Lab:Grinding	7	8.729	183	138.65	0.2727
Cont. level:Grinding	1	1.7555	182	136.89	0.1853
Lab:Cont. level:Grinding	7	0.000	175	136.89	1.0000

. No significant difference 0.05 □ *P* □ 0.1

*** Significant difference with 0 □ *P* □ 0.001

Table 7. Comparison of the ten-fold concentrated seed extract to the undiluted and ten-fold diluted seed extracts after grinding for the suspect Xcc (cfu/ml) of “medium” and “high” levels’ total combinations.

	Combinations	Ten-fold concentrated (10^{+1}) seed extract (X)	Undiluted (10^{0}) seed extract (Y)	Ten-fold diluted (10^{-1}) seed extract (Z)
	51	X>0 and X≥Y and X≥Z		
	25	X>0	Y=0	Z=0
	8	X=0	Y>0	Z>0
Total	208			

ISTA/ISHI comparative test for method 7-021 modification for the identification of *Xanthomonas axonopodis* pv. *phaseoli* (*sensu* Vauterin *et al.*, 2000) on bean seeds 2011

1. Organisation and design

1.1 Test Organiser

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1.2 Pathogen

Xanthomonas axonopodis pv. phaseoli

1.3 Crop

Phaseolus vulgaris (Bean)

1.4 Participating laboratories and contact persons

GEVES-SNES , BioGEVES	FR	Valérie Grimault, Mathieu Rolland	PCR and PT
Plant health laboratory -ANSES	FR	Valerie Olivier, Françoise Poliakoff	PCR and PT
INRA	FR	Marie-Agnès Jacques, Armelle Darrasse	PCR and PT
Naktuinbouw	NL	Harrie Koenraadt	PCR
Monsanto	US	Yumee Kim	PCR
Eurofins STA Labs	US	Narceo Bajet	PCR

Criteria required: Experienced laboratory on bacterial testing

2. Introduction and objective of the method

2.1 Introduction

Xanthomonas axonopodis pv. *phaseoli* (fuscans and non fuscans isolates: Xap) is a very important seedborne bacterial pathogen of bean and the causal organism of common blight. This pathogen is genetically diverse (Birch *et al*., 1997; Lopez *et al*., 2006, Schaad *et al.*, 2006, Vauterin *et al*., 1995 and 2000). Strains of *X. axonopodis* pv. *phaseoli* can be grouped into four distinct genetic lineages, three of them group the non-fuscous strains while fuscous strains are less diverse and formed the remaining lineage (Alavi *et al.*, 2008; Mkandawire *et al.*, 2004). Currently, three valid propositions coexist for this group of pathogens. According to Vauterin *et al.* (1995), strains are grouped in the pathovar *phaseoli* of the *X. axonopodis* species; fuscous strains forming a variant in this pathovar. In 2005, Schaad and colleagues proposed to separate this pathovar into two groups: the fuscous strains forming a subspecies of the newly proposed *X. fuscans* species, while the nomenclature of the others (non-fuscous strains) was not modified and remained

X. axonopodis pv. *phaseoli.* Ah-You and colleagues in 2009, proposed to maintain the fuscous strains as a variant in the pathovar *phaseoli* of the newly proposed *X. citri* species.

In this document, we follow the proposition provided by Vauterin and colleagues (1995 confirmed in 2000) and adopt the name *Xanthomonas axonopodis* pv. *phaseoli* for the pathogen responsible for the common bacterial blight of bean. If necessary, fuscous strains are referred to *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans.* The pathogen has a quarantine status and therefore a reliable test of identification is of outmost importance. Dilution plating on semi selective media, transfer of suspected isolates to non selective media, and then a pathogenicity assay by stem wounding with the suspected isolates is currently used for detection of seedborne Xap (ISTA method 7-021). The ISTA method has been published recently.

In 2010 in the USA several seed lots were sent to different laboratories and conflicting results were obtained. Also in France conflicting data were obtained with the new ISTA method. Research in France (GEVES and INRA) and the Netherlands (Naktuinbouw) showed that isolates that were responsible for the positive result were causing symptoms in the pathogenicity assay but were not identified as Xap based on serological (immunofluorescence microscopy in NL) and/or molecular methods (genetic bacterial fingerprinting in NL and pathogen specific PCR's in France). Some isolates were phenotypically indistinguishable from *Xanthomonas axonopodis* pv. *phaseoli*, were pathogenic with the ISTA test, but were genotypically distinct from *Xanthomonas axonopodis* pv. *phaseoli* (e.i.: Stenotrophomonas spp, Xanthomonas campestris isolates). Therefore we can conclude that the pathogenicity assay used in the ISTA method, a crucial step in the Xap test, is not reliable enough. ISTA needs therefore urgently a modification of its 7-021 method.

In the Netherlands two published primer sets (Toth *et al.,*1998 and Audy *et al.,*1994) have been used for a long time in a routine setting. Recently these sets have been validated in the framework of a validation program between Naktuinbouw and the national plant protection service (NPPS). Toth primers are described as specific for *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* (Toth *et al.,* 1998) whereas Audy primers are described as specific for *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* (Audy *et al.,* 1994). In France completely new primers have been developed and validated by INRA. These primer sets identify bacterial species that cause common blight. The new sets are patented by the Diag-gene company (Boureau et al, 2012) while the Toth and Audy PCR's are in the public domain. Other primer sets also give indication on the identity of suspect isolates: rpfB is specific for Xanthomonadaceae (Simoes *et al.*, 2007) and hrcV (INRA unpublished data) is specific to phytopathogenic xanthomonads. In this program the specificity of the different assays was evaluated using a large number of Xap's and out group strains.

A pathogenicity test was developed at INRA to allow a reliable characterization of aggressiveness of *X. axonopodis* pv. *phaseoli* wild type strains and mutants (Darsonval *et al.*, 2009). This pathogenicity test is based on dipping the first trifoliate leaf in inoculum. After incubation in tropical-humid type conditions (critical point, see protocol attached), watersoaked spots and necrosis developed on the inoculated leaf. This test mimics the conditions of natural contamination of plants. It is not invasive, uses a light inoculum and hence is highly robust. It allows avoiding cross contamination among treatments as inoculum is not sprayed. In preliminary experiments it was shown that results obtained with this test correlate exactly with strains identification and characterization: *X. axonopodis* pv. *phaseoli* strains only induced symptoms while false-positive results were recorded with the ISTA 7-021 pathogenicity test (report attached, third sheet of excel file: confidential). Both the France primer sets and the Toth and Audy PCR's appeared to be good alternatives to replace the actual pathogenicity test.

A comparison study of the new pathogenicity test (protocol attached) and primers was carried out in a collaborative study between INRA laboratory, ANSES and GEVES on a collection of 204 isolates coming from CFBP collection, seed companies, ANSES and GEVES. Primers chosen for this study were:

- Audy
- rpfB
- hrcV
- kit from Diag-gene based on primers developed in a research project conducted by INRA (2 marker bands + internal control)

Toth primers were not included in this study, as they only allow identification of fuscans isolates.

The results of this study showed that the new pathogenicity test was more reliable than the actual one as isolates firstly identified as positive were negative with the new test. The concentration of the bacterial suspensions used for the pathogenicity test was quantified and homogenized among strains at 10^7cfu/ml,

this concentration is a critical control point for the success of this test. More concentrated suspensions (*i.e.* 10^8 cfu/ml or more) could lead to false positive results.

A combination of Audy and Diag-gene primers was chosen for validation study, and compared in one laboratory with Toth primers. Pathogenicity test was kept for validation study as an alternative to PCR, and for laboratories that would not be equipped for PCR or for confirmation of PCR results.

2.2 Objective

The comparative test was done on a common set of 60 isolates, with the Audy and Diag-gene primers (in all laboratories), with Toth primers (in one laboratory) and with the pathogenicity test (in 3 laboratories only in France, due to quarantine constraints for sending isolates of Xap). The 60 isolates were chosen based on the preliminary study. 30 targets (Xap) and 30 non target (non Xap) isolates were chosen.

The objective of this comparative test was to provide an ISHI/ISTA internationally accepted modification of the actual confirmation test (pathogenicity test by wounding) of ISTA 7-021 method, for the identification of *Xanthomonas axonopodis* pv. *phaseoli* on bean seeds.

Materials and methods

3.1 Samples

Samples for PCR were constituted of DNA instead of bacteria, due to the quarantine status of Xap and expected problems of sending/customs if living bacterial cultures were used. For stability purpose during sending of samples, Qiagen extraction was used (it is however important to notify that for routine testing, boiling gives appropriate amplification results). Each participating laboratory received DNA of 30 *Xanthomonas axonopodis* pv. *phaseoli* (Xap, fuscans and non fuscans) isolates and 30 non target isolates (other Xanthomonads, saprophytes of bean seeds, other bean pathogenic bacteria…) (see excel file rough results). For target isolates, isolate 23 corresponded to Xap CFBP 4834 strain which has been sequenced and will be released soon. Isolate CFBP 6546 (isolate 10) is the Xap type strain. Expected results were determined by INRA, by sequencing of housekeeping genes *gyrB* and *rpoD* to confirm genus, species, pathovars and lineages of isolates.

All samples were coded randomly and their correspondence to isolates was known only to the test co-ordinator. One positive Xap DNA (positive control) and one non Xap DNA (negative control) were uncoded.

Audy primers were acquired by each participating laboratory, based on the sequence given in this test plan. Diag-gene kit was sent with DNA samples and kept at -20°C when received.

In order to perform the pathogenicity tests, the same set of 30 Xap and 30 non target (non-Xap) strains was sent as fresh cultures on Petri dishes to French participating laboratories only (because of the quarantine status of Xap and to save time obtaining import permits for other participants).

3.2 Materials needed to perform the test

- Milli Q and chemicals for PCR preparation
- Sterile microtubes (1.5 ml; 0.2 ml)
- Microliter pipettes (*e.g.* Gilson, Finn) with sterile filtered tips (1 µl – 1000 µl)
- Conventional thermocycler
- Electrophoresis equipment (1.5-2% agarose gels)
- DNA visualizing system (BET or analog reagent, UV imaging apparatus)
- Growth chambers capable of operating at 28°C and over 80% humidity or greenhouse, with high humidity, with quarantine status

3.3 Confirmation by Pathogenicity

See attached protocol. Positive and negative controls were added: water, one Xap and one *Xanthomonas axonopodis* pv. *vesicatoria* strains (not sent by test organizer). 2 plants per isolate were inoculated and symptom development was recorded on the first trifoliate leaves.

3.4 Confirmation by PCR

The PCR confirmation was based on two different assays performed on each sample. For each of the two PCR assays, a single amplification per sample was run.

- For the samples and controls the PCR mix was prepared as indicated below, with addition of 4 µl of the received DNA extract. During each amplification run, a PCR negative control (DNA extract replaced by molecular biology grade water) and the positive and negative controls provided (uncoded) with the set of DNA extracts, were added in some participating laboratories.
- Proceed immediately to thermal cycling as indicated below.
- Load 10µl of each PCR product and an appropriate DNA ladder (100 bp ladder recommended) in a 1.5% agarose gel.
- Run electrophoresis (until dye markers have migrated to the end of the gel).
- Stain the gel with ethidium bromide (Safety precautions section) or other appropriate analog reagent.
- Visualize the DNA bands with a UV table (312 nm) (Safety precautions section).
- Record the gel picture.
- Analyze the amplification products as indicated below.

3.4.1: Audy primers (Audy *et al.*, 1994), for the amplification of *Xanthomonas axonopodis* pv. *phaseoli (sensu* Vauterin *et al.*, 2000)

Primers:

p7X4c	ggc aac acc cga tcc cta aac agg
p7X4e	cgc cgg aag cac gat cct cga ag

PCR mix (adapted by INRA from Audy, 1994):

Reagents	Initial Concentration	Final Concentration	Volume µl
Molecular biology grade water			10.02
Buffer*	5x	1x	4
dNTP	2.5 mM each	0.2 mM each	1.6
p7X4c	20 µM	0.15 µM	0.15
p7X4e	20 µM	0.15 µM	0.15
GoTaq Polymerase *	5 U/µl	0.02 U	0.08
DNA			4
Total volume			20

Or equivalent (Platinum Taq from Invitrogen works well too)

Amplification program:

94°C	3 min	
94°C	1 min	35 cycles
72°C**	2 min	
72°C	10 min	
12°C	Until end	

* Concentrated PCR reaction buffer (with $MgCl_2$), from GoTaq DNA polymerase [Promega] (Laboratories using a buffer without $MgCl_2$ will have to add this salt to a final concentration of 1.5 mM).

** Hybridization and elongation temperature

Interpretation:

The used primers have been designed to amplify a sequence of approximately 800 bp. The presence of this band reveals a positive sample; the absence of this band reveals a negative sample.

3.4.2: Diag-gene primers for the amplification of *Xanthomonas axonopodis* pv. *phaseoli (sensu* Vauterin *et al.*, 2000) (Boureau et al, 2012)

PCR mix:

Reagents	Volume µl
Ready to use master mix (provided)*	16
DNA	4
Total volume	20

* Thaw at ambient temperature, do not vortex (mix by pipetting)

Amplification program:

95°C	5 min	
95°C	30 sec	35 cycles
63°C	30 sec	
72°C	45 sec	
72°C	7 min	
4°C	Until end	

Interpretation:

The ready to use master mix contains different sets of primers amplifying three different sequences. Expected results for the positive and negative controls are presented in figure 1. For positive samples, three bands are expected. The upper one (approx 500 bp) corresponds to an internal control and should be present in any amplification product. The absence of this product reveals an amplification issue. The two lower bands (approx 400 and 200 bp) are specific of *Xanthomonas axonopodis* pv. *phaseoli*. Both bands are required to declare a sample positive. If only one band is present, the sample is negative.

Use the following interpretation guidelines:

- Bands 1, 2 and 3 => positive sample
- Bands 1 and 2 => negative sample
- Bands 1 and 3 => negative sample
- Band 1 => negative sample
- No band => amplification issue

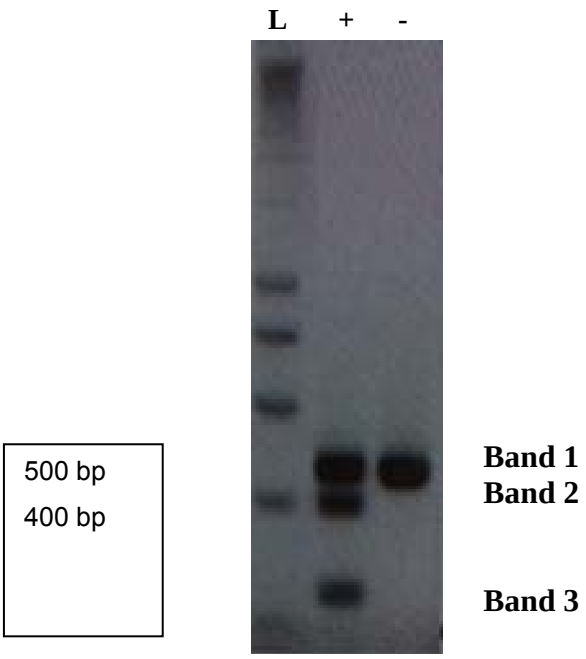

Fig.1: Agarose gel electrophoresis of Diag-gene PCR products. Lane L corresponds to the ladder, lane + to a positive sample, lane – to a negative sample. Three bands are denoted by numbers and by corresponding sizes in base pairs (bp).

4. Statistical analysis

To analyse the results we applied the norms NF EN ISO 16140 to determine the performance criteria of confirmation method through sensitivity, specificity, accuracy and reproducibility.

The analysis consists of a comparison between the expected result and the obtained result in all participating laboratories. This comparison records positive and negative agreements and positive and negative deviations (cf. table 1). In this comparative test the expected result is defined by the known status of target and non target strains.

Table 1:

	expected result + (target)	expected result - (non target)
Obtained result +	positive agreement +/+ (PA)	positive deviation -/+ (PD)
Obtained result -	negative deviation +/- (ND)	negative agreement -/- (NA)

This definition allowed the calculation of sensitivity, specificity, accuracy and reproducibility according to the following mathematical formulas:

Sensitivity = ΣPA/(ΣPA+ΣND)x100

Specificity = ΣNA/(ΣNA+ΣPD)× 100

Accuracy = (ΣNA+ΣPA)/(ΣPA+ΣNA+ΣPD+ΣND)x100

PA = positive agreement

ND = negative deviation

NA = negative agreement

PD = positive deviation

N = total number of possible agreement

A sensitivity of 100% shows that the primers always detect the target pathogen no matter what strain, there were no false negatives.

A specificity of 100% shows that the primers do not give a positive result for a sample that does not contain the target pathogen, there were no false positives.

An accuracy of 100% shows that all the positive or negative results are in agreement with expected ones.

Repeatability and reproducibility were computed assuming that target and non target isolates were respectively identical test materials.

Repeatability was calculated by computing accordance for each laboratory and averaging them over laboratories.

Reproducibility was calculated by computing concordance according to Langton *et al*., (2002).

Results and discussion

- 2 isolates were excluded from analysis because
    - the first one (target 9) was not pure when it was received
    - for the second (target 24), there had been a mistake during DNA preparation
- The results of the PCR tests were indicated by writing "+" if positive or "-" if negative on each primer set column. "?" was indicated if result was not clear (faint band or non specific signal). No extra band at 550bp was observed for fuscans isolates compared to what was described by Audy (1994).
- The result of pathogenicity test were indicated by writing "+" if positive or "-" if negative. "?" was indicated if result was not clear (non typical).

Results	Pathogenicity	Audy primers	Diag gene primers	Toth primers
sensitivity	97.62%	100%	96.43%	46.43% (100% on only fuscans isolates)
specificity	98.89%	96.65%	100%	100% (91.84% if only fuscans isolates are considered)
accuracy	98.28%	98.27%	98.27%	74.14 (93.10%)
repeatability	NT	100%	100%	NT
Reproducibility on target isolates	96.24%	100%	100%	NT
Reproducibility on non target isolates	97.78%	100%	100%	NT

Repeatability was not calculated for pathogenicity. Repeatability and reproducibility was not calculated for Toth primers because only one laboratory performed the test.

Repeatability was 100% for target and non target isolates with both Audy and Diag gene primers.

