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Document OGM26-06

Method Validation Reports on Rules Proposals for the International Rules for Seed Testing 2027 Edition

1. Germination Committee Technical Report

Introduction of a new method for breaking dormancy for sunflower seeds.

Dupont Audrey and Ducournau Sylvie. GEVES-SNES, France.

Summary

The study was conducted to support the introduction of a new dormancy breaking method with Ethephon treatment to the current ISTA methods prescribed for releasing the dormancy of sunflower seeds. Seven laboratories analysed ten lots of *Helianthus annus*. The new Ethrel method was compared to the two official methods, prechilling and preheating. Statistical analysis showed that when the lots were dormant, the Ethrel method was comparable to the preheating method and better than the prechilling method. Results show good repeatability, and results with equal to and even better levels of reproducibility compared with the existing methods for this species in the ISTA Rules. It is therefore suggested that the Ethrel method becomes an additional prescribed method for breaking dormancy on sunflower seeds in the ISTA Rules.

Introduction

The introduction of this method in the ISTA Rules has been suggested by some laboratories in France. Right after harvesting sunflower, seed dormancy is sometimes very deep and ISTA methods recommended to break dormancy of sunflower (prechilling and preheating) do not release completely the dormancy. It is in that case impossible to assess the full germination potential of the seeds with a germination test.

After 2 years of experiment in France, involving 2 seed companies and 2 seed testing laboratories, we have demonstrated that a seed pre-treatment with Ethephon was an effective method to release the dormancy of freshly harvested sunflower seeds exhibiting high levels of dormancy. Ethephon and ethylene are known for their efficacy to break the dormancy of sunflower seeds *(Corbineau et al., 2014; Maiti et al., 2006),* but using ethylene gas is not convenient in a laboratory.

It was therefore proposed to start a validation study in the germination committee, to add this method to the current methods already prescribed in the ISTA Rules for *Helianthus annus*.

This study is conducted between 7 laboratories, 10 seed samples from 2 seed companies (5 seed samples from 2 seed companies each have been analyzed). A total of 4 methods were tested to compare the new method proposed using ethephon, with the dormancy breaking methods proposed by ISTA (prechilling and preheating) as well as a standard germination test to assess the initial dormancy of seed lots.

Material and Methods

a. Seed material

Helianthus annuus L. seed lots were supplied by two different seed companies: 5 seed samples by seed company. The samples are encoded H1 to H10.

Since sunflower dormancy breaks very quickly, samples could not be tested before shipment, which is why a total of 10 samples were analyzed to optimize the probability of observing dormancy.

b. Participant laboratories

A total of seven laboratories participated in the Validation study to add a new dormancy breaking method with Ethephon treatment to the current ISTA methods prescribed for releasing the dormancy of sunflower seeds. The laboratories were located in four countries: Germany, Italy, the Netherlands, and France.

c. Germination methods

All participants in this study compared four methods, three dormancy-breaking methods including the two recommended ISTA methods (prechilling and preheating), the new method proposed with ethephon and the standard germination test to assess the initial dormancy of seed lots.

Method 1 (SD)- ISTA germination test with no dormancy treatment

General directions are those described in ISTA Rules for *Helianthus annuus* (Chapter 5, Table 5A Part 1).

Here are the possible conditions for germination:

- Substrate: BP; TPS; S; O
- Temperature (°C): 20<=>30°C; 25; 20

- First count: 4 days
- Final count: 10 days

Each participant was free to use its own germination method, provided that it is the same used with no dormancy treatment and with dormancy treatment. Details of the methods are shown in Table 1.
Method 2 (PREHEATING) - ISTA germination test after preheating: The dry seeds of the replicates for germination are heated at a temperature of 30 to 35 °C with free air circulation for a period of up to 7 days before they are placed under the prescribed germination conditions (ISTA rules Chapter 5.6.3.1).
Method 3 (PRECHILLING) – ISTA germination test after prechilling: The replicates for germination are placed in contact with the moist substrate and kept at a low temperature. Sunflower seeds are usually kept at a temperature of 5 to 10 °C for an initial period of up to 7 days before they are placed under the prescribed germination conditions (ISTA rules Chapter 5.6.3.1).
Method 4 (ETHREL) – ISTA germination test after Ethephon treatment: the method protocol is as follows.

- Take 250 seeds at random
- Dissolve one bleach tablet in two litres of water. Corresponds to a sodium dichloroisocyanurate concentration of 1.28g/l.
- Soak the seeds in the bleach to disinfect them, shake for few seconds, and remove the solution to keep the seeds.
- Rinse, drain the seeds and place them in a beaker.
- Dilute 0.6 ml of Etheverse (Bayer ©) in one litre of water and shake for 10 seconds. Ethephon concentration in Etherverse is 480 g/l (40% m/m). Refer to safety data sheet for use of this product.
- Pour the solution over the seeds, so that they are completely covered - Soak the seeds for 18 hours (± 1 hour) at room temperature.
- Drain the seeds in a beaker.
- The seeds are then placed onto an absorbent paper and wiped twice. If seeds have already germinated, remove them ¾.
- Immediately after drying, germinate the seeds using the standard ISTA method (4 replicates of 50 seeds are to be sown between paper, normal germination is assessed after the germination period).

All four methods were tested on 2 repetitions of 100 by the participants. Given the number of samples tested, the ISTA Statistics committee has been requested to validate the relevance of testing the lots on 200 seeds and not 400 as usual.

Table 1 : details of the germination methods used by each participant.

Laboratory	Substrate	T°C	Light	Method 2	Method 3
A	BP	20<=>30°C	8H	7 days at 35°C	7 days at **6°C**
B	BP	20<=>30°C	8H	7 days at 35°C	7 days at **7.5°C**
C	BP	20<=>30°C	8H		7 days at **8°C**
D	BP	20<=>30°C	12H	7 days at 35°C	7 days at **7°C**
E	PP	25°C	8H	7 days at 35°C	7 days at **5°C**
F	Sand	20<=>30°C	12H	7 days at 35°C	7 days at **7°C**
G	Sand	25°C	8H	7 days at 35°C	7 days at **7°C**

Raw data of this study was archived in a computer server in GEVES, France.

d. Statistical analyses

Statistical analyses were performed using 'ISTAgermMV', the tool developed by the ISTA Statistics Committee. Boxplots (per lot, per method, per method x lot, and per laboratory), data checking and the repeatability/reproducibility results were generated from this statistical tool. Analysis of variance (ANOVA) and Student Newman-Keuls (SNK) test.

Results and Discussion

e. Germination results by seed lot

f. Figure 1 shows the percentages of normal seedlings obtained for the ten seed lots, by all the laboratories using all the different methods. Boxplots in the figure show the distribution of the data around the median value. In terms of average results lots 7 (72%), 9 (83%) and 10 (90%) are the lots with the lowest percentage of normal seedlings to the standard germination method. These lots are therefore likely to exhibit dormancy. Lots 2, 3, and 4 have a percentage of normal seedlings greater than 94%.

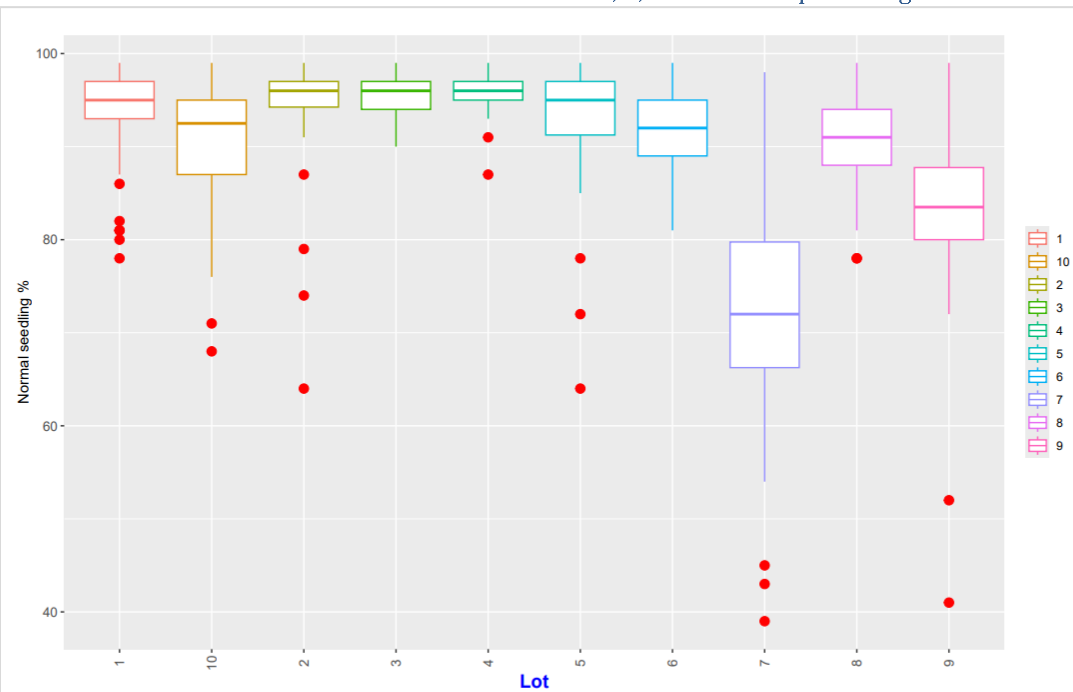

Figure 1 : Boxplots for the ten seed lots grouped across methods and laboratories.

g. Germination results by laboratory

a) For all the participant laboratories

Figure 2 shows the percentages of normal seedlings obtained by all the laboratories, on all seed lots and methods. In terms of laboratory results, lab E, G et A obtained the lowest germination results (lower 90% overall average).

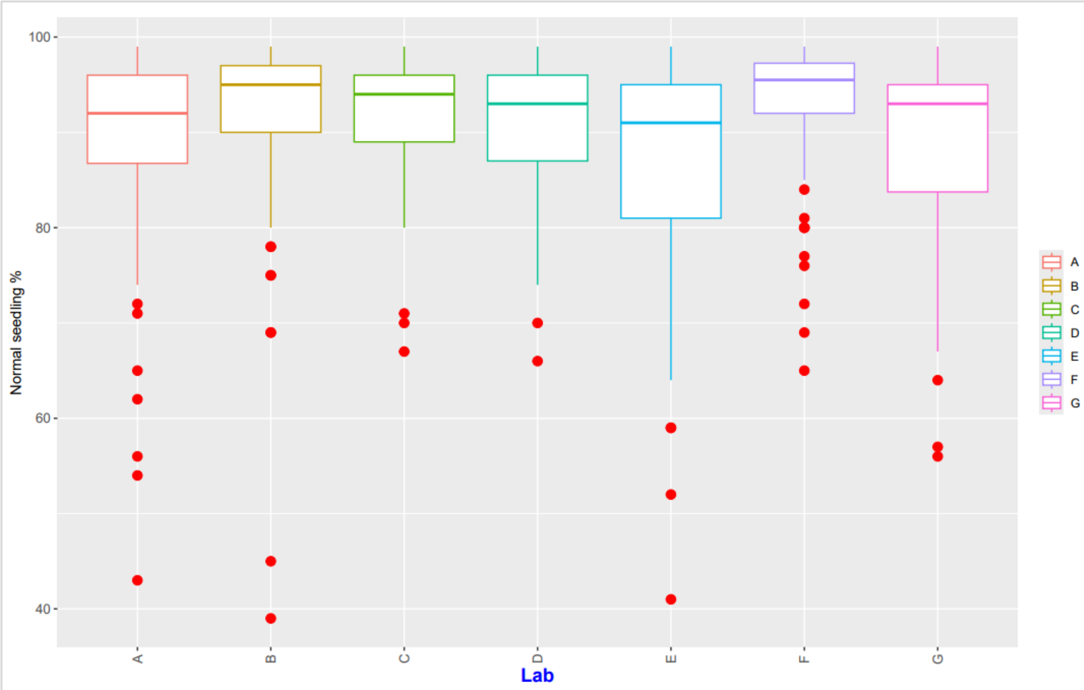

Figure 2 : Boxplots for the 7 laboratories grouped across seed lots and methods.

The aim of this study was to compare the laboratories on the percentage of fresh and therefore dormant seeds. Table 2 shows that laboratories A and B observed dormancy, with an average of 3.5% and 2.5% of fresh seed for all methods and lots. Laboratory E has a high rate of abnormal seedlings and laboratory G has a high rate of dead seeds compared with the other laboratories.

Table 2 : includes the details of the germination classification used by each participant.

Laboratory	Normal seedling (%)	Abnormal seedling (%)	Fresh seed (%)	Dead seed (%)
A	88.7	5.5	3.1	2.8
B	91.0	4.0	2.5	2.6
C	90.9	5.3	0.4	3.4
D	90.8	4.9	1.2	3.0
E	87.1	9.7	0.1	3.1
F	92.9	2.0	0.0	5.2
G	88.5	3.0	0.3	8.2
Mean	**90.0**	**4.9**	**1.1**	**4.1**

Germination results by method.

Figure 3 shows the percentages of normal seedlings obtained with the different methods used by all laboratories on the ten seed lots. The figure shows that results are more homogeneous, with much less outliers, with the prechilling and preheating method.