5.1 Results Pathogenicity tests

The sensitivity of the pathogenicity test was 97.62% meaning that 2 false negative results were obtained on target isolates. The specificity of the pathogenicity test was 98.89% meaning that 1 false positive result was obtained on non target isolates. The accuracy was 98.28% and the reproducibility was 96.24% for targets and 97.78% for non targets, meaning that while values are good (>95%) pathogenicity test is not 100% sure. This is due to the sensitivity of the test to the concentration of the bacterial suspension and to humidity in plant vicinity. These parameters are very important (CCP), but may be difficult to adjust precisely depending on laboratories conditions. Using target and non target controls which can give false results when CCP are not reached would prevent this problem.

5.2 Results Audy, Diag gene and Toth primers

The sensitivity of the Audy primers was 100% meaning that no false negative results were obtained on target isolates. For Diag gene primers, sensitivity was 96.43% meaning that one false negative result was obtained on target isolates in all participating laboratories (isolate 14). For Toth primers, sensitivity was 46.43% meaning that 15 false negative results were obtained on target isolates in all participating laboratory. As Toth primers are described as specific of fuscans isolates only, we calculated the sensitivity on fuscans isolates only. In this case, sensitivity was 100% meaning that no false negative results were obtained on the 9 target isolates tested.

The specificity of the Audy primers was 96.65% meaning that 1 false positive result was obtained in all participating laboratories on non target isolates (isolate 31: *X. axonopodis* pv. *dieffenbachiae)*. The specificity of the Diag gene primers was 100%, there was no false positive results. The specificity of the Toth primers was 100% meaning that no false positive results were obtained on non target (non Xap) isolates in all participating laboratory. As Toth primers are described as specific of fuscans isolates only, we calculated the specificity on non Xap and non fuscans isolates only. In this case, specificity was 91.84% meaning that the primers detect 4 non fuscans Xap isolates.

The accuracy was 98.27% for Audy and Diag gene primers. The accuracy 74.14 and 93.10% for Toth primers on whole collection and only considering fuscans respectively.

Repeatability and reproducibility was 100% for Audy and Diag gene primers. It was not calculated for Toth primers as only one laboratory participated.

Therefore, best results were obtained for Audy and Diaggene primers, with good results (>95%), Audy primers making one false positive result and Diagene one false negative. Therefore, the false positive result given by Audy primers can be considered as negligible as is detects a pathovar which is not infecting bean (pv. *dieffenbachiae).* The false negative result of Diag gene primers show that these primers cannot be used alone for confirmation of suspect Xap isolates. If positive results are correlated between Audy and Diag-gene primers, the presence of Xap is confirmed. If results were not correlated between Audy (+) and Diag-gene (-) no conclusion would be possible and a pathogenicity test would be needed.

6. Conclusion

This comparative test showed that validation criteria of pathogenicity test, Audy and Diag gene primers give very good results for confirmation of suspect Xap isolates, but are not sure at 100%. The Toth primers give less good results for Xap isolates and are informative only for fuscans isolates which are easy to recognize on media due to the pigments they produce.

All suspect isolates (fuscans and non fuscans) should be tested for confirmation tests.

We propose two options for confirmation of suspect isolates:

Option 1: pathogenicity tests, for laboratories not equipped or experienced with PCR. In this case, CCP must be followed and target and non target controls added (*X. vesicatoria*, Xap, water). This option is also valuable and less time consuming when few suspect isolates have been detected but need climatic chamber or greenhouse equipped for high hygrometry.

Option 2: PCR test with Audy primers. If positive with Audy and a low risk of false positive result is desired, a pathogenicity test or PCR test with Diag-gene primers can be used as a confirmation step. This option can be used for laboratories experienced and equipped for PCR, when a short delay is needed for obtaining results and/or a high number of suspect isolates have been detected.

The comparative test was carried out on DNA extracted with a Qiagen kit because we needed stable DNA for transport. A bacterial suspension with boiling step (95°C for 5 min) can also be used as primers are used on isolates and not on seed macerate. For routine use, we advise to use a boiling step.

7. Critical points

- Prevent DNA contamination.
- With Diag-gene primers, bands 1 and 2 are close together, be careful to run the electrophoresis enough time to be able to separate the bands
- Concentration of isolates for pathogenicity test must be at 10^7cfu/ml. If the concentration is higher, false positive necrosis can be observed.

- Humidity must be high (>95%) during the test to obtain typical symptoms
- Xap is a quarantine organism: manipulate in appropriate conditions of quarantine agreement to avoid spread.

8. Safety precautions

Ethidium bromide

Ethidium bromide is carcinogenic. Use ethidium bromide according to safety instructions. It is recommended to manipulate solution instead of powder. Some considerations are mentioned below.

- Consult the Material Safety Data Sheet on ethidium bromide before using the chemical.
- Always wear personal protective equipment when handling ethidium bromide. This includes wearing a lab coat, nitrile gloves and closed toe shoes.
- Leave lab coats, gloves, and other personal protective equipment in the lab once work is complete to prevent the spread of ethidium bromide or other chemicals outside the lab.
- All work with ethidium bromide is to be done in an "ethidium bromide" designated area in order to keep ethidium bromide contamination to a minimum.

UV light

UV light must not be used without appropriate precautions.

Ensure that UV protective eyewear is utilized when visualizing ethidium bromide.

9. Acknowledgements

Participating laboratories: SNES and BioGEVES (France), LSV-ANSES (France), INRA (France), Naktuinbouw (The Netherlands), Monsanto (USA) and Eurofins (USA) are gratefully acknowledged for providing bacterial isolates and carrying out this peer validation study.

10. Bibliography

Audy P., Laroche A., Saindon G., Huang H.C. and Gilbertson R.L. 1994. Detection of the Bean Common Blight Bacteria, Xanthomonas campestris pv. phaseoli and X. c. phaseoli var. fuscans, Using the Polymerase Chain Reaction. Molecular Plant Pathology 84(10): 1185-1192

Birch, P. R. J., Hyman L. J., Taylor R., Opio A. F., Bragard C., and Toth I. K.. 1997. RAPD PCR-based differentiation of Xanthomonas campestris pv. phaseoli from Xanthomonas campestris pv. phaseoli var. fuscans. Eur. J. Plant Pathol. 103:809–814.

Boureau T., Chhel F., Hunault G., Kerkoud M., Lardeux F., Manceau C., Poussier S., Saubion F. 2012 Procédé de dépistage de Xanthomonas axonopodis pv. phaseoli. Patent. Publication 2970480, 20 juillet 2012

Darsonval A., Darrasse A., Durand K ., Bureau C., Cesbron S. et Jacques M.A.. 2009. Adhesion and fitness in the bean phyllosphere and transmission to seeds of Xanthomonas fuscans subsp. fuscans. Molecular Plant-Microbes Interactions 22:747-757.

Langton, S.D., Chevennement, R., Nagelkerke , N., and Lombard , B. 2002. Analysing collaborative trials for qualitative microbiological

methods: accordance and concordance. International Journal of Food Microbiology 79: 175– 181

Lopez, R., Asensio C., and Gilbertson R. L.. 2006. Phenotypic and genetic diversity in strains of common blight bacteria (Xanthomonas campestris pv. phaseoli and X. campestris pv. phaseoli var. fuscans) in a secondary center of diversity of the common bean host suggests multiple introduction events. Phytopathology 96:1204–1213.

Mkandawire, A. B. C., Mabagala R. B., Guzman P., Gepts P., and Gilbertson R. L. 2004. Genetic diversity and pathogenic variation of common blight bacteria (Xanthomonas campestris pv. phaseoli and X. campestris pv. phaseoli var. fuscans) suggests pathogen coevolution with the common bean. Phytopathology 94:593–603.

Schaad, N. W., Postnikova E., Lacy G. H., Sechler A., Agarkova I., Stromberg P. E., Stromberg V. K., and Vidaver A. K. 2006. Emended classification of xanthomonad pathogens on citrus. Syst. Appl. Microbiol. 29:690–695.

Simoes T.H.N., Gonçalves E.R., Rosato Y.B. and Mehta A. 2007. Differentiation of Xanthomonas species by PCR-RFLP of rpfB and atpDgenes. FeMS Microbiol Lett 271: 33-39

Toth I.K., Hyman L.J., Talylor R. and Birch P.R.J. 1998. PCR-based detection of Xanthomonas campestris pv. phaseoli var. fuscans in plant material and it differentiation from X. c. pv. phaseoli . Journal of Applied Microbiology 85: 327–336

Vauterin, L., Hoste B., Kersters K., and Swings J. 1995. Reclassification of Xanthomonas. Int. J. Syst. Bacteriol. 45:472–489.

Vauterin L., Rademaker J. and Swings J. 2000. Synopsis on the taxonomy of the genus xanthomonas. Phytopathology 90(7): 677-82.

Validation of a new method for the detection of *Pseudomonas syringae* pv. *pisi* on Pea (*Pisum sativum*) seed

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Summary

Pseudomonas syringae pv. *pisi* (Psp) is an important seed-borne and seed-transmitted pathogen of pea (*Pisum sativum*). A detection method of Psp on pea seed was evaluated in a comparative test between nine laboratories organized by the ISHI-Veg, ISF. The method includes a seed wash-dilution-plating on the KBBCA and SNAC semi-selective media, optional biochemical tests on Psp suspects and a pathogenicity test for their confirmation. Three untreated naturally contaminated seed lots with various levels of contamination and one untreated pathogen-free seed lot resulting in forty 1000-seed subsamples were used. A characterization and a stability test on the seed lots were additionally conducted by the reference laboratory before and after the comparative test respectively. Results of all three tests showed that the pathogen's distribution in the seed lots was heterogeneous and that the seed lots' contamination level decreased in time depending on the lot. Yet, all laboratories in the comparative test detected the expected number of positive subsamples, distinguished the contamination levels and didn't record any false positives. The detection method was shown repeatable and reproducible. It is considered a reliable method for the detection of Psp on pea seeds and is therefore recommended in routine pea seed testing.

Introduction

Pseudomonas syringae pv. *pisi* (Psp), causal organism of bacterial blight on pea seeds (Grondeau *et al*., 1993), is a significant seed-borne (Hollaway *et al*., 2007) and seed-transmitted (Grondeau *et al*., 1993; 1996; Roberts *et al*., 1992, 1996) bacterial pathogen. Epidemiological studies have shown that Psp infected pea debris is also an important source of inoculum. (Halloway *et al*., 1997, 2007; Grondeau *et al*., 1996). No effective chemical foliar or seed treatments are currently available for control of this disease. Therefore the use of healthy seed is a critical aspect in the disease management strategy (Grondeau and Samson, 1992; Lawyer and Chun, 2001; Hollaway *et al*., 2007; Martin-Sanz *et al.,* 2012).

Several studies have been carried out on the characterization of Psp (Grondeau *et al*., 1996; Elvira-Recuenco and Taylor 2001; Schaad *et al.* 2001) and distinction between *P.s*. pv. *syringae* and *P. s*. pv. *pisi* (Malandrin and Samson 1998; Martin-Sanz *et al.* 2011) for identification purposes, as well as on the development of seed health assay tests for the detection of Psp on pea seed (Lyons and Taylor 1990; Fraaije *et al*., 1993). Yet, the serological assays can not provide information on the bacterium's viability and pathogenicity (Schaad 1982). So, the available methods that are being used by seed health laboratories are based on seed soaking-dilution-plating assays on semi-selective media (Fraaije *et al*., 1993; Grondeau *et al*., 1993; Mohan and Schaad 1987) with addition of boric acid and confirmation of suspect colonies by a pathogenicity test (Grondeau *et al*., 1992; Malandrin and Samson 1998).

A survey on the various methods currently in use by the seed health testing laboratories of the International Seed Health Initiative - Vegetables, ISF showed that they were both suitable for the Psp recovery and detection. As no difference in Psp recovery on the media was observed in initial evaluations, the KBBCA and SNAC semi-selective media were proposed. The low limit od detection of this method is 10 cfu/ml. Several biochemical tests based on the profile of Psp were found useful for the identification of Psp suspects because they aided in reducing not only the number of Psp suspects to be confirmed but also the time and labor needed for the pathogenicity tests. Moreover, two pathogenicity test methods, giving no difference in results but a range of freedom in the way to operate in different laboratory conditions, were proposed. In this comparative test organised by the ISHI-Veg, nine seed health testing laboratories from France, The Netherlands and U.S.A. participated.

Aim and objective of the comparative test

The aim of this comparative test was to evaluate the proposed method for the Psp detection on pea seed. The ultimate objective was to develop an internationally accepted seed health testing method for detecting Psp on untreated pea seed lots.

Materials and Methods

Seed lots and subsamples

Four pea (*Pisum sativum*) untreated, seed lots with variable levels of natural Psp contamination and saprophytes were selected by the GEVES-SNES laboratory in France. Prior to the comparative test the seed lots were characterized with the proposed detection method by testing 5 subsamples of 1000 seeds of each. The final characterization was given based on the number of positive subsamples and the Psp confirmed population (cfu/ml) obtained per semi selective medium for each lot (Table 1). The Psp confirmed population (cfu/ml) per semi-selective medium and lot was calculated by multiplying the average number of Psp recorded suspects with the proportion of Psp positive colonies of the total tested in the pathogenicity test (Asma 2005; Kurowski and Remeeus 2007) as shown in the following formula:

Confirmed Psp colonies (no.) = (Av. number of suspects) × (number of positives ÷ total number tested)

For instance, if an average of 100 suspect colonies were recorded in the SNAC medium from the five subsamples tested of the lot and 5 suspect colonies were tested for pathogenicity, 4 positives in the confirmation test would give 100*4/5 or 80 confirmed colonies.

Thus, the seed lots E 22061 and E 16736 were characterized “healthy” and “low”. Although all five tested subsamples of the E 16737 lot were found positive in both media a lower number of Psp confirmed colonies (cfu/ml) compared to the one of E16741 was detected. Therefore, the E 16737 lot was characterized “medium” and the E16741 “high” (Table 1).

Each participating laboratory received five subsamples from the healthy and high level seed lots and fifteen seed subsamples from the low and medium level seed lots. The forty in total subsamples were of 1000 seeds size and they were prepared by the sampling department of GEVES-SNES with the use of the rotary sample divider apparatus based on the thousand seed weight of each seed lot. The subsamples were coded randomly to ensure a blind comparative test.

Reference culture

Each laboratory used a known *P. syringae* pv. *pisi* reference culture.

Media

Each laboratory prepared plates with KBBCA and SNAC semi-selective media. The final concentration per liter of demineralised water of the KBBCA semi-selective medium is: 20.0g proteose peptone, 10.0g glycerol, 1.5g K_2HPO_4, 0.73g anhydrous $MgSO_4$, 1.0g H_3BO_3, 2.0ml NaOH 1N and 15g agar. The medium was autoclaved at 121°C, 15 psi for 15 min. When the mixture cooled down to 50°C the following sterile antibiotics were added: 40 mg cephalexin monohydrate and 35mg nystatin or 100 mg cycloheximide.

The final concentration per liter of demineralised water of the SNAC semi-selective medium is: 5.0g tryptone, 3.0g peptone, 5.0g NaCl, 50.0g sucrose, 10ml H_3BO_3 and 15g agar. The medium was autoclaved at 121°C, 15 psi for 15 min. After cooling to 50°C the following sterile antibiotics were added: 80mg cephalexin monohydrate and 35mg nystatin.

Bacteria extraction from seed subsamples

All 1000-seed subsamples were suspended in 2.5 ml sterile saline (0.85% NaCl autoclaved at 121°C, 15 psi for 15 min) per gram of seed and were incubated overnight (18h to 24 h) at 4°C under agitation. From each undiluted seed wash two series of a ten-fold dilution was prepared. 100 µl of the undiluted seed extract and the two dilutions (10^{-1} and 10^{-2}) was plated in duplicate on the KBBCA and SNAC media. Positive control plates were prepared by plating 100 µl of serial ten-fold dilutions of the reference culture suspensions (prepared in sterile saline) on each medium. Serial ten-fold dilutions of the sterile saline were plated on each medium which served as sterility control plates. All plates were incubated at 28°C +/- 2°C.

On the SNAC medium the Psp colonies after 4 days of incubation appear circular, white to transparent, mucoid, dome shaped and produce levan. On the KBBCA medium the Psp colonies after 4 days of incubation appear creamy and half-translucent.

On the last day of incubation colonies extracted from seed subsamples were visually compared to colonies of the reference culture grown on the same medium and were considered suspects if appeared to have similar morphology. Sterility check plates were also examined for Psp colonies' presence. The number of suspect Psp colonies on each plate was counted.

Biochemical tests on Psp suspects

If present, at least one Psp suspect colony per subsample grown on KBBCA medium was sub-cultured on sectored plates of SNAC medium. Likewise and if present, at least one Psp suspect colony per subsample grown on SNAC medium was sub-cultured on sectored plates of KBBCA medium. Colonies of Psp reference culture were similarly sub-cultured to reversed medium plates. All sub-cultured plates were incubated at 28°C (± 2°C) for 24-48h.

Sub-cultured colonies on SNAC medium were examined for levan production in comparison to the Psp reference culture colonies on the same medium. Some Psp strains grown on the KBBCA medium produce a blue fluorescent pigment under UV light or not due to a variation in their genus. As both types of strains may be present the sub-cultured colonies on KBBCA medium were examined for blue fluorescence under UV light and were compared to the Psp reference culture colonies.

All sub-cultured colonies on both media were tested in an oxidase test. Laboratories used a ready to use oxidase test or placed a drop of 1% aqueous N,N-dimethyl paraphenylene diamine oxalate solution on a filter paper. Bacteria from each suspect colony were then added in the drop of the solution and an emulsion was made on the filter paper. The emulsion was compared to the emulsion of the Psp reference culture which is oxidase negative (no cytochrome C oxidase: no red staining).

All oxidase negative, typical blue fluorescent or non-fluorescent colonies on KBBCA and all oxidase negative colonies that produced levan on SNAC medium were confirmed in a pathogenicity test.

Pathogenicity tests for confirmation of Psp suspects

Two pathogenicity test methods were proposed to the participating laboratories for the confirmation of the Psp suspect colonies.

In the first method, pea seeds of a susceptible cultivar (*e.g*. Kelvedon Wonder) (Bevan *et al.* 1995; Martin-Sanz *et al.,* 2012) were spread over the surface of a wet blotter paper. The blotter paper with the seeds was rolled, placed in a plastic bag and incubated at room temperature for two days to allow seed germination. A prolonged incubation of two more days was followed in case seeds had not germinated. A suspension of each suspect bacterial culture and the reference culture from KBBCA and SNAC media was prepared in sterile demineralised water to the 10^8 cfu/ml concentration. Three seedlings with cut root tips were placed in each suspension and three pea seedlings were placed in sterile demineralised water which served as negative control. The seedlings were incubated for 15 min.

The incubated seedlings were sown in a potting substrate and incubated in a growth chamber at 20°C with saturating humidity. Five to nine days post sowing, the developed symptoms were compared to symptoms of positive control plants. Typical Psp symptoms are greasy lesions on the stems and leaves of pea seedlings. The positive colonies were recorded and their correspondence to the subsamples was identified.

In the second method, pea seeds of a susceptible cultivar were sown in potting soil and incubated in a growth chamber at 20°-25°C with sufficient light until the stage of two true leaves (aprox. 8-10 days). A suspension of each suspect bacterial culture from KBBCA and SNAC and the reference culture was prepared in sterile demineralised water to a 10^6 cfu/ml maximum concentration. Each bacterial suspension was injected with the needle of a syringe in the stem of at least two pea seedlings. Sterile demineralised water was similarly injected in the stem of two pea seedlings which served as negative control plants.

Inoculated seedlings were incubated in a growth chamber at 20°C +/- 5°C with saturating humidity. Five to nine days post sowing, symptoms were compared to symptoms of positive control plants. Typical Psp symptoms are the extended greasy lesions developed in the inoculation point. The positive colonies were recorded and their correspondence to the subsamples was identified.

Data analysis

All 9 participating laboratories submitted their generated results and these were archived by the test organisers. Yet, results of laboratories 7 and 9 were not included in the analysis due to significant deviations from the protocol (e.g. Laboratory 7 reported dried out medium in most plates and Laboratory 9 reported not good growth of plants. Subsequently, these laboratories didn't confirm any Psp suspects with the pathogenicity test).

For each combination (laboratory x lot x subsample x medium x dilution x plate) the number of Psp suspects in cfu/ml was recorded. The number of positive colonies in the pathogenicity test was used to estimate the number of confirmed Psp cfu/ml with the formula used in the characterization test. However, this data was indicative and therefore not analysed statistically.

The results of the pathogenicity test were also used to record the number of positive subsamples that each laboratory detected per level lot. A subsample was considered positive if at least 1 Psp suspect colony isolated either from KBBCA or SNAC medium - after giving the expected results in the biochemical tests - was confirmed in the pathogenicity test. A subsample was considered negative if there were no Psp suspect colonies recorded in *any* of the two media. A subsample was considered to be missing value if there was no pathogenicity test performed on the Psp suspects (laboratory 2, for instance, didn't perform the pathogenicity test in one subsample from the low level lot).

Following the seed lots' characterization, the "Quality Impurity Estimation" tab of the SeedCalc Version 8 (7) spreadsheet application was used to calculate the contamination rate of Psp in the low, medium and highly contaminated lots. The estimate of the 95% upper limit was considered the contamination rate of each lot. The number of expected positive subsamples in the comparative test at a probability higher than 5% was then calculated for each lot with the spreadsheet application developed by J. L. Laffont (ISTA Statistics Committee Chair).

For the low contaminated lot, the 2 positive subsamples of the 5 tested (Table 1) revealed a 0.17% contamination rate and 10-15 expected positive subsamples of the 15 distributed to laboratories (Table 2). Regarding the medium contaminated lot, all 5 tested subsamples were positive in this lot's characterization. Based on *%GM estimate* spreadsheet of SeedCalc, the calculation of a contamination rate higher than 0.16% is not feasible when 5 subsamples of 1000 seeds are tested. Therefore, the comparative test result of laboratory 6 on the medium lot (Table 2) - reference laboratory that conducted the seed lots' characterization - was used to calculate its contamination rate (0.37%). For this rate, 13-15 expected positive subsamples of the 15 distributed to laboratories were shown by the spreadsheet application developed by J. L. Laffont at a probability higher than 5% (Table 2). Due to the same limitation, the result of the high contaminated lot on SNAC medium - 4 positives of the 5 tested - was used to calculate the contamination rate of this lot. The revealed 0.46% contamination rate showed the expected number of 4-5 positive subsamples of the 5 distributed to laboratories (Table 2).

The number of positive and negative subsamples using data from the (laboratory x lot x subsample) combination of the three contaminated lots was analysed using a Generalized Linear Model on the binary results (positive or negative result) (positive or negative result) with a logit link function. The same type of analysis was conducted for each contaminated lot using data from the (laboratory x subsample) combination. Both analyses were implemented in the *R* version 2.13.1 (6) statistical program.

At the end of the comparative test, a stability test was conducted by the reference laboratory on the three contaminated seed lots to study the effect of time on their Psp contamination level. In this test, five 1000-seed subsamples per lot were tested which were prepared simultaneously to the subsamples of the comparative test with the use of rotary sample diviser machine.

Results

None of the 7 laboratories whose results were analysed recorded any Psp suspect colonies in the healthy subsamples or in the sterility control plates. All laboratories but Laboratory 6 followed the second pathogenicity test method proposed to confirm the tested Psp suspect colonies per subsample and medium. Furthermore, all laboratories confirmed all tested Psp suspect colonies whatever the pathogenicity test was used.

In the low contaminated lot, the average number of Psp confirmed colonies (cfu/ml) that was recorded by the laboratories was lower than the average number recorded in the medium and high contaminated lots (Data not shown). However, a higher number of Psp confirmed colonies (cfu/ml) was recorded by most laboratories in the medium than in the high contaminated lot (Data not shown).

In the stability test, a higher average number of Psp confirmed colonies (cfu/ml) was recorded in the medium than in the high contaminated lot (Table 3) which is contradictory to the result of the characterisation test of these two lots (Table 1). Regarding the low contaminated lot, the average number of Psp confirmed cfu/ml was higher than the one of the medium and high contaminated lots (Table 3). In an overall comparison to results of the characterisation test, a decreased average number of Psp confirmed colonies (cfu/ml) was detected in the medium and high contaminated lots (Table 3). Yet, the reverse was shown for the low contaminated lot in the same comparison (Table 3).

Regarding the number of positive subsamples, all laboratories detected the number that was expected in the low contaminated lot except Laboraroty 2 that detected a lower than the expected number (Table 2). For the medium contaminated lot, all laboratories detected the expected number (Table 2). Finally for the high contaminated lot all laboratories but Laboratory 8 detected the expected number of positive subsamples (Table 2).

The analysis of deviances for the number of positive and negative subsamples detected by all laboratories in the three contaminated lots using the Generalized Linear Model showed a laboratory and a lot effect and no laboratory x lot interaction (Table 4). A laboratory effect was also shown by the results of the analysis with data of the low contaminated lot (Table 5). However, no laboratory effect was shown by the results of the analysis with data of the medium and high contaminated lots (Tables 6, 7).

A decreased number of positive subsamples per semi selective medium was shown in the stability test for the medium and high contaminated lots (Table 3) compared to the results of the characterisation test (Table 1). However, in the low contaminated lot there was a higher number of positive subsamples shown in the stability test (Table 3) compared to the characterisation test results (Table 1).

Discussion

The compared results of the characterisation and stability tests demonstrated an effect of time on the level of the contaminated seed lots. They also demonstrated that the distribution of the Psp population (cfu/ml) in the contaminated seed lots used in this comparative test was heterogeneous. This finding is in agreement with the studies of Grondeau (1992) and Grondeau *et al*. (1993) where the heterogeneous distribution of Psp in pea seed lots was shown. Furthermore, this outcome confirms laboratories' findings with regards to the Psp population on the medium and high contaminated lots. When the comparative test was conducted the homogeneity test had not been developed by the ISTA Statistics Committee. Therefore, there was no means to generate information on the distribution of the pathogen in the three contaminated lots. In addition, the number of subsamples tested in the characterization and stability tests might have been rather low regarding the number of subsamples that was sent to the labs. A higher number of tested subsamples would have allowed for a better estimation of the contamination rate of each lot.

The sampling procedure that was used for the preparation of subsamples, the pippetting of seed extracts during preparation of dilutions and saprophytic presence are known to affect the detection of target bacteria. Thus, it was expected by the test organisers that the recorded Psp population (cfu/ml) would differ between laboratories. However, this is considered usual for naturally contaminated seeds. The similar number of cfu/ml that was detected on KBBCA and SNAC media in each contaminated lot demonstrated that they were both suitable for the Psp recovery and detection.

Nevertheless, the healthy and the contaminated lots were distinguished by the laboratories as the lot effect showed in the Generalized Linear Model analysis with results of all laboratories and lots. In the medium contaminated lot all laboratories not only detected the expected number of positives but they arrived at a similar number. The same finding was shown in the high contaminated lot with the only exception being results of Laboratory 8. These were justified by the low experience of this laboratory with the method. The similar number of detected positives is further supported by the absence of laboratory effect in the Generalized Linear Model analysis for both of these lots. The laboratory effect that was shown in the Generalized Linear Model analysis in the low lot is attributed to the heterogeneous distribution of the pathogen and to the effect of sampling for the preparation of subsamples on this low lot.

Repeatability and reproducibility was not possible to calculate with Langton *et al*. (2002) (qualitative data) or ISO 5725 (quantitative data-cfu/ml) because the pathogen was not homogeneously distributed in the subsamples which subsequently were not considered identical. However, the final protocol of this method recommends a minimum sample size of 5000 seeds with a maximum subsample size of 1000 seeds per seed lot to be tested in order to detect the Psp pathogen. According to this recommendation there were no false positives recorded in the healthy lot and no false negatives in the three contaminated lots. This

recommendation in conjunction with the absence of interactions in the Generalized Linear Model analysis shows that the method is repeatable and reproducible.

Conclusions and recommendations

The method described in this report for the Psp detection on pea seed contains biochemical tests which follow the classic seed soaking- dilution-plating method on semi-selective media for a pathogen's detection. These tests allow for a more precise identification and a reduced number of Psp suspects for confirmation in the pathogenicity test. Subsequently, the time and labor for the performance of the pathogenicity test is also reduced. However, the biochemical tests are considered optional. Regarding the pathogenicity test, both proposed test methods have been shown equivalent based on laboratories' results of the comparative test and therefore are both considered suitable for the confirmation of Psp suspect isolates.

The proposed Psp detection method does not lead to false positive results as the target bacterial pathogen is isolated on the semi-selective media and confirmed in a pathogenicity test. The method has been shown to be reliable for detecting Psp in pea seed lots and it is therefore highly recommended to seed health testing laboratories for routine testing.

In the second pathogenicity test method a higher than the 10^6cfu/ml concentration of the reference culture increased the risk of not having typical symptoms develop on the inoculated pea seedlings. The pathogenicity test in such a case would be considered invalid and should be repeated.

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Bibliography

Asma, M. (2005). Proposal for a new method for detecting *Xanthomonas hortorum* pv.*carotae* on carrot seeds. ISTA Method Validation Reports 2, 1-17.

Bevan J. R., Taylor J. D., Crute I. R., Hunter P. J., Vivian A. (1995) Genetics of specific resistance in pea (*Pisum sativum)* cultivars to seven races of *Pseudomonas syringae* pv. *pisi. Plant Pathology* 44, 98-108.

Elvira-Recuenco, M. and Taylor, J.D. (2001) Resistance to bacterial blight (*Pseudomonas syringae* pv. *pisi*) in Spanish pea (*Pisum sativum*) landraces. *Euphytica*, 118 (3), 305-311.

Fraaije, B.A., Franken, A.A.J.M., van der Zouwen, P.S., Bino, R.J. and Langerak, C.J. (1993) Serological and conductimetric assays for the detection of *Pseudomonas syringae* pathovar *pisi* in pea seeds. Journal of Applied Microbiology, 75, 409–415.

Grondeau, C. (1992) La graisse bactérienne du pois proteagineux due à *Pseudomonas syringae* pv. *pisi*: identificaion, épidémiologie et méthodes de lutte. Thèse de l'Institut National Polytechnique de Toulouse, 146 pp.

Grondeau, C., Saunier, M., Poutier, F., Samson, R. (1992) Evaluation of physiological and serological profiles of *Pseudomonas syringae* pv. *pisi* for pea blight identification. *Plant Pathology*, 41 (4), 95–505.

Grondeau, C., Olivier, V. and Samson, R. (1993) Détection de Pseudomonas syringae pv. pisi dans les semences de pois: Méthodes, limites et controverses. *Phytoma* 455, 45-47.

Grondeau, C., Mabiala, A., Ait-Oumeziane., R. and Samson., R. (1996) Epiphytic life is the main characteristic of the life cycle of *Pseudomonas syringae* pv. *pisi*, pea bacterial blight agent. *European Journal of Plant Pathology* 102, (4) 353-363.

Hollaway, G. J., Bretag T. W. (1997) Survival of *Pseudomonas syringae* pv. *pisi* in soil and on pea trash and their importance as a source of inoculum for a following field pea crop. *Australian Journal of Experimental Agriculture* 37, 369–375.

Hollaway, G. J., Bretag, T. W. and Price, T. V. (2007) The epidemiology and management of bacterial blight (*Pseudomonas syringae* pv. *pisi*) of field pea (*Pisum sativum*) in Australia: a review. *Australian Journal of Agricultural Research* 58, 1086–1099.

ISO 5725 (http://www.seedtest.org/en/statistical-tools-for-seed-testing-_content---1--1143--279.html)

Kurowski, C. and Remeeus, P.M. (2007). Proposal for a new method for detecting *Pseudomonas savastanoi* pv. *phaseolicola* on bean seeds. *ISTA Method Validation reports 4*, 1–22.

Langton, S.D., Chevennement, R., Nagelkerke, N. and Lombard, B. (2002) Analysing collaborative trials for qualitative microbiological methods: accordance and concordance, *International Journal of Food Microbiology* 79: 175-181.

Lawyer, A.S., Chun, W. (2001) Foliar diseases caused by bacteria. In: Kraft JM, Pfleger FL, eds. *Compendium of Pea Diseases.* St. Paul, MN, USA: APS Press, 22-4.Lyons N.F., Taylor J.D. (1990) Serological detection and identification of bacteria from plants by conjugated *Staphylococcus aureus* slide agglutination test. *Plant Pathology* 39, 584-590.

Martin-Sanz, A., PalomboJI, Perez de la Vega M, Caminero C, (2011) Identification of pathovars and races of *Pseudomonas syringae,* the main causal agent of bacterial diseases in pea in North-Central Spain, and the search for resistance. *European journal of Plant Pathology* 129*,* 57-69.

Martin-Sanz, A., Perez de la Vega, M., Murillo, J. and Caminero, C. (2012) Genetic, biochemical and pathogenic diversity of *Pseudomonas syringae* pv*. pisi* strains. *Plant Pathology* 61, 1063-1072

Malandrin and Samson (1998) Isozyme analysis for the identification of *Pseudomonas syringae* pathovar *pisi* strains. *Journal of Applied Microbiology*, 84: 895–902.

Mohan, S.K. and Schaad, N.W. (1987) An improved agar plating assay for detecting *Pseudomonas syringae* pv. *syringae* and *Pseudomonas syringae* pv. *phaseolicola* in contaminated bean seeds. *Phytopathology* 77, 1390-1395.

Roberts, S. J. (1992) Effect of soil moisture on the transmission of pea bacterial blight (*Pseudomonas syringae* pv. *pisi*) from seed to seedling. *Plant Pathology* 41, 136–140.

Roberts, S.J., Ridout, M.S., Peach, L. and Brough, J. (1996) Transmission of pea bacterial blight (*Pseudomonas syringae* pv. *pisi*) from seed to seedling: effects of inoculum dose, inoculation method, temperature and soil moisture. *Journal of Applied Microbiology* 81; (1), 65–72.

Schaad, N. W. (1982) Detection of seedborne bacterial plant pathogens. *Plant Disease*, 66, 885-890.

Schaad, N. W., Jones J. B., Chun W. (2001) *Laboratory Guide for Identification of Plant Pathogenic Bacteria.* St. Paul, MN, USA: The American Phytopathological Society.

Table 1. Results of seed lot characterisation.

Seed lots	Contamination level	KBBCA		SNAC		
		Positive subsamples/total tested	Psp log10(cfu/ml)	Positive subsamples/total tested	Psp log10(cfu/ml)	Average Psp log10(cfu/ml) of both media
E 22061	Healthy	0+/5	0	0+/5	0	0
E 16736	Low	2+/5	1.95	1+/5	1.6	1.77
E 16737	Medium	5+/5	3.67	5+/5	3.61	3.64
E 16741	High	5+/5	3.95	4+/5	3.85	3.89

Table 2. Number of detected and expected positive subsamples of the total tested per lot and laboratory.

	Low lot		Medium lot		High lot	
Laboratories	Positive detected subsamples/total tested	Expected positive subsamples with a probability□	Positive detected subsamples/total tested	Expected positive subsamples with a probability □	Positive detected subsamples/total tested	Expected positive subsamples with a probability □
		5%		5%		5%
1	14+/15		15+/15		5+/5	
2	8+/15		14+/14*		5+/5	
3	15+/15	10-15+/15 for 0.17% cont.rate	15+/15	13-15+/15 for 0.37%cont.rate	5+/5	4-5+/5 for 0.46% cont.rate
4	10+/14*		14+/14*		4+/5	
5	14+/15		15+/15		4+/5	
6	11+/15		13+/15		5+/5	
8	10+/15		15+/15		2+/5	

*Missing value; pathogenicity test was not performed.

Table 3. Results of seed lots' stability test.

Seed lots	Contamination level	KBBCA	SNAC

		Positive subsamples/total tested	Psp log10(cfu/ml)	Positive subsamples/total tested	Psplog10(cfu/ml)	Average Psp log10(cfu/ml) of both media
E 22061	Healthy	N/A	N/A	N/A	N/A	
E 16736	Low	3+/5	2.38	3+/5	2.68	2.53
E 16737	Medium	4+/5	2.26	5+/5	1.97	2.12
E 16741	High	4+/5	2.17	4+/5	1.88	2.02

Table 4. Statistical output of the Generalised Linear Model analysis on the binary results (total number of Psp positive and negative subsamples) of low, medium and high lots, of all laboratories with a logit function.

Response: Final Score

	LR Chisq	dDf	Pr(>Chisq)
Lab	21.867	6	0.00128**
Lot	22.864	2	1.084e-05***
Lab:Lot	16.708	12	0.16093

** Significant difference with 0.001 □ *P* □ 0.01

*** Significant difference with 0 □ *P* □ 0.001

Table 5. Statistical output of the Generalised Linear Model analysis on the binary results (number of Psp positive and negative subsamples) of the low lot of all laboratories.