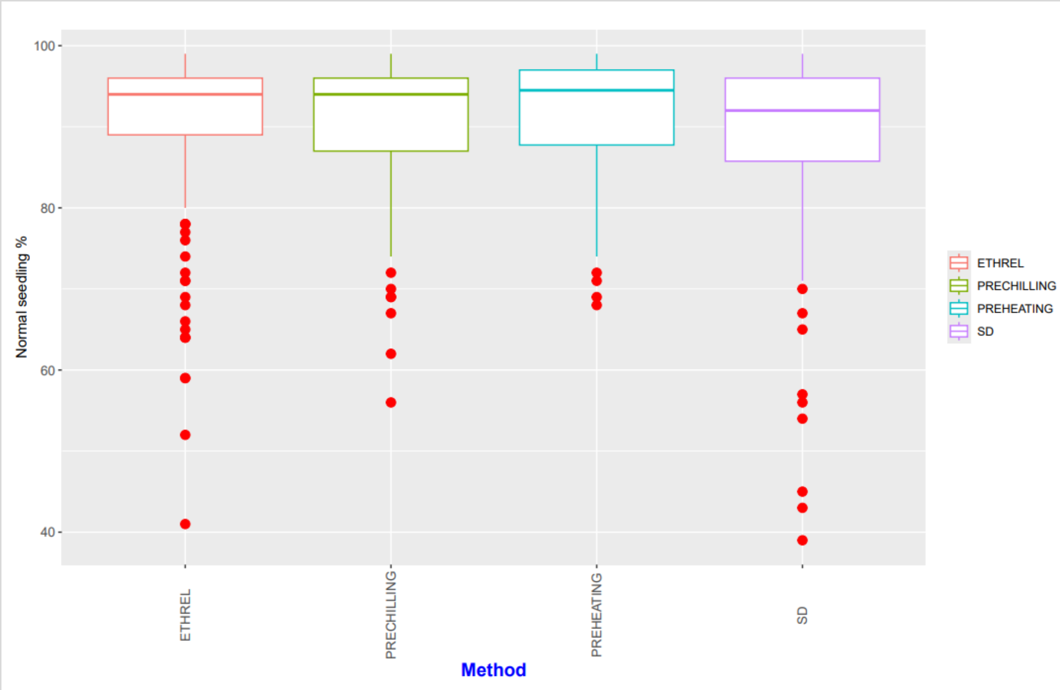

Figure 3 : Boxplots for the 4 methods grouped across seed lots and laboratories on the normal seedlings.

Actually, laboratory E obtained 24% of abnormal seedling with Ethrel method, it is the only laboratory to obtain a strong increase in the percentage of abnormal seedlings with the Ethrel method (Table 3).

Laboratory E is the only one to have used a PP method, so we decided to carry out few tests to compare the impact of the substrate on the development of seedlings after Ethrel. The results showed that with a PP substrate, the percentage of abnormal seedlings was much higher, demonstrating a toxic effect observed with PP, but not with sand or BP.

Table 3 : details of the germination results (in %) obtained by each participant and by method. Legend: NS = normal seedling, ANS = abnormal seedling, FS = fresh seed, DS = dead seed.

	ETHREL				PRECHILLING				PREHEATING				SD			
Lab	NS	ANS	FS	DS	NS	ANS	FS	DS	NS	ANS	FS	DS	NS	ANS	FS	DS
A	92	4	2	2	87	7	3	3	91	6	1	3	86	5	6	3
B	95	3	1	1	91	4	2	3	93	4	1	2	85	5	7	3
C	93	5	0	3	91	6	0	3					89	5	1	4
D	93	4	0	3	90	5	2	3	92	5	0	3	89	6	3	3
E	74	24	0	2	93	4	0	3	90	6	0	4	92	4	0	4
F	94	2	0	5	93	2	0	6	93	2	0	5	92	3	0	5
G	89	5	1	5	89	2	0	9	90	2	0	8	86	3	0	11
Mean	**90**	**7**	**1**	**3**	**90**	**4**	**1**	**4**	**91**	**4**	**0**	**4**	**88**	**4**	**2**	**5**

b) For the participants without laboratory E

Figure 4 shows the percentages of normal seedlings obtained with the different methods on the ten seed lots used by laboratories without laboratory E. The figure shows that the results for Ethrel are better to the preheating method and therefore better than the prechilling method.

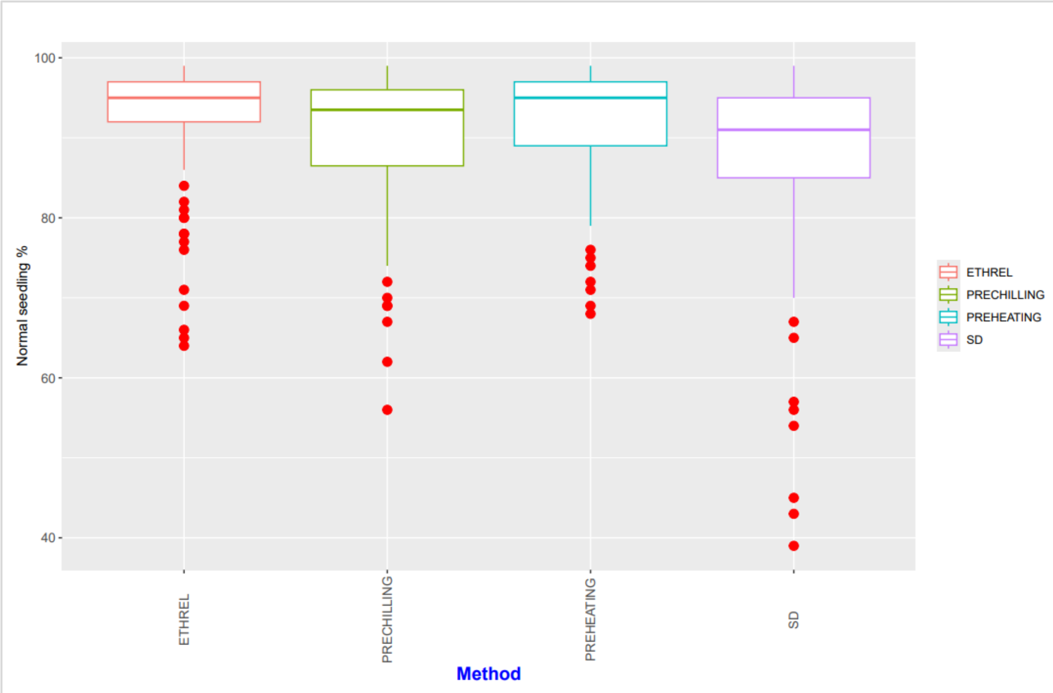

Figure 4 : Boxplots for the 4 methods grouped across seed lots on the normal seedling, for all the laboratories except laboratory E.