Response: Final Score

	Df	Deviance residual	Df	Residual deviance	P(>|Chi|)
NULL			103	107.325	
Lab	6	18.658	97	88.668	0.004783**

** Significant difference with 0.001 □ *P* □ 0.01

Table 6. Statistical output of the Generalised Linear Model analysis on the binary results (number of Psp positive and negative subsamples) of the medium lot of all laboratories.

Response: Final Score

	Df	Deviance residual	Df	Residual deviance	P(>|Chi|)
NULL			102	19.727	
Lab	6	7.947	96	11.780	0.242

Table 7. Statistical output of the Generalised Linear Model analysis on the binary results (number of Psp positive and negative subsamples) of the high lot of all laboratories.

Response: Final Score

	Df	Deviance residual	Df	Residual deviance	P(>|Chi|)
NULL			34	28.708	
Lab	6	11.97	28	16.738	0.06264

Peer validation for detection of three fungal pathogens infecting *Linum* seeds by a single method

Organisation:

GEVES-SNES rue Georges Morel – BP 90024 – 49071 Beaucouzé CEDEX, France.

Organiser:

Grimault V., Sérandat I., Brochard C., Kohen R., Brière S.

Participants:

Three laboratories: GEVES-SNES, Volcani Center A.R.O., Canadian Food Inspection Agency.

Pathogens:

Alternaria linicola, Botrytis cinerea and *Colletotrichum lini*

Crop:

Flax (*Linum usitatissimum*)

1. Introduction

The International Seed Testing Association (ISTA) currently uses different methods (7-007, *7*-017, 7-018) to detect the three main pathogens of flax seeds, *Botrytis cinerea, Alternaria linicola* and *Colletotrichum lini*.

ISTA's Seed Health Committee decided to start a research project with the aim of proposing a single standard method for the detection of the three pathogens on flax seeds. A working group, with French and Israeli participants, was created and established a three step approach:

1. Analyze differences between the existing three methods.
2. Perform a pretest to compare conditions and select some for further testing.
3. Perform a peer validation to propose a new validated method.

The first two steps were carried out in a former study in GEVES-SNES laboratory and the results are presented in the attached document.

It was very difficult to obtain seed lots infected with the three pathogens and to be sure of the percentage of infection due to variability in distribution of the pathogens, so it was decided to make an artificial infection. The method used was adapted from that described by Machado *et al*. (2004) and Sousa (2006) for *Fusarium oxysporum* f.sp. *vasinfectum* based on growth of the fungus under water restriction. The principle of this method is based on addition of a high concentration of mannitol to the media to increase the osmotic potential to 1.0 MPa. The pathogen cultivated on these media penetrates the seeds which have a lower osmotic potential. This method allows an artificial infection close to the natural infection to be obtained.

The differences between the three existing ISTA methods were: sample size, temperature, light conditions during incubation, and number of evaluations. In comparison with the routine tests carried out in GEVES-SNES and Volcani Center A.R.O. laboratories the differences were: medium (PDA, addition of 50 ppm or 200 ppm streptomycin) and light conditions during incubation, In other ISTA methods streptomycin can be added at 50 to 130 ppm.

A pretest was carried out in one laboratory at SNES to define the conditions to be tested for the peer validation. Different conditions were tested on four replicates of 100 seeds, which included medium, concentration of streptomycin and temperature and light conditions during incubation.

All conditions tested allowed the detection of the three pathogens, and addition of streptomycin at 50 mg/L in the media was able to restrict the development of bacteria but did not affect the detection of the target pathogens.

The threshold of detection was studied in another pretest with two levels of infection (1% and 2%), on malt-agar with addition of streptomycin at concentration 50 mg/L, with incubation at 20°C and in darkness for 9 days. The results of this pretest (presented in the attached document) showed that this method allowed detection of *Alternaria linicola*, *Botrytis cinerea* and *Colletotrichum lini* at a threshold of 1%. Sensitiviy of the

method varied from 73% for *Botrytis cinerea* to 77% for *Colletotrichum lini*. Sensitiviy was 100% for *Alternaria linicola*. The specificity was 100% for each pathogen.

The aim of this peer validation test, third step, was to compare three existing ISTA methods (7-007, 7-017, 7-018) and three proposed protocols in order to be able to propose a single method for detecting the three pathogens. Canadian Food Inspection Agency laboratory joined this study, allowing a peer validation between three laboratories.

2. Materials and methods

Reference strains:

Botrytis cinerea (MAT/REF No. 01-02-01-01*), Colletotrichum lini* (MAT/REF n° 01-05-04-02) and *Alternaria linicola* (MAT/REF n° 01-05-01-04) were used as reference strains to obtain artificially infected seeds.

Seed lots:

Three infection levels for each pathogen were tested: one healthy, one low and one medium.

Infection levels were estimated by a pretest on the naturally infected seed lot and by estimation of number of artificially infected seeds to be added to a healthy seed lot to obtain the expected infected percentage.

- Healthy: This level is coded A in the description of results.
- Low: naturally infected lot for *Alternaria linicola* (8.5%) and *Colletotrichum lini* (3.5%) and artificially infected for *Botrytis cinerea* (2.0%). This level is coded B in the description results.
- Medium: Artificially infected lot for the 3 pathogens (15% of each pathogen). This level is coded C in the description of results.

For each condition, 12 samples corresponded to four random replicates of 100 seeds of the three initial seed lots. Each lab received a total of 60 samples of 100 seeds corresponding to the 12 samples for the five conditions.

Media

- Malt agar: medium containing 10g malt and 20g agar (If using a commercial preparation ensure that it contains 2% agar and 1% malt extract).
- PDA: i.e. Difco, 39g.
- 1000 ml distilled/de-ionized water.
- Streptomycin: 50 mg/L added after autoclaving.

Equipment

- Autoclave operating at 121°C and 15 p.s.i.
- Incubator operating at 20°C± 2°C and 22°C ± 2°C, equipped with day light and near UV.
- A specific equipment allowing to decrease rapidly the temperature after autoclaving to 55 ± 2 °C (to add antibiotic).

Safety precautions

Ensure you are familiar with hazard data and take appropriate safety precautions, especially during preparation of media, autoclaving and weighing out of ingredients. It is assumed that this procedure is carried out in a microbiological laboratory by persons familiar with the principles of Good Laboratory Practice, Good Microbiological Practice, and aseptic technique. Dispose all waste material in an appropriate way (e.g. autoclaving or disinfection) and in accordance with local health, environmental and safety regulations.

Proposed protocols

Seeds were plated on media as described in the ISTA methods (7-007, 7-017, 7-018).

Based on the results of the pretest, the following conditions were tested. They are listed in the same order in the Results.

1. Malt-agar without streptomycin, incubation at 20°C, in darkness as described in the ISTA 7-007 method.
2. Malt-agar with streptomycin at concentration 50mg/L, incubation at 20°C, in darkness for 9 days followed by 12h near ultraviolet (NUV)/12h dark to induce sporulation, if problem for pathogen identification arised.
3. Malt-agar without streptomycin, incubation at 22°C, under 12h NUV/12h dark as described in the ISTA 7-017 and 7-018 methods.
4. PDA with streptomycin at concentration 50mg/L, incubation at 20°C, in darkness for 9 days followed by 12h NUV/12h dark to induce sporulation if problem for pathogen identification arised.
5. PDA with streptomycin at concentration 50mg/L, incubation at 20°C, 12h day light, 12h dark.

Evaluation of the results

Generally, evaluation was carried out after 9 days of incubation, only one laboratory performed the evaluation after 5 and 7 days. The incubation was not prolonged to 14 days because sporulating fungi was identified after 9 days. Criteria for identification of the three pathogens are described in the ISTA methods already mentioned.

All results were recorded as a number of seeds infected by each pathogen per plate in the report sheets provided. When using the Excel files provided to the participating laboratories, the calculation of percentage of infected seeds was made automatically.

The final result of an analysis corresponded to the last evaluation, so the final values, after 9 days of incubation, were analysed.

3. Results

3.1 Duration of incubation

One laboratory performed evaluations after 5, 7 and 9 days to determine the incubation period to be used for final evaluation and analysis of results. Results of evaluations are shown in figures 1, 2 and 3 for *Alternaria linicola, Botrytis cinerea* and *Colletotrichum lini* respectively and for the three levels of infection, healthy coded A, low coded B and medium coded C.

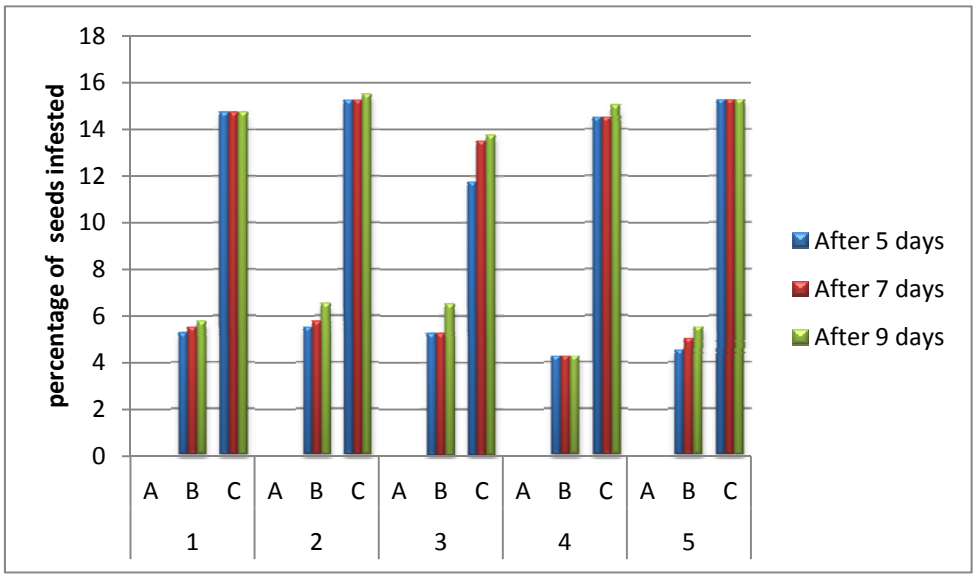

Figure 1. Average percentage of seeds infected with *Alternaria linicola* detected after 5, 7 and 9 days of incubation for the three seed lots (A, B and C).

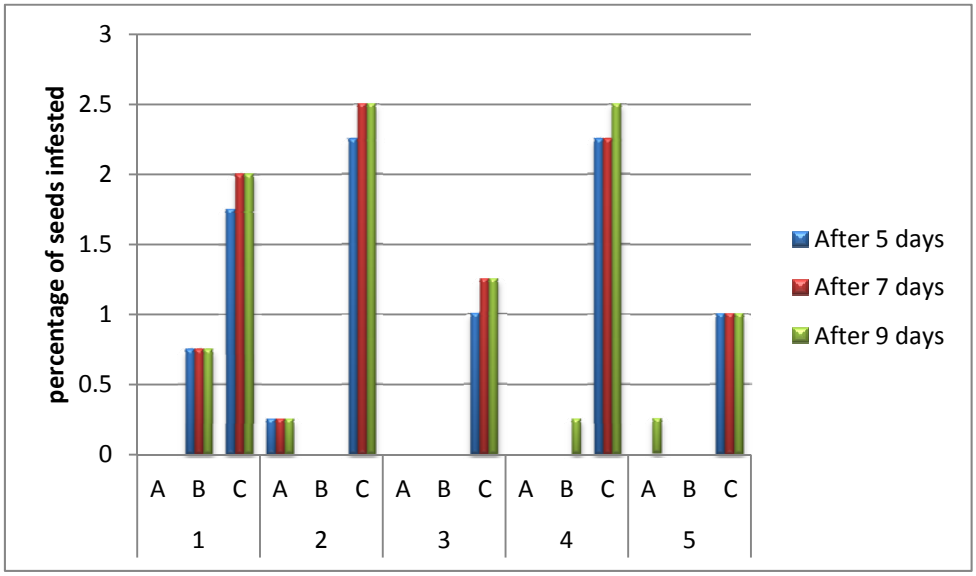

Figure 2. Average percentage of seeds infected with *Botrytis cinerea* detected after 5, 7 and 9 days of incubation for the three seed lots (A, B and C).

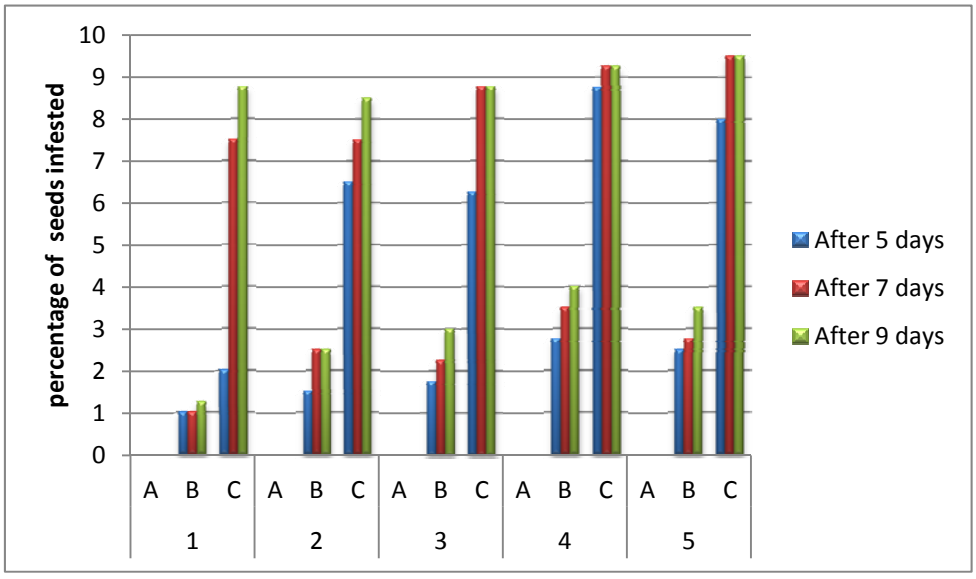

Figure 3. Average percentage of seeds infected with *Colletotrichum lini* detected after 5, 7 and 9 days of incubation for the three seed lots (A, B and C).

Figure 1 shows that *Alternaria linicola* was well detected after 5 days of incubation, with a slight better detection after 9 days. Figure 2 shows that detection of *Botrytis cinerea* was better after 7 and 9 days of incubation than after 5 days. Figure 3 shows that detection of *Colletotrichum lini* was better after 9 days of incubation compared to 5 and 7 days. This result is not surprising as this pathogen takes time to sporulate.

3.2 Comparison of lots and laboratories for all conditions

The results corresponding to the mean of the 4 replicates of 100 seeds, are shown per pathogen, for each condition and each level of infection (Lot A, B and C).

Results for *Alternaria linicola* are shown in figure 4, for *Botrytis cinerea* in figure 5 and for *Colletotrichum lini* in figure 6.

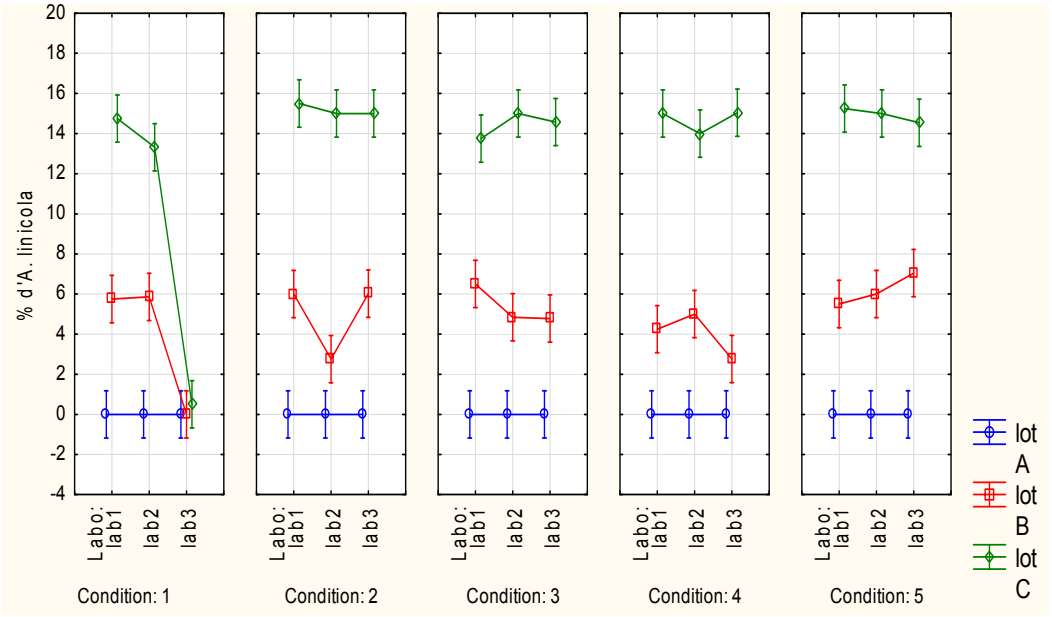

Figure 4. Mean of the four replicates per condition and level of *Alternaria linicola*

Result for the healthy lot (A) was 0% for all laboratories and conditions. There was no false positive.

The mean of infection was equivalent or very close to the expected results, i.e. 8.5% for lot B and 15% for lot C, for all conditions, except for the laboratory 3 for condition 1, which was the only condition tested with incubation only in darkness. This laboratory indicated that suspect colonies were detected, but not recorded, because they did not sporulate.

This result is not surprising as *Alternaria linicola* does not sporulate easily in darkness.