Fresh seed results by method.

a) For all the laboratories

Figure 5 shows the percentages of fresh seeds obtained with the different methods used by all laboratories on the ten seed lots. The figure shows that results are similar between the different methods for breaking dormancy.

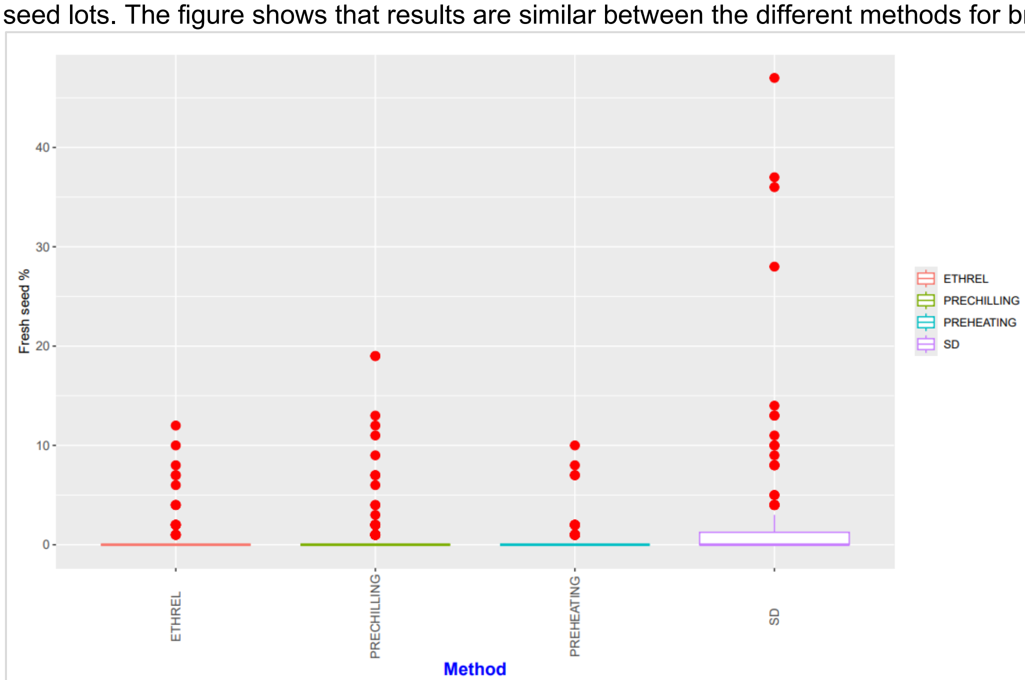

Figure 5 : Boxplots for the 4 methods grouped across seed lots and laboratories on the fresh seed.

Figure 6 shows the percentages of fresh seeds obtained with the different methods for each lot. Lots 7 and 10 present seed dormancy, in particularly for laboratories A and B as previously mentioned.

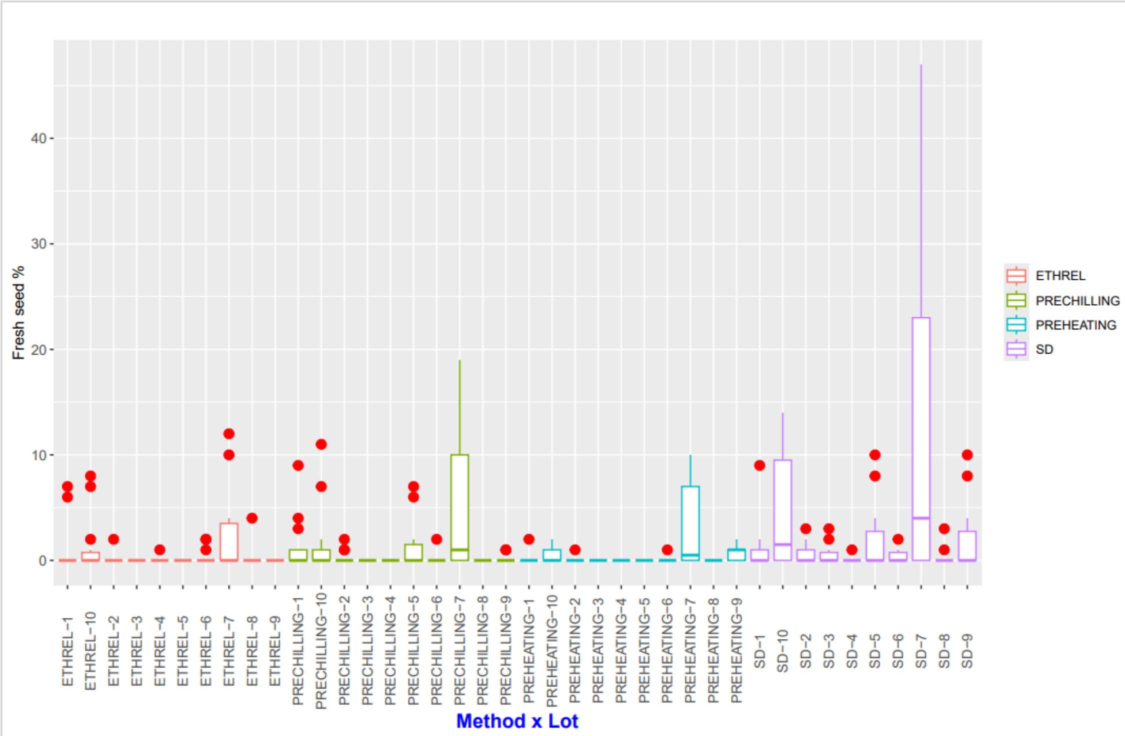

Figure 6 : Boxplots for the 4 methods per seed lot and grouped by laboratory on fresh seed.

b) Results for dormant lots observed in laboratories A and B.

- <u>Fresh seed results</u>

Figure 7 shows the percentages of fresh seeds obtained with the different methods used by laboratories A and B for 2 seed lots. Lot 7 showed 37% fresh seed on average in the two laboratories for the standard germination method, 8% for the preheating method, 16% for the prechilling method and 5.5% for the Ethrel method. The same observations were made for lot 10, although it was less dormant.

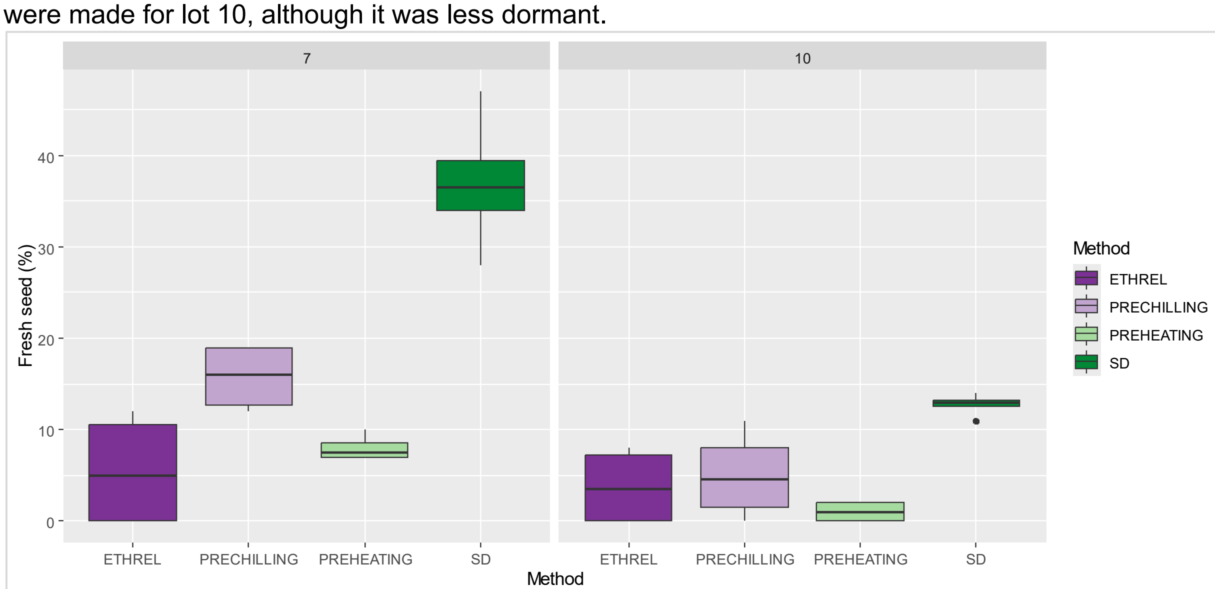

Figure 7 : Boxplots for the 4 methods per dormancy seed lot (lot 7 and lot 10) and grouped by laboratory on fresh seed.

On average for the two lots with dormancy, the Ethrel method is similar to the preheating method and better than the prechilling method.

Table 4 shows the analysis of variance with a significant effect of the method (p = 0.0001), there is also an interaction between the batch and the method.

Table 4 : Analysis of variance on fresh seed.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Mod	3	2206.1	735.4	35.78	5.09e-09	***
Mod:Lot	4	1511.4	377.8	18.39	4.95e-07	***
Residuals	24	493.2	20.6			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table 5 shows the SNK test to classify the method. The same classification was obtained for preheating and the Ethrel method with the lowest rate of fresh seeds. The prechilling method is classified in group b with a higher rate of fresh seeds than the other two breaking dormancy methods.

Table 5 : Classification method with Student Newman-Keuls test (SNK).

	Fresh_seed	groups
SD	24.875	a
PRECHILLING	10.375	b
ETHREL	4.625	c
PREHEATING	4.500	c

- Normal seedling results

Figure 8 shows the percentages of normal seedling obtained with the different methods used by laboratories A and B for the 2 dormant seed lots. The average percentage of normal seedlings is 88% on average for the Ethrel method, compared with 85% for the preheating method, 77% for the prechilling method and 62% for the standard germination test.

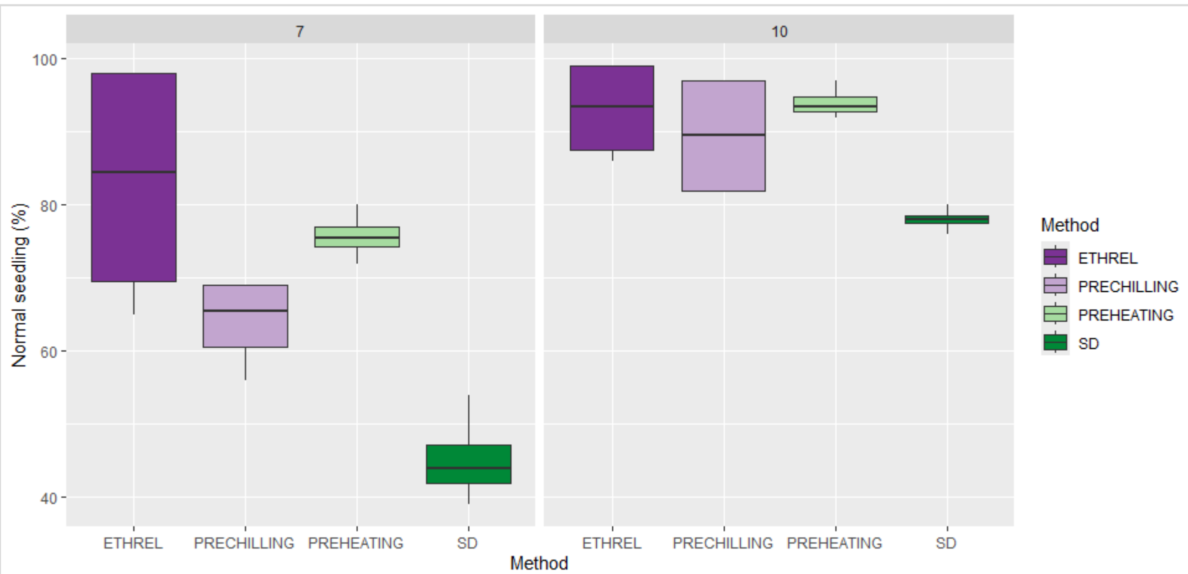

Figure 8 : Boxplots for the 4 methods per dormancy seed lot and grouped by laboratory on normal seedling.

Table 6 shows the analysis of variance with a significant effect of the method (p = 0.004), and no laboratory effect.

Table 6 : Analysis of variance on normal seedling.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Mod	3	3335	1111.5	16.86	4.17e-06	***
Mod:Lot	4	4312	1077.9	16.35	1.37e-06	***
Residuals	24	1582	65.9			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table 7 shows the SNK test to classify the method. The same classification was obtained for preheating and the Ethrel method, Ethrel method obtain the highest percentage of normal seedlings. Prechilling method is classified in group b with 76.7% of normal seedling. This demonstrates the effectiveness of the Ethrel method.

Table 7 : Classification method with Student Newman-Keuls test (SNK).

	Normal_seedling	groups
ETHREL	88.000	a
PREHEATING	84.875	ab
PRECHILLING	76.750	b
SD	61.625	c

Results of data checking

Data checking of the normal germination percentages was performed according to the ISTA rules by computing tolerances for germination test replicates.
Some results were out of tolerance.
Lab D : lot 6 method prechilling lot 1 method prechilling.
Lab E : lot 3 method prechilling and lot 8 method Ethrel.
Lab F : lot 7 method SD and method preheating.

Repeatability and reproducibility of the results for the different methods on normal seedlings, only on the two lots with dormancy

a) For all the participants laboratories

Table 8 : Repeatability and reproducibility of the different methods.