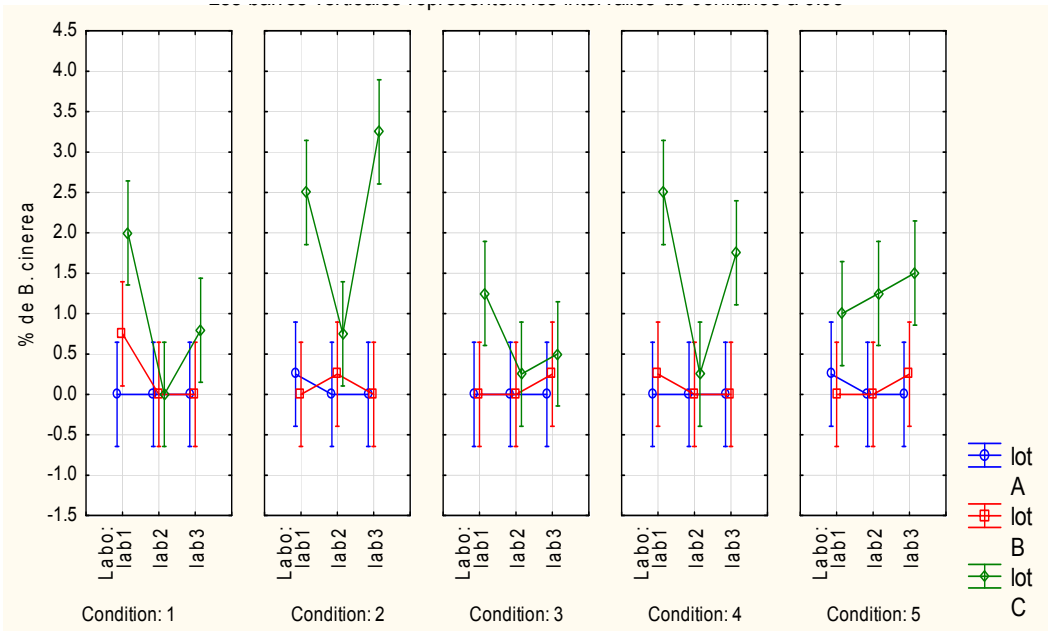

Figure 5. Mean of the four replicates per condition and level of *Botrytis cinerea*

Result for the healthy lot (A) was 0% for all laboratories and conditions, except for laboratory 1 in the conditions 2 and 5, due to the detection of one colony of *Botrytis cinerea* per 100 seeds. For this comparative test the homogeneity of the lots was not tested, so it is possible that the lot was not completely healthy. Homogeneity test is from now on run for each comparative tests.

The expected value was 2% for lot B and 15% for lot C. The mean obtained was lower than the expected value for both lots. For this pathogen, the level of infection can decrease rapidly. As artificially infected seeds were used, instability of artificial infection could explain why no laboratory obtained the expected value. For this comparative test stability of the lots was not tested, so there is no data determine if the rate of *B.cinerea* decreased. Stability test will be included from now for all comparative testing.

In parallel to these comparative tests, seeds infected with the three pathogens were analysed so the evolution of the infection could be followed over time. There was a strong decline in the percentage infection of *Botrytis cinerea*. This could explain the results obtained by the three laboratories for this pathogen. The pretest was undetakenin 2009 and the comparative testing began in 2010, which could explain the decrease in infection.

Nevertheless, the results for lots A and C can give information for the comparison of the different methods and are confirmed by pretest and validation study.

Except for one condition (condition 5), laboratory 2 detected fewer infected seeds than laboratories 1 and 3 irrespective of the method used. For method 1, this laboratory did not detect *B. cinerea* although it is an ISTA method. This laboratory started the analysis one month later than the other two laboratories which could explain the lower level of detection, due to the decrease in infection.

This result is confirmed by the report on validation study of the method presented in the report attached, which shows that the repeatability and sensitivity of the method for *Botrytis cinerea* is lower than that for *Alternaria linicola.*

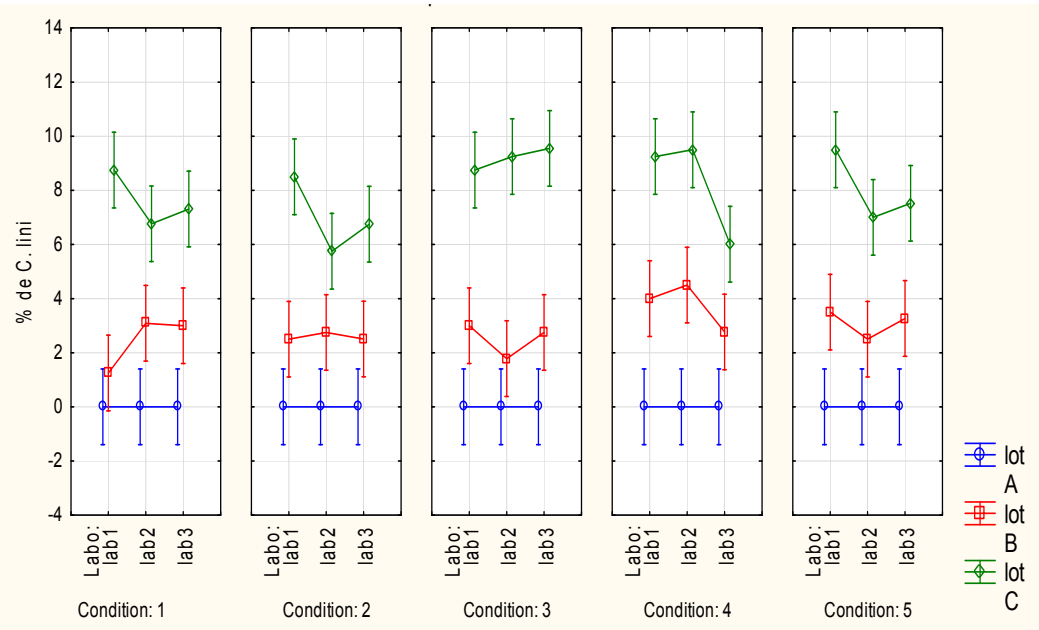

Figure 6. Mean of the four replicates per condition and level for *Colletotrichum lini*

Result for the healthy lot (A) was 0% for all laboratories and conditions. There was no false positive.

The mean of level of infection was equivalent to the expected result for the lot B (3.5%) except for the laboratory 1 for condition 1, which was the only condition tested with incubation only in darkness.

The mean of infection was lower than expected for lot C (15%) for all laboratories. Similarly to *B.cinerea* homogeneity was not tested, so it is possible that the actual level of infection was lower than found during the pretest.

Nevertheless levels of infection of lots B and C were differentiated.

3.3 Comparison of conditions

The comparison of conditions was performed for each pathogen and each level of infection, except for healthy lot. A factorial analysis of variance (ANOVA) was run to determine the P value and a multiple range

test (Fisher) used to separate means where a significant effect was identified in the ANOVA. Raw results are shown in Appendix in tables 1- 12.

3.3.1 Detection of *Alternaria linicola* for lots B and C

Table 1. Summary of statistical analysis for all lots for *Alternaria linicola*

	P Value		
Lot	Lab	Condition	Lab*condition
B	0.65	0.67	0.90
C	0.55	0.65	0.59

There was no difference between the conditions for detection of *Alternaria linicola* at low level of infection (P=0.67) and medium level of infection (P=0.65).

3.3.2 Detection of *Botrytis cinerea* for lots B and C

Table 2. Summary of statistical analysis for all lots for *Botrytis cinerea*

	P Value		
Lot	Lab	Condition	Lab*condition
B	0.01	0.12	0.73
C	0.14	0.85	0.40

There was a difference between laboratories (P=0.01) for detection of *Botrytis cinerea* for a low level of infection whereas there was no difference for a medium level infection (P=0.14). As shown in figure 5, the level of *Botrytis cinerea* detection, for laboratory 2, was lower than for the other laboratories, principally for conditions 1 and 3 and less so for conditions 4 and 5.

The multiple range test (table 3) showed 2 groups and a difference between conditions without streptomycin (1 and 3) and with streptomycin (2, 4 and 5).

Conditions 2, 4 and 5 gave better results for detection of *Botrytis cinerea* at a low level of infection.

Table 3. Multiple range test *Botrytis cinerea/* lot B

	Test LSD ; variable % de B. cinerea (tout_Labo.sta) Groupes Homogènes, alpha = .05000 Erreur : MCE Inter = 1.1859, dl = 105.00 Condition d'exclusion : Lot="B"			
N°Cellu.	Condition	% de B. cinerea Moy.	1	2
3	3	0.333754	****	
1	1	0.465686	****	
5	5	0.667088	****	****
4	4	0.750421	****	****
2	2	1.125000		****

3.3.3 Detection of *Colletotrichum lini* for lots B and C

Table 4: Summary of statistical analysis for all lots for *Colletotrichum lini*

	P Value		
Lot	Lab	Condition	Lab*condition

B	0.72	0.94	1.00
C	0.99	0.71	0.93

There was no difference between the conditions for detection of *Colletotrichum lini* at low and medium levels of infection.

3.4 Repeatability and reproducibility

Repeatability and reproducibility for each pathogen were calculated with ISTA ISO 5725 tool.

3.4.1 Alternaria linicola

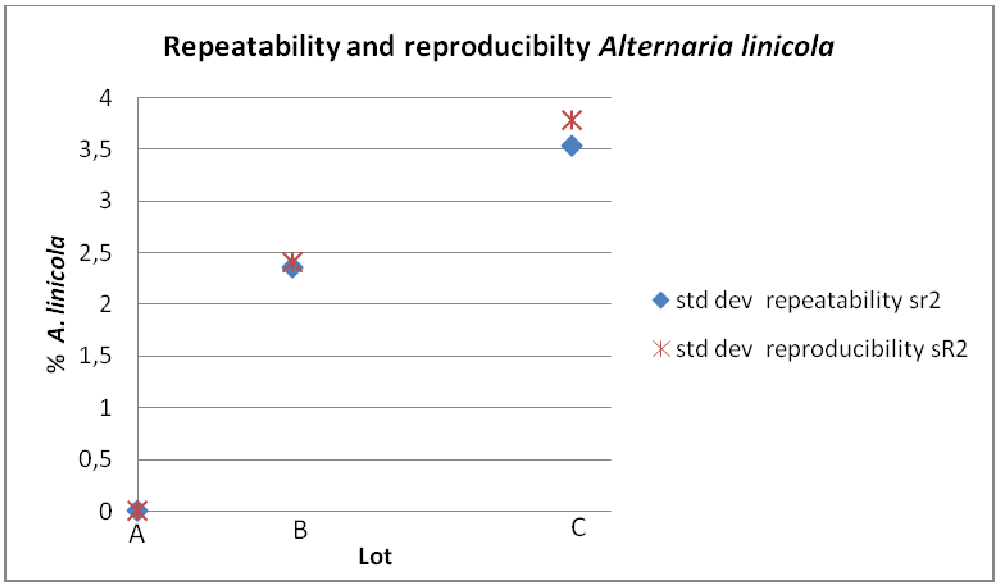

Figure 7. Repeatability and reproducibility for *Alternaria linicola* for the three levels of infection and the five conditions

Repeatability and reproducibility increased with an increase in the level of infection. As expected reproducibility was not lower than repeatability.

Repeatability and reproducibility for the 3 levels of infection were equal which indicates that for each level of infection, there was no difference between results obtained by the three laboratories for detection of *Alternaria linicola* irrespective of the condition.

3.4.2 Botrytis cinerea

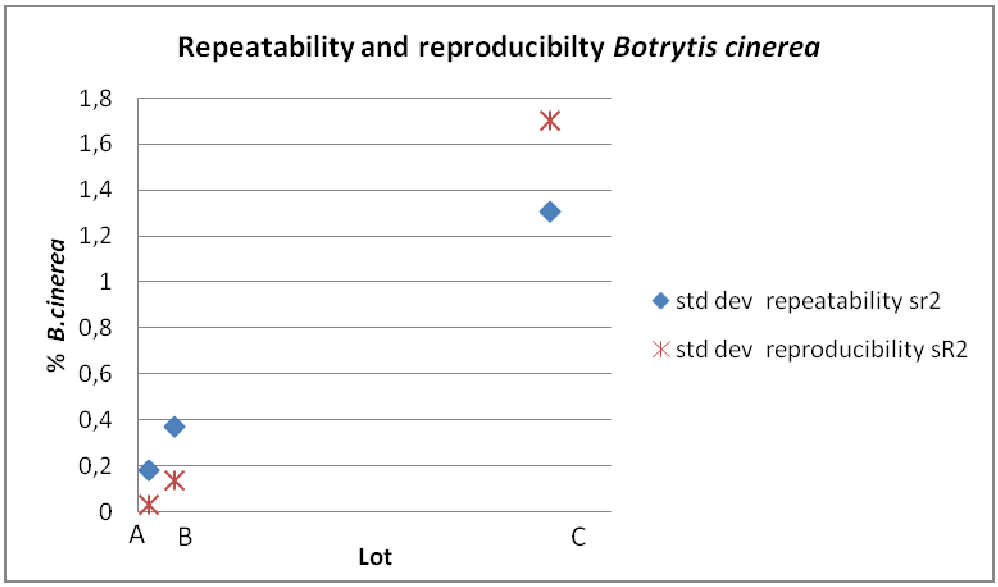

Figure 8. Repeatability and reproducibility of *Botrytis cinerea* for the three levels of infection and the five conditions

Repeatability and reproducibility increased with an increase in the level of infection which was the expected result.

Repeatability and reproducibility show that for the healthy lot and lot with the low level of infection, there was variability between replicates in each laboratory. This could be explained by the decrease in the level of infection with *B.cinerea* infection for the low level lot and the *B. cinerea* being detected the healthy lot.

Repeatability and reproducibility show that for a medium level of infection, there was variability between laboratories. This could also be explained by the decrease in the level of *B.cinerea* infection.

3.4.3 Colletotrichum lini

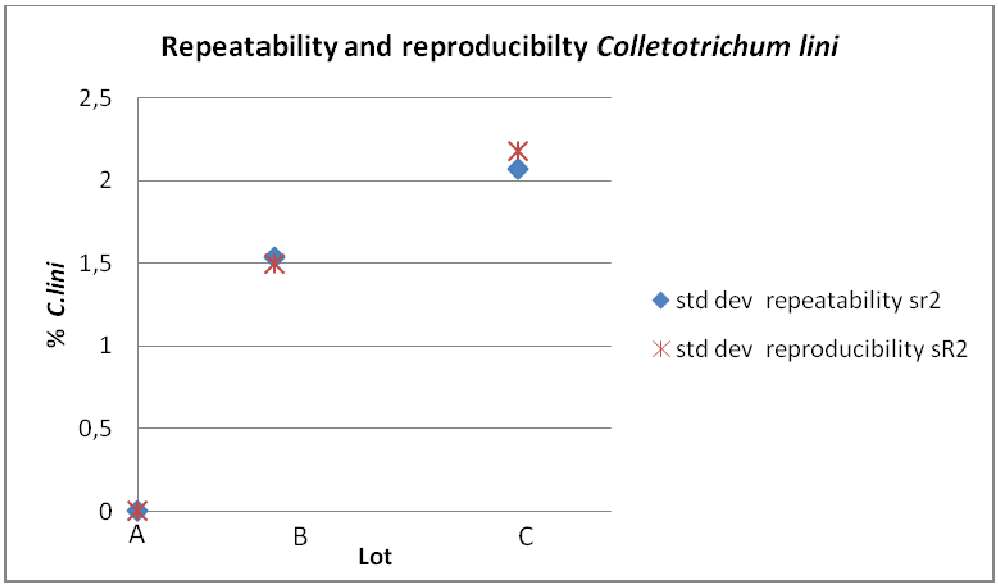

Figure 9. Repeatability and reproducibility of *Colletotrichum lini* for the three levels of infection and the five conditions

Repeatability and reproducibility increased with an increase in the level of infection. As expected reproducibility was not lower than repeatability.

Repeatability and reproducibility for the three levels of infection are equal which indicates that for each level of infection, there was no difference between results obtained by the three laboratories for detection of *Colletotrichum lini* whatever the condition.

5. Conclusion

Results of repeatability and reproducibility show that the detection of *Alternaria linicola* and *Colletotrichum lini* was reproducible between the three laboratories irrespective of the conditions used. Condition 1 gave lower results for the detection of *Alternaria linicola* because this pathogen does not sporulate easily in darkness and the sporulation was taken into consideration by the laboratories as decisive criterion. Therefore, this method has been discarded as it could lead to problems for laboratories not used to identify non-sporulating fungi or colonies.

The detection of *Botrytis cinerea* was less reproducible between the three laboratories. This can be explained by the low infection level, possibly due to the decrease in the level of infection. For this comparative test the stability of the lots in terms of pathogen viability over time was not tested. Pathogen stability should be checked as part of future comparative testing.

Conditions 2, 4 and 5, with addition of streptomycin, gave better results than without streptomycin for *Botrytis cinerea* Conditions 2 and 4 gave a higher percentage of seed infection.

This comparative testing showed that sporulation was not the only diagnostic criterion for pathogen detection.

Based on these results and to have uniformity with other ISTA methods, in terms of temperature of incubation and using streptomycin, we are in favour of describing a single ISTA method for detecting *Alternaria linicola, Botrytis cinerea* and *Colletotrichum lini* by using the following conditions corresponding to conditions 2 and 4 tested during this PT:

- Medium: Malt-agar or PDA with streptomycin at concentration 50mg/L.
- Incubation: at 20°C for 9 days in darkness followed by 12h NUV/12h dark to induce sporulation if problem for pathogen identification arises.

6. Bibliography

International Seed Testing Association (2012). International rules for Seed Testing, annexe to Chapter 7 Seed Health Testing Methods, 7-007 - *Linum usitatissimum; Botrytis cinerea*, p.1-7.

International Seed Testing Association (2012). , International rules for Seed Testing, annexe to Chapter 7 Seed Health Testing Methods, *7*-017 - *Linum usitatissimum; Alternaria linicola*, p.1-6.

International Seed Testing Association (2012)., International rules for Seed Testing, annexe to Chapter 7 Seed Health Testing Methods, *7-018 - Linum usitatissimum; Colletotrichum lini*, p.1-6.

Machado, J.C., Oliveira, J.A., Vieira, M.G.G.C. and Alves, M.C. (2004). Uso de restrição hídrica na inoculação de fungos em sementes de algodoeiro (*Gossypium hirsutum* L.). *Revista Brasilieira de Sementes*, 26, 62-67.

Sousa, M. de. (2006). Metodologias de inoculaçao de *Fusarium oxysporum* f. sp. *vasinfectum* em sementes de algodoeiro (*Gossypium hirsutum* L.). Dissertaçao (Mestrado em agronomia/Fitopatologia) – Universidade Federal de Lavras. 68 p.

7. Appendix

Table 1. ANOVA *Alternaria linicola/* lot B

Effet	Tests Univariés de Significativité de % d'A. linicola (tout_Labo.sta) Paramétrisation sigma-restreint Décomposition efficace de l'hypothèse Condition d'exclusion : Lot="B"				
	SC	Degré de Liberté	MC	F	p
Ord.Orig.	5670.170	1	5670.170	97.68375	0.000000
Labo	50.329	2	25.165	0.43353	0.649374
Condition	135.621	4	33.905	0.58411	0.674825
Labo*Condition	199.833	8	24.979	0.43033	0.900489
Erreur	6094.850	105	58.046		

Table 2 Multiple range test *Alternaria linicola/* lot B

N°Cellu.	Test LSD ; variable % d'A. linicola (tout_Labo.sta) Groupes Homogènes, alpha = .05000 Erreur : MCE Inter = 58.046, dl = 105.00 Condition d'exclusion : Lot="B"		
	Condition	% d'A. linicola Moy.	1
1	1	4.761667	****
3	3	7.220539	****
4	4	7.339646	****
5	5	7.464646	****
2	2	7.583333	****

Table 3. ANOVA *Alternaria linicola/* lot C

Effet	Tests Univariés de Significativité de % d'A. linicola (tout_Labo.sta) Paramétrisation sigma-restreint Décomposition efficace de l'hypothèse Condition d'exclusion : Lot="C"				
	SC	Degré de Liberté	MC	F	p
Ord.Orig.	711.6864	1	711.6864	78.07339	0.000000
Labo	10.9304	2	5.4652	0.59954	0.550931
Condition	22.3768	4	5.5942	0.61370	0.653704
Labo*Condition	59.6428	8	7.4554	0.81787	0.588503
Erreur	957.1388	105	9.1156		

Table 4. Multiple range test *Alternaria linicola* / lot C

N°Cellu.	Condition	% d'A. linicola Moy.	1
	Test LSD ; variable % d'A. linicola (tout_Labo.sta) Groupes Homogènes, alpha = .05000 Erreur : MCE Inter = 9.1156, dl = 105.00 Condition d'exclusion : Lot="C"		
1	1	1.934583	****
4	4	2.002104	****
2	2	2.461700	****
3	3	2.687238	****
5	5	3.090909	****

Table 5. ANOVA *Botrytis cinerea/* lot B

Effet	SC	Degré de Liberté	MC	F	p
	Tests Univariés de Significativité de % de B. cinerea (tout_Labo.sta) Paramétrisation sigma-restreint Décomposition efficace de l'hypothèse Condition d'exclusion : Lot="B"				
Ord.Orig.	53.6094	1	53.60939	45.20517	0.000000
Labo	11.2622	2	5.63109	4.74832	0.010613
Condition	8.8390	4	2.20976	1.86334	0.122358
Labo*Condition	6.2136	8	0.77669	0.65493	0.729755
Erreur	124.5208	105	1.18591		

Table 6. Multiple range test *Botrytis cinerea/* lot B

N°Cellu.	Condition	% de B. cinerea Moy.	1	2
	Test LSD ; variable % de B. cinerea (tout_Labo.sta) Groupes Homogènes, alpha = .05000 Erreur : MCE Inter = 1.1859, dl = 105.00 Condition d'exclusion : Lot="B"			
3	3	0.333754	****	
1	1	0.465686	****	
5	5	0.667088	****	****
4	4	0.750421	****	****
2	2	1.125000		****

Table 7. ANOVA *Botrytis cinerea/* lot C

Effet	SC	Degré de Liberté	MC	F	p
	Tests Univariés de Significativité de % de B. cinerea (tout_Labo.sta) Paramétrisation sigma-restreint Décomposition efficace de l'hypothèse Condition d'exclusion : Lot="C"				
Ord.Orig.	0.675000	1	0.675000	7.767123	0.006316
Labo	0.350000	2	0.175000	2.013699	0.138617
Condition	0.116667	4	0.029167	0.335616	0.853423
Labo*Condition	0.733333	8	0.091667	1.054795	0.400512
Erreur	9.125000	105	0.086905		

Table 8. Multiple range test *Botrytis cinerea/* lot C

N°Cellu.	Test LSD ; variable % de B. cinerea (tout_Labo.sta) Groupes Homogènes, alpha = .05000 Erreur : MCE Inter = .08690, dl = 105.00 Condition d'exclusion : Lot="C"		
	Condition	% de B. cinerea Moy.	1
4	4	0.041667	****
3	3	0.041667	****
5	5	0.083333	****
2	2	0.083333	****
1	1	0.125000	****

Table 9. ANOVA *Colletotrichum lini/* lot B

Effet	Tests Univariés de Significativité de % de C. lini (tout_Labo.sta) Paramétrisation sigma-restreint Décomposition efficace de l'hypothèse Condition d'exclusion : Lot="B"				
	SC	Degré de Liberté	MC	F	p
Ord.Orig.	1925.032	1	1925.032	94.16565	0.000000
Labo	13.493	2	6.746	0.33001	0.719661
Condition	15.711	4	3.928	0.19213	0.942033
Labo*Condition	21.217	8	2.652	0.12973	0.997825
Erreur	2146.519	105	20.443		

Table 10. Multiple range test *Colletotrichum lini/* lot B

N°Cellu.	Test LSD ; variable % de C. lini (tout_Labo.sta) Groupes Homogènes, alpha = .05000 Erreur : MCE Inter = 20.443, dl = 105.00 Condition d'exclusion : Lot="B"		
	Condition	% de C. lini Moy.	1
2	2	3.500000	****
1	1	3.803971	****
5	5	4.003367	****
4	4	4.127104	****
3	3	4.591751	****

Table 11. ANOVA *Colletotrichum lini*/ lot C

Effet	Tests Univariés de Significativité de % de C. lini (tout_Labo.sta) Paramétrisation sigma-restreint Décomposition efficace de l'hypothèse Condition d'exclusion : Lot="C"				
	SC	Degré de Liberté	MC	F	p
Ord.Orig.	248.3470	1	248.3470	71.52927	0.000000
Labo	0.0332	2	0.0166	0.00477	0.995237
Condition	7.3342	4	1.8336	0.52810	0.715308
Labo*Condition	10.2736	8	1.2842	0.36988	0.934318
Erreur	364.5561	105	3.4720		

Table 12. Multiple range test *Colletotrichum lini*/ lot C

N°Cellu.	Test LSD ; variable % de C. lini (tout_Labo.sta) Groupes Homogènes, alpha = .05000 Erreur : MCE Inter = 3.4720, dl = 105.00 Condition d'exclusion : Lot="C"		
	Condition	% de C. lini Moy.	1
1	1	1.222917	****
3	3	1.255417	****
2	2	1.292508	****
5	5	1.544192	****
4	4	1.877946	****

Comparative Test for a better "New improved A-PAGE method for the Verification of *Triticum*"

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Background

A-PAGE method is used for testing the alcohol soluble storage protein profiles of *Triticum* varieties (gliadins).

The gliadins are extracted from seeds and separated by electrophoresis. The pattern of the protein bands produced is related to the genetic constitution and can be considered as a "fingerprint" of a variety. The "fingerprints" can be used to identify unknown samples and mixtures, by single seed analysis.

The **AIM** of the Comparative Test was to evaluate the performance of the laboratory validated A-PAGE method for wheat in comparison with ISTA A-PAGE method.

Materials and methods used for the comparative test

Seed samples

During the Comparative Test, three different wheat varieties were used, two from Germany and one from Argentina. For each variety, 100 individual seeds were tested for their protein profile using

- A: the ISTA method (A-PAGE) and the
- B: Laboratory validated method (A-PAGE)

The seeds used were pre-basic seeds from breeders.

About 300 seeds of each variety were sent, so there were enough seeds to do the test with both methods.

Equipment chemicals and procedure

All equipment, chemicals and procedures needed to perform the test are listed in **Table 1: A: ISTA method**.

Each participating laboratory compared its own method with the ISTA method.

The new proposal is in **Table 2: Method description: equipment, chemicals and procedure to perform the test.**

Evaluation and reporting of results

Data was analysed together with the STACOM. Results are qualitative.

Laboratories measured the Relative Electrophoretic Mobility (REM) for each band.

For each gel and gel track ALL visible bands were considered in the analysis, in order to know whether there were bands missing or whether there were any changes regarding the Relative Electrophoretic Mobility between them, using any of the methods. This was done for the most frequent pattern only. In case any biotype appears (this is a different pattern among the most frequent pattern), it should be recorded but not measured the bands, so not to introduce background noise in the comparison.

Participants reported these results by filling a specified table of results. For each variety, 100 seeds were analyzed, so, in total, there were data for 100 tracks per variety.

The experts were also requested to send JPG files of 2-3 scanned gels per method used to the test organiser. For each gel the experts indicated:

- Sample name
- Gel number
- Method used to obtain the gel
- A numerical identification for each seed
- Bands number

- Identify the off-types if any

The experts from each laboratory were allowed to make any comment about the correspondence between profiles obtained using any of the methods (regarding bands pattern, bands sharpness and bands intensity or other).

The experts were requested to make a general comment about the performance, expenses and time needed to carry out both tests.

Participating Laboratories

Laboratories wishing to participate fulfilled the Registration Form and sent it to ISTA.

Five laboratories participated in this comparative test.

Results obtained after the comparative test

Methods were evaluated in its reproducibility and repeatability.

As this is a qualitative method, there was no reason to evaluate uncertainty.

See document: "1st CT on wheat -Stat analysis 1- 121511.doc" by Jean-Louis Laffont for detailed results.

Proposal of the "New improved A-PAGE method for the Verification of *Triticum*"

The statistical analysis revealed that different methods give similar results compared with the actual ISTA method in the Rules. Methods B, from laboratories with the most similar patterns compared with method A (ISTA method), were selected for this proposal.

The scientists involved in this validation consider that each step (1 to 8 of this new method) is independent from the others. So, the proposed strategy consists on merging some solutions and procedures that were understood go together.

For this new method, laboratories will have options for some of the steps of the procedure. When there are options given, the laboratories will have to select one of them, but not necessarily all the time the same one. For example: a given laboratory will select option 1 for the "extraction solution and extraction procedure", select option 1 also for the "gel preparation", while selecting option 2 for the "electrophoresis" and for "fixing-staining".

Table 1: A: ISTA method

	ISTA method (as described in the Rules)
Equipment	Any suitable vertical electrophoresis system
Chemicals	analytical reagent grade or better acrylamide (AA) bisacrylamide (BIS) urea glacial acetic acid glycine ferrous sulphate ascorbic acid hydrogen peroxid or APS and TEMED pyronin G or methyl green trichloroacetic acid (TCA) ethanol 2-chlorethanol PAGE Blue G-90 or G- 83 or any equivalent to the Commassie Blue
Sample preparation	Single seeds are crushed with pliers and transferred to 1.5ml polyproylene centrifuge tubes.
Extraction solution	2-chlorethanol (25 %) - keep cold.
Extraction procedure	Add the extraction buffer and thoroughly mix the sample. Stand over night at room temperature. Before loading the gel the tubes are centrifuged at 18.000 x g. Extracts can be stored at 4°C for 3-4 days.
Gel cassettes	Silcon treatment of the glass plates is recommended. The gel cassettes can incorporate a plastic backing sheet.
Gel preparation	Stock gel buffer solution: keep cold (4 - 6 °C). Use hydrogen peroxide 0.6 %, freshly prepared and keep cold at 4 -6 °C. Polymerisation should be complete in 5 - 10 minutes.
Amount of the samples loaded	10 - 20 microliter.
Electrophoresis	Constant voltage at 500 V. Water should be circulated through the buffer tank to maintain the temperature at 15 - 20 °C
Fixing and staining	1 step (fixing and staining). This takes about 1-2 days.
Destaining	Not usually needed.
Storage of the gels	Gels can be stored in polythene bags at 4 °C for many months without deterioration

Table 2: Method description: equipment, chemicals and procedure to perform the New improved A-PAGE method

GENERAL INFORMATION	
Equipment	- Any suitable vertical electrophoresis system. - Cooling system. - Power Supply. - Hood. - Mixer - Centrifuge - Shaker - Transiluminator - Oven or a Drying Equipment (gel-dryer or just glass plates and cellophane sheets)

Chemicals	Analytical reagent grade or better (acrylamide and bisacrylamide specially purified for electrophoresis)

STEPS	**OPTION 1**	**OPTION 2**
1. Sample preparation	Seeds can be ground, crushed or halved with pliers or a razor blade and transferred to microcentrifuge tubes (1.5 ml) or microtiterplates (200 µl).	
2. Extraction solutions and extraction procedure		
Extraction solution		
2-chlorethanol	-	25-30%
PyroninG or methyl Green	-	0.05%
Ethanol	70% prepared just before the use	-
Water	-	To final volume
Acetone	Concentrated	-
Sample buffer		
Glycerol	30% w/v	-
Urea	6M	-
Acetic acid	25mM	-
Pyronina G	0.05%	-
Water	To the final volume	
Extraction procedure	Keep the solutions at room temperature	Keep the solution cold (4 ºC)
	70% ethanol (200µl/seed or 50-60 mg flour) mix by vortex. Let the sample in the dark at room temperature for1hour.	Add 150-200 µl extraction buffer and mix the samples with e.g. a vortex when using microcentrifuge tubes. In the case of using microtiterplates you must not mix the samples.
	Centrifuge, recover the clarified supernatant in 1.5 ml tube then add 1 ml acetone stored at room temperature. Proteins will	Incubate the samples over night at room temperature.
		If it is necessary, before loading the gel centrifuge the samples

STEPS	OPTION 1	OPTION 2
	precipitate in few minutes (keep at 4°C if not used). Centrifuge, discard the acetone, dry the pellet under the hood for 5 minutes. Add 150µl of sample buffer. The extraction is finished in about two hours. Extracts can be stored at 4°C for some weeks.	at 13000 rpm 15 minutes. Extracts can be stored at 4°C for some days.
3. Gel Preparation and buffer tank solutions **Gel Mix**		
Acrylamide (AA)	12% (From 40% solution)	10% Final concentration (from solution or powder) Note: The powder forms of acrylamide and bisacrylamide are much more readily inhaled, as they are very light and highly electrostatic, so the powder floats in the air as soon as the bottle is opened. Handle in a fume hood.
Bisacrylamide (BIS)	0.4% (From 2% solution)	0.4% Final concentration (from solution or powder). Note: The powder forms of acrylamide and bisacrylamide are much more readily inhaled, as they are very light and highly electrostatic, so the powder floats in the air as soon as the bottle is opened. Handle in a fume hood.
Acetic acid		- 6% 0.005%

STEPS	OPTION 1	OPTION 2
Urea	0.75%	0.005 - 0.1%
Ferrous sulphate	12%	
Ascorbic acid	0.0014%	Add the following buffer: 0.1% Glycine (w/v), 2% Glacial acetic acid (v/v) and water to final volume.
	0.1%	Mix until complete dissolution.
	Add water to final volume (for example 80 ml for 2 gels of 16x18cm x 1.5mm thik)	0.002-0.003% (v/v) hydrogen peroxide 100 vol, 30%, for the final gel concentration.
	Mix until complete dissolution.	
Polymerisation Starter	Hydrogen peroxide 100 vol, 0.001% (v/v), final gel concentration.	Gel preparation should be done quickly because polymerization takes place very fast. Furthermore, place the cassettes at 4 ºC for a while before filling with the gel mix, helps to delay the polymerization.
		Only one buffer: 0.4% Glacial acetic acid (v/v) + 0.04% Glycine (w/v) + water to final voumen
Buffer tank solution	**Upper tank buffer :** Water 700ml +1ml Acetic Acid (0.143% v/v)	
	Lower tank buffer : Water 4000ml + 10ml Acetic Acid (0.25% v/v)	
4. Loading samples	5-20 µl depending on the equipment used loading could be performed using a syringe, a multichannel syringe, a pipette or a multichannel pipette.	
5. Fixing- Staining solutions and procedure	One step: Fixing and Staining:	Two possibilities: One step: Fixing and Staining. Stock commassie:

STEPS	**OPTION 1**	**OPTION 2**
	Stock Commassie: Commassie R250 1g/100 ml ethanol. Store this solution at 4ºC in a dark bottle. Fixing and Staining 2.5% Stock Commassie Blue R250 (v/v) + 6.25% TCA (w/v), water to 400ml. This solution is enough for 2 gels 16x18cm x 1.5mm thik. Shake over night with orbital shaker. The solution use: one time only.	0.25% (w/v) Commassie Blue G250 + 0.75% (w/v) Commassie Blue R250 + water to complete volume Staining solution: 8.3% (w/v) TCA + 5.8 (v/v) Acetic Acid + 12.5 % (v/v) Ethanol + 2% (v/v) stock commassie. Staining is complete after 1 day, but at the earliest after 4 hours. Solutions can be used six times. Two steps: 1) Fixing: 10% TCA. Store at room temperature under hood. 2) Staining: Stock commassie: 0.25% (w/v) commassie blue G250 + 0.25% (w/v) commassie blue R250, complete volume with ethanol 100%. Store this solution at 4ºC in a dark bottle. Staining solution: 20% stock solution (v/v) + 8% Acetic Acid (v/v). Add water to complete volume. Store under hood at room temperature in a dark bottle. First: fixing gels in TCA 10% for one hour. Gels can be saved in this solution for few days. Second: stain the gels for about three hours or over-night using the staining solution. Fixing and staining solutions can be used six times.
6. Electrophoresis	Constant Voltage: 500 V for the chamber. Water should be circulated through the buffer tank to maintain the buffer temperature at 18°C.	Constant current: 40 mA for each gel. Water should be circulated through the buffer tank to maintain the buffer temperature at 10-20°C.

STEPS	OPTION 1	OPTION 2
	Running time: 2 times the time required for the dye to leave the gel	Running time: 2 times the time required for the dye to leave the gel
7. Destaining	Destaining with tap water: rinse the gels 1-2 times (30 min each). For a slow destaining use a 10% TCA solution.	
8. Storage of the gels	Gels can be kept in 10% TCA solution and then dried between two cellophane sheets or photographed. After drying they can be stored for years.	Previous to drying, gels could be soaked in a glycerol solution (3%). Then, gels can be dried between two cellophane or photographed. After drying they can be stored for years.

Validation of a new method: Use of SDS-PAGE technique for the Verification of Triticum and XTriticosecale

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Introduction

Species and variety identification by morphological traits of seeds may be difficult or even impossible. In seed testing the identification of *Triticum* spp. and related species such as x*Triticosecale* by seed traits could be difficult because of the high level of seed morphological similarity among these species Therefore, there is a need for more reliable tools for species and varietal verification of seed lots. SDS-PAGE (Laemmli discontinuous buffer system) is a well known electrophoretic technique widely used in seed protein separation in order to perform variety characterization. The method is used for testing glutenin profiles of varieties belonging to Triticum spp. and related species such as xTriticosecale. The HMW-glutenins are extracted from seeds and separated by SDS-PAGE, the protein profile is related to a well known genetic background. For wheat varieties the interpretation of the results is based on a alleles catalogue compiled by Payne and Lawrence, catalogues for alleles recognition in XTriticosecale are also available.

The electrophoretic pattern can be considered as a “fingerprint” of the variety and can be used to identify unknown samples and mixtures, by single seed analysis.