Method	Mean	s_repetability	Disp.	s_Reproductibility	s_Lab	s_LotxLab
ETHREL	82	2.69	0.70	12.14	10.98	4.81
PRECHILLING	83	4.06	1.07	7.41	5.51	4.03
PREHEATING	85	3.81	1.07	2.72	0.35	0.00
SD	75	5.13	1.18	11.03	9.59	4.07

b) For the participants without laboratory E

Table 9 : Repeatability and reproducibility of the different methods without the laboratory E.

Method	Mean	s_repetability	Disp.	S_Reproductibility	s_Lab	s_LotxLab
ETHREL	85	2.84	0.79	10.2	8.5	5.26
PRECHILLING	81	4.38	1.12	6.19	4.88	2.21
PREHEATING	85	4.09	1.14	2.89	0.00	0.00
SD	73	5.48	1.23	10.63	8.93	4.27

In Tables 8 and 9, summarized by the method, s.repeatability and s.Reproducibility corresponds to the repeatability and reproducibility standard deviations. Disp. is the dispersion factor; it applies to the results of repeatability and indicates an over dispersion when its value is greater than 1.
The dispersion factors are below 1 except for the standard germination test, which was slightly worse, since two laboratories observed dormancy on certain lots. Ethrel methods of breaking dormancy give a low value for the dispersion factor, indicating good repeatability of the results obtained with this method in the participating laboratories. Repeatability is therefore better than for the other two methods of lifting dormancy.
The values of the standard deviation of reproducibility are different depending on the methods. The lowest values and therefore the best reproducibility is for the method preheating and prechilling when lab E is not included.
Without the results from lab E (see tables 8 and 9), the germination results are better using the method with Ethrel, on average, 85% of seedlings are normal. Including the results from lab E, the best method is the preheating method with 85% of normal seedlings, and Ethrel method and prechilling method are the same with 82-83% of normal seedlings.

General conclusion

This study shows the value of adding the dormancy breaking method using Ethrel to the ISTA rules. When dormancy is observed, as in lots 7 and 10, the two laboratories demonstrate the effectiveness of the Ethrel method. The Ethrel method is just as effective as the preheating method and better than the prechilling method.

Across all lots and laboratories, the Ethrel method was found to be as effective as the two official methods for lifting dormancy.

Given the preliminary studies showing the effectiveness of the Ethrel method *(Ducournau S. and Dupont A., 2023 and 2024),* we propose to add this new method to the international ISTA rules.

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Thanks to Jean-Louis Laffont, Statistics Committee vice-chair, for the ISTAgermMV stat program to run the data and the support he gave for interpretation.

Thanks to Takayuki Okuda (Takii & Co. Ltd. – Japan), member of the ISTA Germination Committee, who sent us an additional lot which unfortunately did not present any dormant seeds.

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2. ISTA validation study for viability test of *Glycine max* (L.) Merr.

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Summary

The objective of this validation study was to develop a tetrazolium viability procedure to test *Glycine max* (L.) Merr. seeds to be included in chapter 6 of the ISTA Rules. The experiment was carried out by seven Labs (6 ISTA accredited laboratories and 1 TCOM Member´s lab) using three seed lots with different quality. The seeds were tested hydrating the sample replicate overnight (18 hours) between moist rolled filter paper / paper towels at 20 °C ± 2 °C within sealed plastic bags to avoid evaporation. After that, the seeds were stained 6 h at 30 °C ± 2 °C in a 1 % of concentration of 2,3,5 triphenyl tetrazolium chloride solution in the dark. Statistical analysis of the results of all laboratories showed that the method tested meets the ISTA performance criteria. Hence, the viability method proposed to be included in the ISTA Rules to test *Glycine max* seeds is: BP/18, 1% of Staining solution, 6h optimum staining time.

Introduction

Soybean [*Glycine max* (L.) Merr.] serves as one of the most valuable crops in the world, not only as an oil seed crop and feed for livestock and aquaculture, but also as a good source of protein for the human diet and as a biofuel feedstock. The use of the tetrazolium viability test on soybean reaches its pinnacle when it is necessary to obtain fast results of soybean seed lots to take preliminary decisions.

Material and methods

Seed material

Three seeds lots of *Glycine max* with different quality levels have been selected for this ISTA comparative test.

Participant laboratories

The following laboratories expressed their interests and met the criteria required (indicated in the "ISTA Method Validation for seed Testing") to conduct the tests of this validation study: Laboratorio Central de Análisis de Semillas del INASE, Argentina (ISTA Accredited Lab), Embrapa Soybean, Brazil (ISTA TCOM Member´s Lab), SGS Sherwood Park, Canada (ISTA Accredited Lab), GEVES-SNES Station Nationale d'Essais de Semences, France (ISTA Accredited Lab), Landwirtschaftliches Technologiezentrum Augustenberg, Germany (ISTA Accredited Lab), CREA DC, sede di Tavazzano, Italy (ISTA Accredited Lab) and OSTS, NIAB, United Kingdom (ISTA Accredited Lab).

Testing method.

Three lots of seed were used. For each lot, 400 pure seeds were tested following the PSD 11 described in chapter 3 when preparing seed to be used in the viability test.

This proposal was based on the ISTA Working Sheet on Tetrazolium Testing (Volume 1) for *Glycine max.* The proposed viability method is:

Pre-treatment: The seeds were hydrated 18 hours between moist rolled filter paper / paper towels at 20 °C ± 2 °C within sealed plastic bags to avoid evaporation.

Staining: From each seed lot 4 replicates of 100 seeds were stained 6 h at 30 °C ± 2 °C in a 1 % of concentration of 2,3,5 triphenyl tetrazolium chloride solution in the dark.

Preparation for evaluation: Before evaluation, the seeds in the tetrazolium solution were rinse seeds with water and remain submerged in water during the evaluation to avoid dehydration and discoloration. The seed coat was removed by hand to expose the embryo and was cut down through the middle of the cotyledons, and the hypocotyl-radicle axis with a sharp device (Figures 1 and 2).

Figure 1. Cutting procedure of a soybean seed with a razor blade.

Figure 2. Internal view of a viable soybean seed, after a perfect cutting.

The evaluation must be done checking the colour, tissue turgidity and location of damage or dead areas both internally and externally all over the seed.

Seeds with close fitting seed coat are directly related with sound and vigorous tissues, which will always appear turgid, externally in red colour (Figure 3) and brilliant white inside of cotyledons (Figure 4). The Tetrazolium solution can penetrate only to a shallow depth, and internally this seed shows a brilliant white colour surrounded by an area of red colour (Figure 4).

Figure 3. External view. Viable seed in red colour

Figure 4. Internal view. Viable seed in brilliant light red colour.

Deteriorated tissues will always appear in dark red colour and dead tissues, a dull white colour (Figure 5).

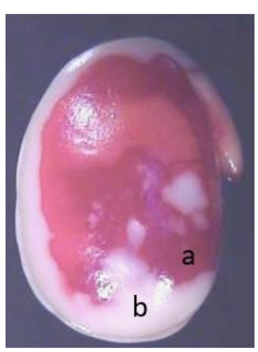

Figure 5. External view of Non-Viable seed.

Seed with deteriorated red tissue (a) and dead tissues in white dull colour (b).

Other colours like yellowish, and/or greyish and/or purplish-red and green must be considered as non-living tissues (Figure 6).

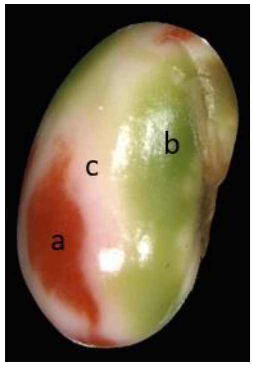

Figure 6. External view of Non-Viable seed.
Seed with deteriorated dark red tissue (a) and green (b) and white dull dead tissues (c).

Evaluation:

Viable seeds:

- Completely turgid and stained seed of a normal red colour externally and brilliant white inside (Figures 3 and 4).
- Presence of minor area of dark red colour, unstained, flaccid, or necrotic tissues with limited extension and superficial depth localized on any site of the seed (including embryo axis and joining area on the embryo axis and the cotyledons) (Figures 7 and 8).
- Presence of major or multiple areas of dark red colour, unstained, flaccid or necrotic tissues extending from ⅓ to the whole of the cotyledon area at the distal end of the cotyledon(s), and a depth from ½ of the cotyledon to entire cotyledon (Figure 9).
- Figure 10: Hypocotyl-radicle axis with tissues up to 1/3 deteriorated, unstained or lost (A); joining area embryo axis-cotyledons with deteriorated dark red tissues (B); cotyledons with tissues up to ½ deteriorated, unstained or lost (C); cotyledons with tissues up to ¼ deep deteriorated or unstained (D); cotyledon with tissues up to ¾ deteriorated, unstained or lost (E).

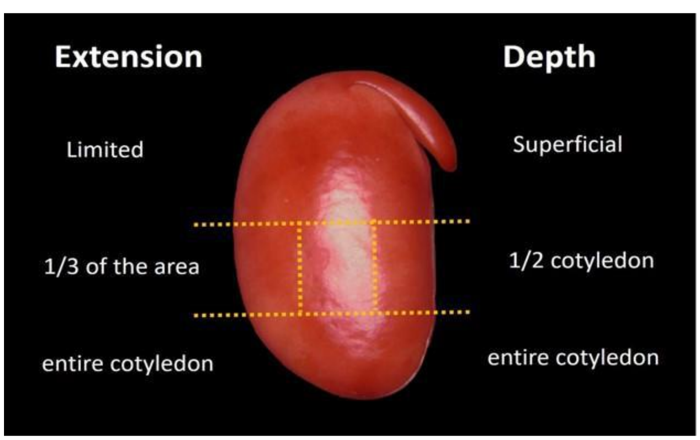

Figure 7. Location of damaged areas on the seed.

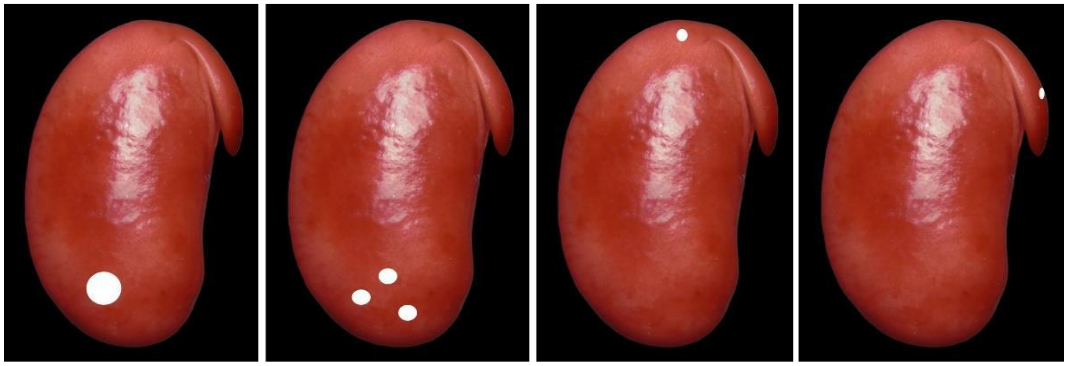

Figure 8. Viable seeds: minor areas of dark red colour, unstained, flaccid, or necrotic tissues with limited extension and superficial depth.

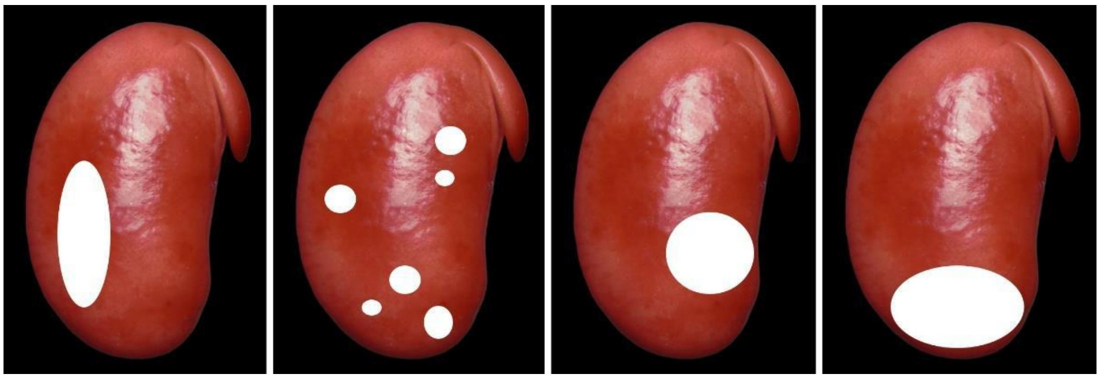

Figure 9. Viable seeds: Major or multiple areas of dark red colour, unstained, flaccid, or necrotic tissues with an extension of 1/3 of the cotyledon area (A) to 3/3 of the cotyledon area at the distal end of the cotyledon(s) (B); and a depth of ½ of the cotyledon to entire cotyledon.