The technique is described in the “TG/3/11 UPOV Guideline for the conduct of tests for distinctness, uniformity and stability in wheat”; the aim of this Comparative Test is to evaluate the performance of the SDS-PAGE technique in seed testing to confirm varietal identity of seed lots and species verification of *Triticum* spp. and related species such as *XTriticosecale*

Background

Triticum sp.

Glutenins are large, heterogeneous molecules built up from different subunits connected by disulfide bonds falling into two different groups, the low molecular weight subunits (LMW) and high molecular weight subunits (HMW). The high molecular subunits in hexaploid (AABBDD) and tetraploid (AABB) wheat species are coded by genes at three and two loci respectively designated *Glu-A1*, *Glu-B1* and *Glu-D1* as well as *Glu-A1*, *Glu-B1* which occur on the long arms of the chromosomes A1, B1 and D1.

Fig 1 shows the catalogue of the most frequent *Glu-1* alleles compiled by Payne and Lawrence (1983) and used for variety description.

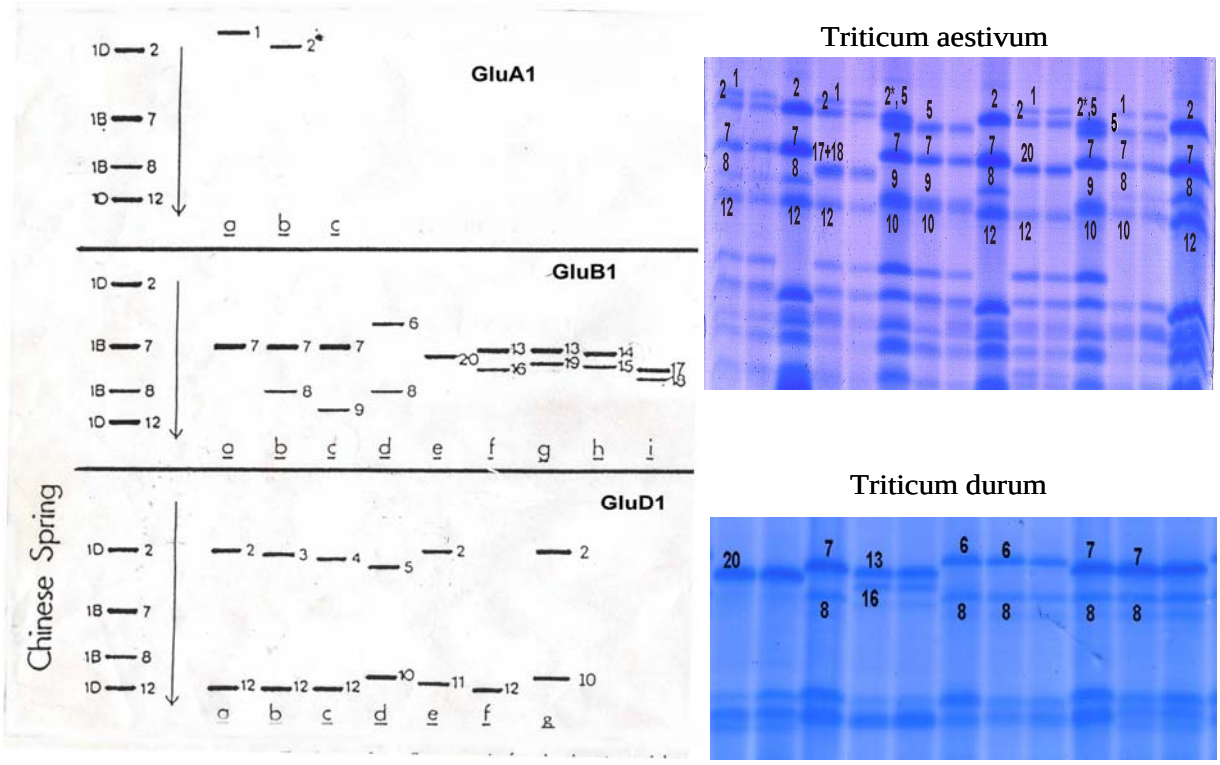


Fig.1: catalogue of the most frequent *Glu-1* alleles in hexapoloid (AABBDD genome) and tetraploid (AABB genome) wheat species and description of some glutenin profiles.

Species identification in seed testing-1

Moreover glutenin profiles are very useful in *Triticum* species verification: the absence of the D genome in tetraploid wheat (AABB) can be easily detected by SDS-PAGE (fig.2)

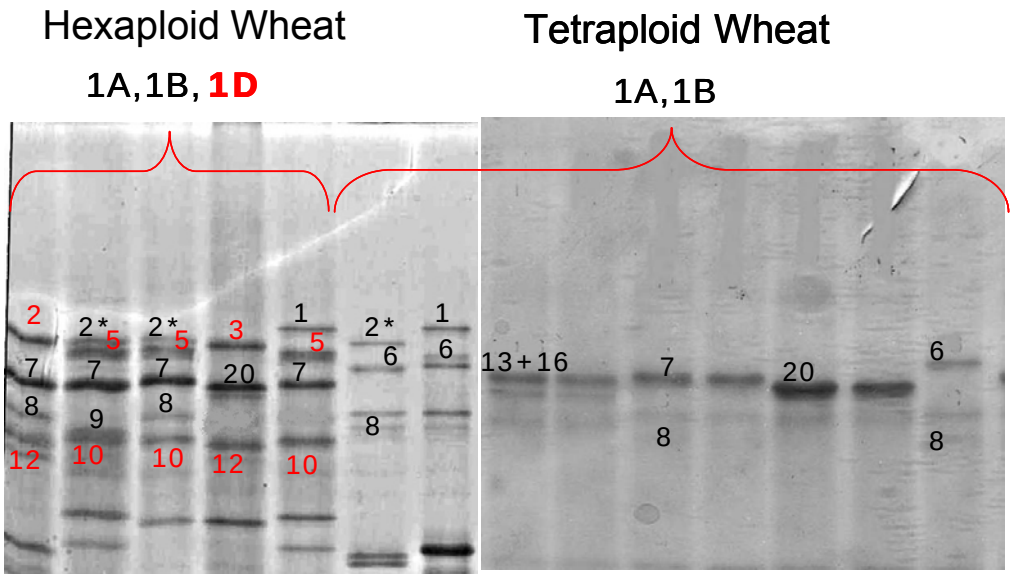


Fig.2: HMW glutenin profiles of different hexaploid and tetraploid wheat varieties.

xTriticosecale

The hexaploid triticale, (AABBRR) x*Triticosecale*, is a synthetic cereal crop, and is a combination of the A and B genome from durum wheat and the R genome from rye (*Secale cereale*). The two species wheat and rye, have striking similarities, particularly with group 1 chromosomes, where the mayor loci encoding storage proteins are located. *Glu-R1* encodes the high molecular weight secalins, another important locus *Gli-R2* is located on chromosome 2RS and is responsible for the genetic control of the 75Kγ-secalin fraction. SDS-PAGE technique can be used for varietal identification based on polymorphism of seed storage proteins encoded by these alleles. (fig.3)

HMW glutenins			75Kγ-secalin
Glu-A1	*Glu-B1*	*Glu-R1*	*Gli-R2*
1	6+8	0.8^{R} +6^{R}	d1
2*	7+8	$5,8^{R}$	d2
N	13+16	1^{R}+4^{R}	t2
	17+18	2^{R} +$6,5^{R}$	t1
	6,8+20y	6^{R} +13^{R}	
	23+18	2^{R} +9^{R}	
		6.5^{R}	

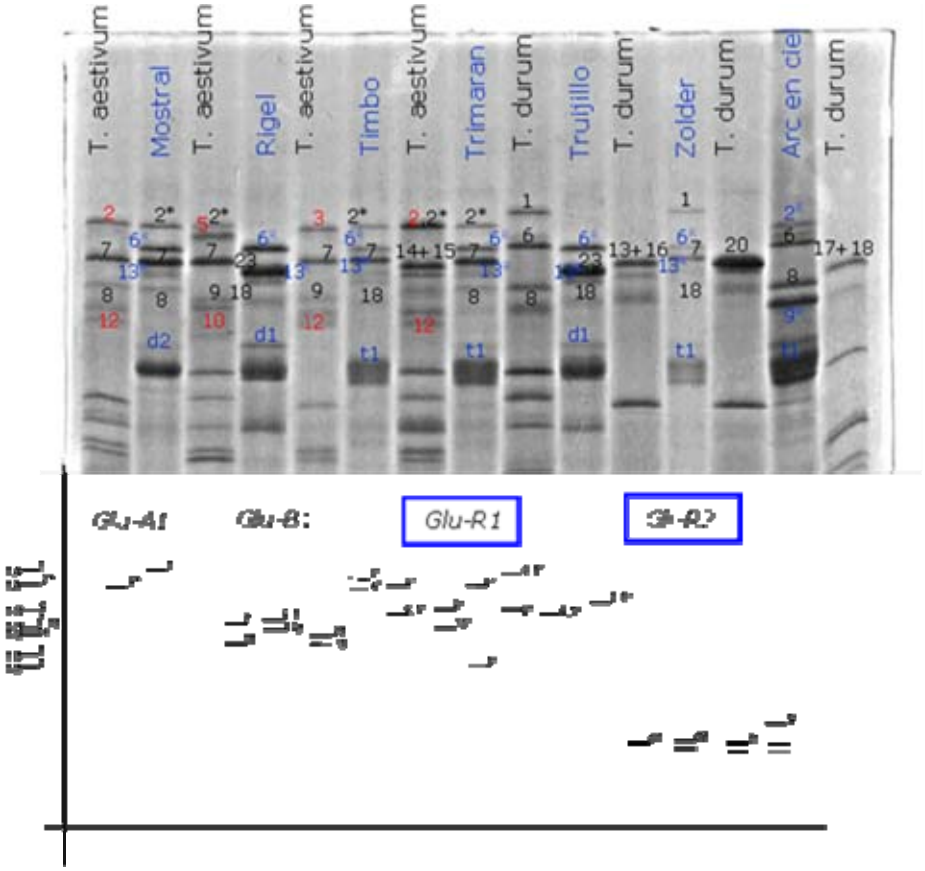


Fig.3: catalogue of the most frequent *Glu-1* and *Gli-R2* alleles in triticale and general description of their electrophoretic mobility; some triticale varieties are shown in blue, with their electrophoretic patterns.

Species identification in seed testing-2

In seed testing the identification of *Triticum* spp. and related species such as x*Triticosecale* by seed traits could be difficult because of the high level of seed morphological similarity among these species (fig. 4).

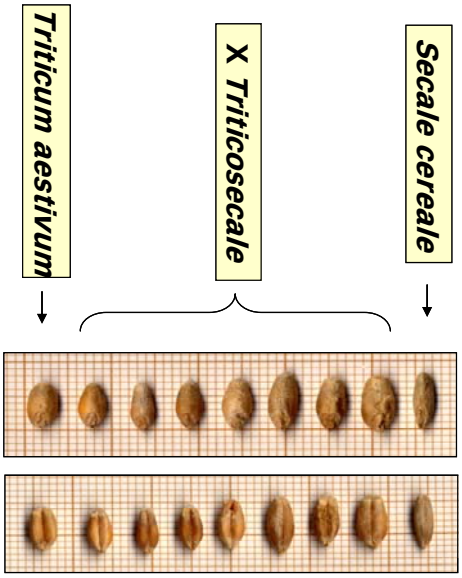


Fig.4: Seeds of some x*Triticosecale* varieties compared to seeds of *Triticum aestivum* and *Secale cereale*.

Electrophoresis by SDS-PAGE of seed proteins represents a reliable tool to point out the differences among the species due to their genome composition.

The alleles (3+12; 2+12; 5+10) encoded by D genome of *Triticum aestivum* are not present in x*Triticosecale* varietes and most of the x*Triticosecale* varieties are characterized by the presence of 75 γ Secalin fraction encoded by R genome (fig.5).

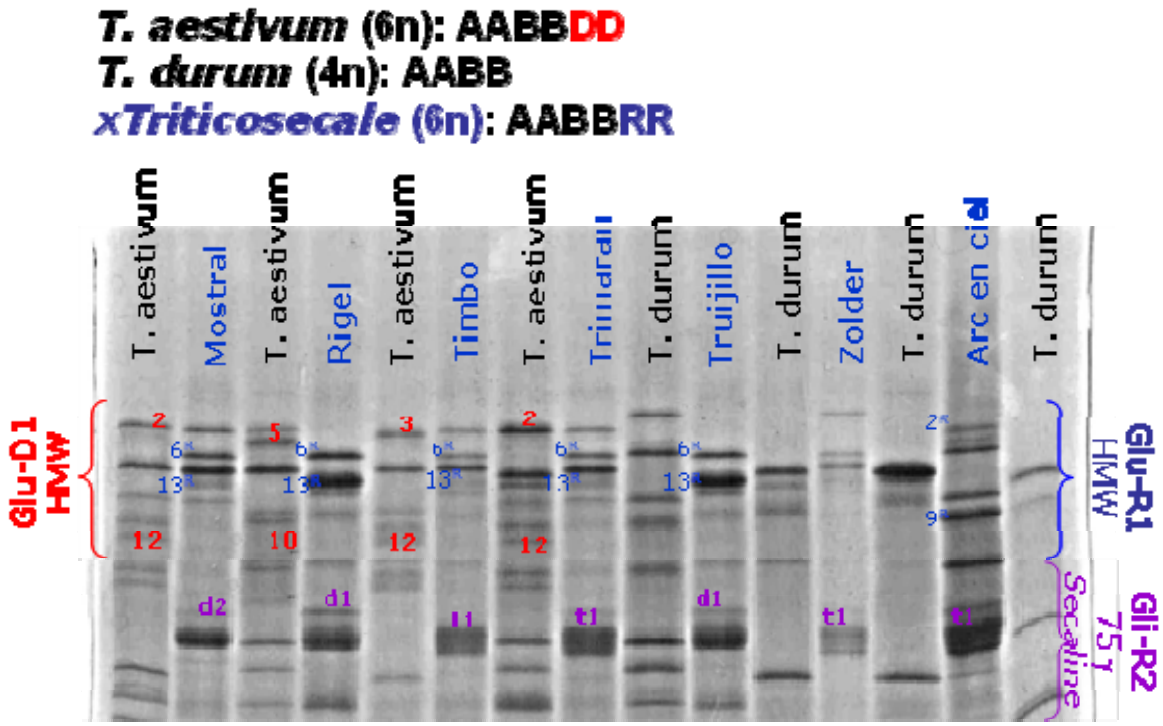


Fig.5: Different allele compositions of seed storage proteins of *Triticum aestivum*, *T. durum* and x*Triticosecale* related to their genome composition.

Materials and methods

Participating laboratories

Five international laboratories, 2 ISTA and 3 non ISTA from five different countries were involved. The participating labs are invited to perform the CT by using the UPOV SDS-PAGE stated method for wheat: The supplementary use of in house validated method (if different) is also allowed (tab. 1)

Table 1: Laboratory involved and methods used.

Laboratory	UPOV method	Lab validated method
Lab 1	X	X
Lab 4	X	X
Lab 5	X	X
Lab 6	X	
Lab 7	X	

Seed samples

Three different wheat varieties named A, B, C and three triticale varieties D, E, F were used. For each species, three reference varieties with known alleles composition Std.1, Std.2, Std.3 for wheat and Std.4, Std.5, Std.6 for triticale, were included. The analysed seeds were basic seeds from the breeders.

Due to the genetic interpretation of the protein profiles, 50 seeds were considered enough for testing variety or species identity. More precise estimates of varietal purity may require a larger sample, at least 100 seeds.

SDS methods

The seeds were tested for their protein profile using

- the UPOV stated SDS-PAGE method (all the laboratories)
- the laboratory validated SDS-PAGE method (three laboratories)

Method description

The suggested protocol refers to the Laemmli SDS discontinuous buffer system and to the extraction method as described in the UPOV guidelines:

Stacking gel: 3% acrylamide/bis-acrylamide, 0.125 M Tris-HCl pH 6.8

Resolving gel: 10% acrylamide/ bis-acrylamide, 0.375 M Tris-HCl pH 8.8

Reservoir buffer: 0.025 M Tris , 0.187 M glycine pH 8.3

Glutenins Extraction solution: Urea 4.5 M, 3% 2-mercaptoethanol, 10 % SDS.

SDS-PAGE ISTA/UPOV

Equipment*:* Any suitable vertical electrophoresis system

Chemicals*:* (All analytical reagent grade or better):

acrylamide 40% solution(AA)

bisacrylamide 2% solution(BIS)

urea

glycine

APS and TEMED

2-mercaptoethanol

Sodium dodecyl phosphate (10% stock solution)

TRIS

pyronin G/Bromophenol Blue

Coomassie Blue R-250

Coomassie Blue G-250

Water quality: purified water

Sample preparation:

Single seeds crushed with pliers or alternatively 50-70 mg of flour are transferred to 1.5mL polypropylene centrifuge tubes.

Extraction buffer:

Urea 4.5 M, 3%

2-mercaptoethanol, 10 % SDS.

Extraction procedure:

Add 500 µL of the extraction buffer and thoroughly mix the sample

Stand overnight at room temperature.

Heat the samples in a boiling water bath for ten minutes and then allow to cool. Before loading the gel the tubes are centrifuged at 18.000xg.

Gel preparation 2 gel 16x18 cm, 1.5 mm thickness:

Stacking gel: Acrylamide 3%, 0.125 M Tris-HCl pH 6.8

acrylamide 40% solution(AA): 1.5 mL

bisacrylamide 2% solution(BIS) : 0.43 mL

Tris-HCl 1 M pH 6.8 2.5 mL

SDS 10% 0.16 mL

Water: 14.87 mL

For polymerization:

APS 1% 0.75 mL

TEMED 20 µL

Add the reagents to a 19.46 mL of stacking gel solution.

Resolving gel: Acrylamide 10%, 0.375 M Tris-HCl pH 8.8

acrylamide 40% solution(AA):20 mL

bisacrylamide 2% solution(BIS)5.2 mL

Tris-HCl 1 M pH 8.8 30 mL

SDS 10% 0.8 mL

Water: 20.8 mL

For polymerization:

APS 1% 2 mL

TEMED 40 µL

Add the reagents to a 76.80 mL of resolving gel solution.

Tank buffer: Tris 0.0250 M, Glycine 0.187 M SDS 1 % pH 8.3

Stock solution:

Glycine 141.1 g

TRIS 30.0 g

SDS 10.0 g

Made up to 1000 mL with water

Dilute the stock solution 1:10 before use.

Electrophoresis

Samples loading: 10-15 µL

Running condition 2 gel 16x18 cm, 1.5 mm thickness

Stacking gel : Constant voltage

at 100 V. (about 40 mA)

Running gel: 80 mA (max 400 mA)

Water should be circulated through the buffer tank to maintain the temperature at 15 - 20 °C.

Stop the run 40 minutes after the tracking dye has reached the bottom of the gel.

Fixing and staining

Fixing: T

TCA 15% about 30 minutes

Staining:

Solution A: Coomassie Blue G-250 0.25 g

Coomassie Blue R-250 0.75 g

made up to 100 mL with water.

Solution B: TCA 27.5 g

Acetic acid 32.5 mL

Ethanol 90 mL

Solution A: 12.5 mL

Water to 400 mL

Stain overnight at room temp.

Destaining: water

Storage of the gels

Gels can be stored in polythene bags at 4-6 °C for many months without deterioration

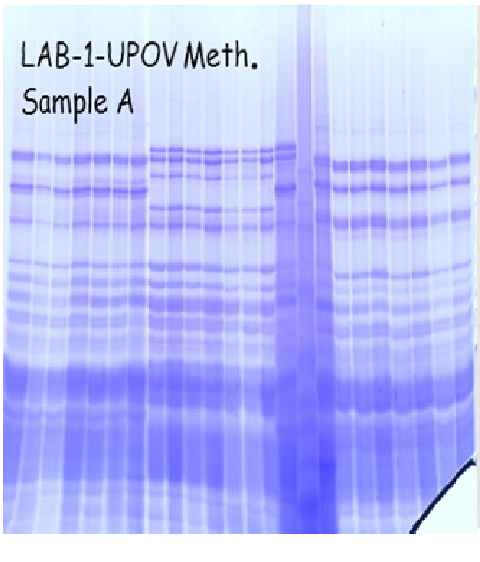
Results

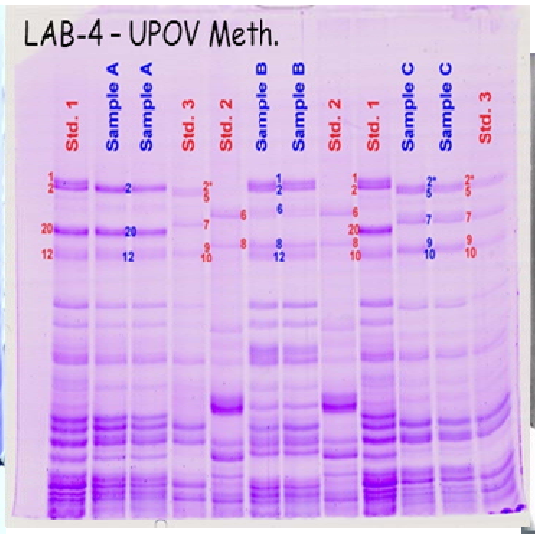
For the interpretation of the banding pattern the international agreed system of band nomenclature stated by Payne and Lawrence was used. To check the running condition and as an aid for the alleles recognition reference varieties with known profiles were included in the gels.

Table 2: Glutenin profiles recognized by the participating laboratories.

Laboratory	Wheat glutenin profiles	xTriticosecale glutenin profiles
Lab 1	Sample A: N, 20, 2+12 Sample B: 1, 6+8, 2+12 Sample C: 2*, 7+9, 5+10	Sample D: 6^R, 23, d1 Sample E: 1, 6^R, 7+18, d1 Sample F: 2*, 6^R, 7+8, t1
Lab 4	Sample A: N, 20, 2+12 Sample B: 1, 6+8, 2+12 Sample C: 2*, 7+9, 5+10	Sample D: 6R, 23, d1 Sample E: 1, 6R, 7+18, d1 Sample F: 2*, 6R, 7+8, t1
Lab 5	Sample A: N, 20, 2+12 Sample B: 1, 6+8, 2+12 Sample C: 2*, 7+9, 5+10	Sample D: 6R, 23, d1 Sample E: 1, 6R, 7+18, d1 Sample F: 2*, 6R, 7+8, t1
Lab 6	Sample A: 1, 2*, 20, 2+12 Sample B: 2*, 6+8, 2+12 Sample C: 2*, 7+9, 5+10	Sample D: N, 6R, 23, d1 Sample E: 1, 2*, 6R, 7+18, d1 Sample F: 2*, 6R, 7+18, t1
Lab 7	Sample A: N, 20, 2+12 Sample B: 1, 6+8, 2+12 Sample C: 2*,7+9, 5+10	Sample D: 6R, 23, d1 Sample E: 1, 6R, 7+18, d1 Sample F: 2*, 6R, 7+8, t1

Different labs give the same banding pattern (tab 2) and comparable protein separation both for wheat and triticale (fig. 6a, fig. 6b respectively). Only lab 6 didn't give the right interpretation of the profile for sample A and B for wheat, and E and F for XTriticosecale, probably due to the low quality of the gel separation. Lab validated methods with slight differences from UPOV technique give the same banding pattern as UPOV technique (data not shown).





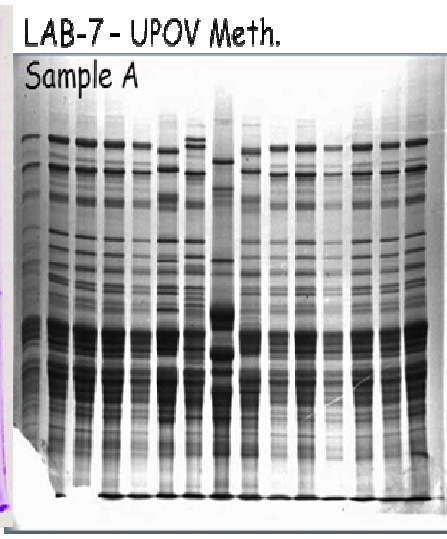


Fig. 6a: An example of the electrophoretic separation of wheat glutenins performed by the different laboratories.

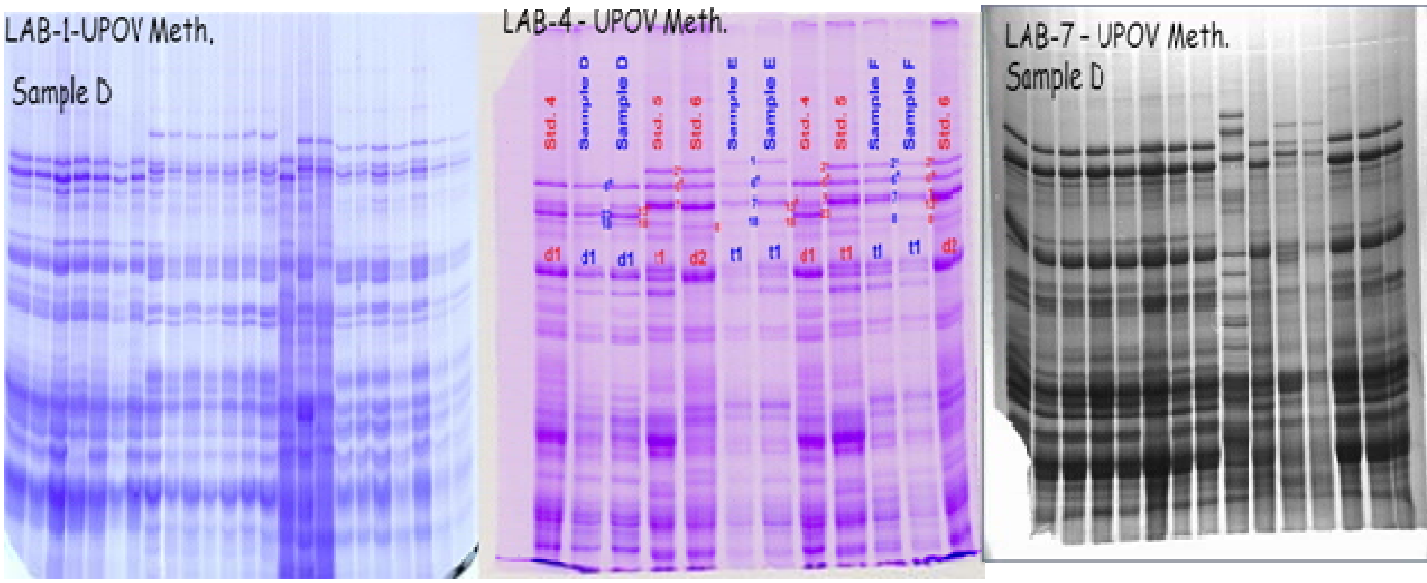


Fig. 6b: An example of the electrophoretic separation of XTriticosecale glutenins performed by the different laboratories.

Conclusion

Based on the results of this Comparative Test the SDS-PAGE technique is a reliable tool in seed testing and as consequence the UPOV method is proposed for the inclusion in the ISTA Rules:

- to confirm varietal identity of Triticum spps
- for species verification of Triticum spps. and Xtriticosecale

References:

Payne, P.I, Lawrence G,J. 1983. Catalogue of alleles for the complex gene loci , Glu-A1, Glu-B1, and Glu-D1, which code for the high-molecular-weight subunits of glutenin in hexaploid wheat. Cer. Res. Comm. , 11: 229-241.

Amiour N., Dardevet M., Khelifi D., Bouguennec A., G. Branlard. 2002 . Allelic variation of HMW and LMW glutenin subunits, HMW secalin subunits and 75K gamma-secalins of hexaploid triticale. Euphytica, 123: 179-186.

Amiour N., Bouguennec A. , Marcoz C.,Sourdille P., Bourgoin M., Khelifi D., G. Branlard. 2002. Diversity of seven glutenin and secalin loci within triticale cultivars grown in Europe. Euphytica, 123: 295-305.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

TG/3/11 UPOV Guidelines (www.upov.int)

Appendix 1. Methods description: equipment, chemicals and procedure used in the test.

	SDS-PAGE ISTA/UPOV	Lab method description 1	Lab method description 2	Lab method description 3
Equipment	Any suitable vertical electrophoresis system	Bio-Rad Mini-Protean 3 system; glass cassette with 0.5 mm spacers	Any suitable vertical electrophoresis system	Any suitable vertical electrophoresis system
Chemicals (All analytical reagent grade or better)	acrylamide 40% solution(AA) bisacrylamide 2% solution(BIS) urea glycine APS and TEMED 2-mercaptoethanol Sodium dodecyl phosphate (10% stock solution) TRIS pyronin G/Bromophenol Blue Coomassie Blue R-250 Coomassie Blue G-250 Water quality: purified water	acrylamide 40% solution(AA) bisacrylamide 2% solution(BIS) glycine APS and TEMED 2-mercaptoethanol (2-ME) Sodium dodecyl sulphate (10% stock solution) TRIS Bromophenol Blue Coomassie Blue R-250 Water quality: purified water	acrylamide 40% solution(AA) bisacrylamide 2% solution(BIS) glycine APS and TEMED 2-mercaptoethanol Sodium dodecyl phosphate (10% stock solution) TRIS Isopropanol Acetone Glycerol pyronin G/Bromophenol Blue Comassie Blue R-250 Coomassie Blue G-250 Water quality: purified water	The same as UPOV Method
Sample preparation	Single seeds crushed with pliers or alternatively 50-70 mg of flour are transferred to 1.5 ml polyproylene centrifuge tubes	The same as UPOV Method	The same as UPOV Method	Single seeds are not crushed (only cut in two halves) and transferred to a microtiterplate (200 µl)
Extraction buffer	Urea 4.5 M, 3% 2-mercaptoethanol, 10 % SDS.	Solve 15 g Tris, 19 g SDS and 0.025g Bromophenol blue in 750 ml Water. Add 96ml Glycerol and 54 ml 1N HCl. Before use add 5% (v/v) 2-ME	*Reducing solution*: Isopropanol 2.5 ml Tris HCl 1 M pH 8.8 0.4 ml 2-mercaptoethanol 250 µl Water 2.1 ml Prepare just before the use *Sample buffer*: Tris HCl 1 M pH 6.8 12.5 ml SDS (powder) 4 g Water 24 ml Glicerol 20ml Pyronin G 0.0 5% (the solution can be stored at room temperature for 1 month)	The same as UPOV method
Extraction procedure	Add 500 µl of the extraction buffer and thoroughly mix the sample Stand over night at room temperature. Heat the samples in	Add 500 µl of the extraction buffer and thoroughly mix the sample. Heat the samples in a boiling water bath for three minutes	Add 150 µl of the Reducing buffer and thoroughly mix the sample Heat the samples in a water bath at 60°C for thirty minutes Centrifuge at 14.000xg for 15 minutes.	Add 150 µl extraction buffer, no mixing, no heating, no centrifugatin, only standing over night

	SDS-PAGE ISTA/UPOV	Lab method description 1	Lab method description 2	Lab method description 3
	a boiling water bath for ten minutes and then allow to cool. Before loading the gel the tubes are centrifuged at 18.000xg.	and then allow to cool. Leave to stand for min. 30 min, then centrifuge at 13.000 x g.	Transfer the surnatant and add 1ml Acetone stored at -20°C. Proteins will precipitate in few minutes (keep at 4°C). Centrifuge, discard the Acetone and dry the pellet under the hood for 1 hour then add 250 µl of sample buffer	
Gel preparation 2 gel 16x18 cm 1.5 mm thickness	Stacking gel: *Acrylamide 3%, 0.125 M Tris-HCl pH 6.8*. acrylamide 40% solution(AA): 1.5 ml bisacrylamide 2% solution(BIS) : 0.43 ml Tris-HCl 1 M pH 6.8 2.5 ml SDS 10% 0.16 ml Water: 14.87 ml *For polymerization*: APS 1% 0.75 ml TEMED 20 µl Add the reagents to a 19.46 ml of stacking gel solution. Resolving gel: *Acrylamide 10%, 0.375 M Tris-HCl pH 8.8* acrylamide 40% solution(AA):20 ml bisacrylamide 2% solution(BIS)5.2 ml Tris-HCl 1 M pH 8.8 30 ml SDS 10% 0.8 ml Water: 20.8 ml *For polymerization:* APS 1% 2 ml TEMED 40 µl Add the reagents to a 76.80 ml of resolving gel solution. Tank buffer: *Tris 0.0250 M, Glycine 0.187 M SDS 1 %* pH 8.3 Stock solution: Glycine 141.1 g TRIS 30.0 g SDS 10.0 g Made up to 1000 ml with water	Stacking gel (stock solution): acrylamide 40% solution (AA): 6.75 ml bisacrylamide 2% solution (BIS): 2.8 ml Tris-HCl 1 M (60.5g Tris + 225 ml 1N HCl, water to 500 ml) 5.6 ml SDS 10% 0.7 ml Water: 49 ml *For polymerization*: Stacking gel solution 2 ml APS 20% 80 µl TEMED 10 µl Resolving gel: *(for preparation 4 cassettes)* acrylamide 40% solution(AA): 3.3 ml bisacrylamide 2% solution(BIS) 0.75 ml Tris-HCl 1 M 2.5 ml SDS 10% 115 µl Water: 5 ml *For polymerization*: APS 20% 80 µl TEMED 10 µl Tank buffer: *Tris 0.0250 M, Glycine 0.187 M SDS 1 %* pH 8.3 the same as UPOV	Stacking gel: *Acrlamide 3%, 0.125 M Tris-HCl pH 6.8*. acrylamide 40% solution(AA): 1.5 ml bisacrylamide 2% solution(BIS) : 0.43 ml Tris-HCl 1 M pH 6.8 2.5 ml SDS 10% 0.16 ml Water: 14.87 ml *For polymerization*: APS 1% 0.75 ml TEMED 20 µl Add the reagents to a 19.46 ml of stacking gel solution. Resolving gel: *Acrylamide 12%, 0.375 M Tris-HCl pH 8.8* acrylamide 40% solution(AA):24 ml bisacrylamide 2% solution(BIS)6.24 ml Tris-HCl 1 M pH 8.8 30 ml SDS 10% 0.8 ml Water: 15.8 ml *For polymerization:* APS 1% 2 ml TEMED 40 µl Add the reagents to a 76.80 ml of resolving gel solution. Tank buffer: *Tris 0.0250 M, Glycine 0.187 M SDS 1 %* pH 8.3 the same as UPOV	The ratio of AA and BIS is different, we have more BIS than AA TEMED 2.5-times APS 4-times TEMED 2-times The polymerization is faster More TRIS (0.043M), less Glycine (0.046M)

	SDS-PAGE ISTA/UPOV	Lab method description 1	Lab method description 2	Lab method description 3
	Dilute the stock solution 1:10 before use.			
Loaded samples	10-15- microliter.	1 – 2 microliter	10-15- microliter	
Electrophoresis 2 gel 16x18 cm 1.5 mm thickness	Stacking gel : Constant voltage at 100 V. (about 40 mA) Running gel: 80 mA (max 400 mA) Water should be circulated through the buffer tank to maintain the temperature at 15 - 20 °C. Stop the run 40 minutes after the tracking dye has reached the bottom of the gel.	Stacking and running gel : Constant voltage at 280 V. Stop the run when the tracking dye reach the bottom of the gel.	Stacking gel : Constant voltage at 100 V. (about 40 mA) Running gel: 80 mA (max 400 mA) Water should be circulated through the buffer tank to maintain the temperature at 15 - 20 °C. Stop the run 40 minutes after the tracking dye has reached the bottom of the gel.	
Fixing and staining	Fixing: TCA 15% about 30 minutes Staining: Sol A: Coomassie Blue G-250 0.25 g Coomassie Blue R-250 0.75 g made up to 100 ml with water. Sol B: TCA 27.5 g Acetic acid 32.5 ml Ethanol 90 ml Sol A 12.5 ml Water to 400 ml Stain overnight at room temp.	Fixing and staining: Solve 0.56g Coomassie Blue R-250 in 250 ml Methanol Add 125 ml 50% TCA, 160 ml glacial Acetic acid and 1000 ml water. Stain 2 hours or more at room temp.	Fixing: TCA 15% about 30 minutes Staining: Sol A: Coomassie Blue G-250 0.25 g Coomassie Blue R-250 0.75 g made up to 100 ml with water. Sol B: TCA 27.5 g Acetic acid 32.5 ml Ethanol 90 ml Sol A 12.5 ml Water to 400 ml Stain overnight at room temp.	No fixing Sol. A is the same Methanol
Destaining	water		water	Tap water
Storage of the gels	Gels can be stored in polythene bags at 4-6 °C for many months without deterioration		Gels can be stored in polythene bags at 4-6 °C for many months without deterioration	Gels can be dried between cellophane sheets, scanned or photographed. Soake them first in a 3% glycerol solution and then dry them at room temperature. The gels can be stored for years.