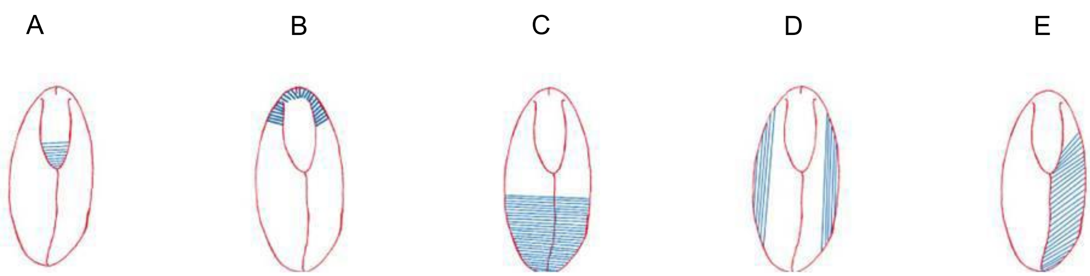

Figure 10. Viable Seeds This information is identical to the one mentioned before when describing "Figure 10"

*NOTE***:** Seeds with obvious genetic damage development of the embryo shall be regarded as non-viable whether stained or not.

Non-Viable seeds: Figure 11: Hypocotyl-radicle axis with more than 1/3 deteriorated, unstained, or lost tissues (F); joining area embryo axis-cotyledons unstained (G); plumule deteriorated or lost (H); cotyledons with more than ½ deteriorated, unstained or lost tissues (I); cotyledons with more than ¼ deep deterioration or unstained tissues (J); cotyledon with more than ¾ deteriorated, unstained or lost tissues (K); entire seed unstained (L).

F G H I J K L

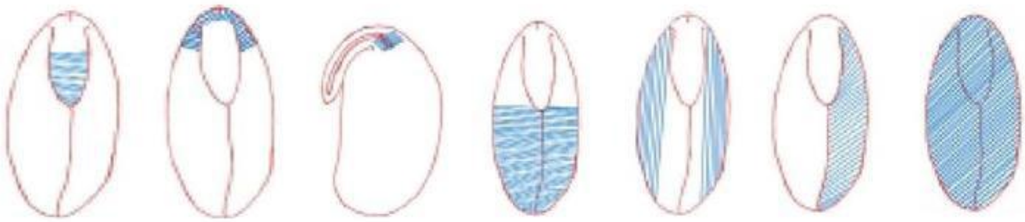

Figure. 11. Non-Viable Seeds This information is identical to the one mentioned before when describing "Figure 11"

Photos of Viable and Non-Viable seeds

Viable seeds

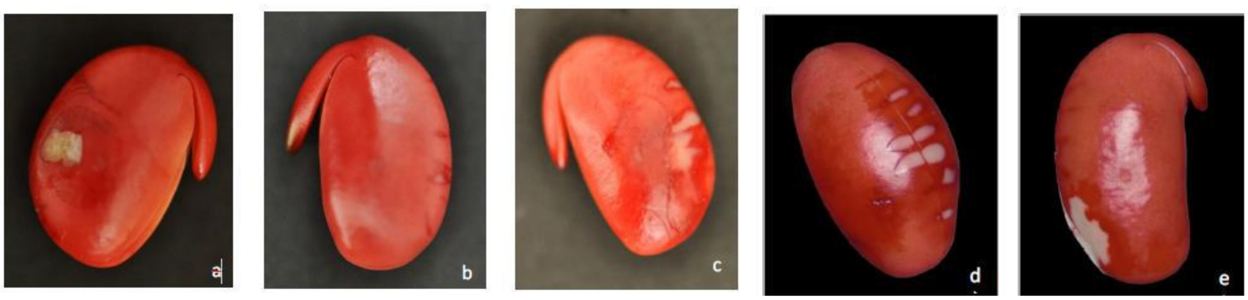

Figure. 12. Viable seeds: damages located in areas away from the hipocotyl-radicle axis and joining area (the point of insertion of both cotyledons with the embryo axis). a) stink-bug bite; b to e) environmental damages.

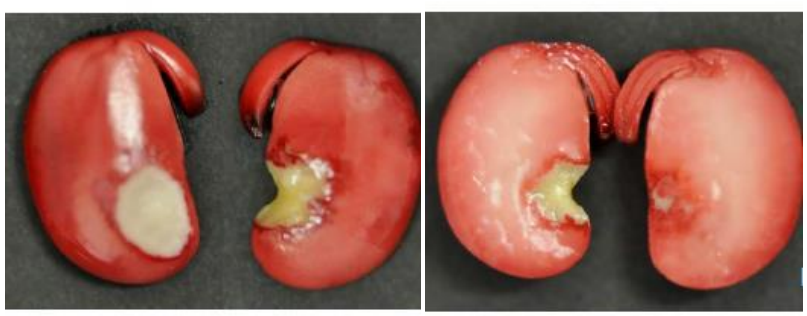

Figure. 13. Viable seeds: deep stink-bug damages located on cotyledons, in areas away from the hipocotyl-radicle axis and joining area (the point of insertion of both cotyledons with the embryo axis).

Non-Viable seeds

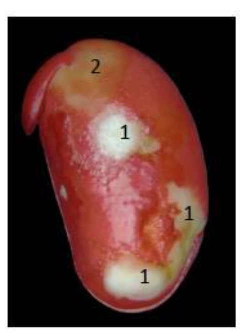

Figure. 14. Non-viable seed: extensive stink-bug damages (1) located on cotyledon

and environmental damage (2) on the joining area (the point of insertion of both cotyledons with the embryo axis).

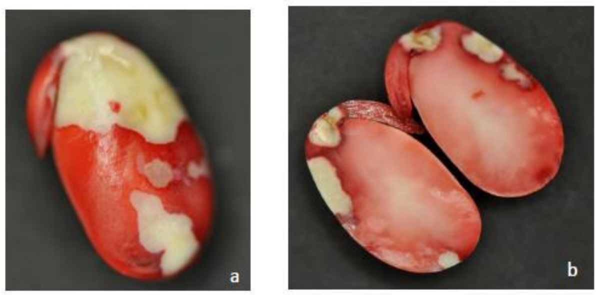

Figure. 15. Non-viable seeds: extensive and deep stink-bug damages located on cotyledons (a) and plumule (b).

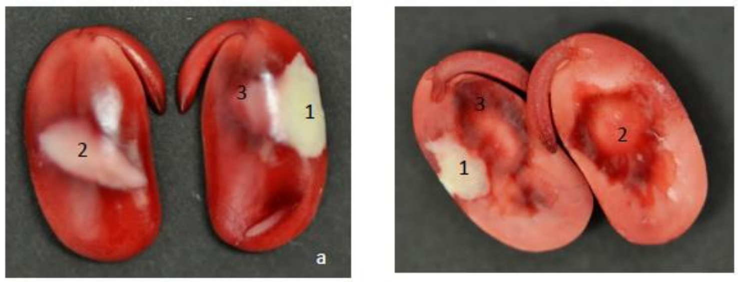

Figure. 16. Non-viable seeds: extensive and deep stink-bug damage located on cotyledon (1) and extensive and deep environmental damage on cotyledons (2 and 3).

Statistical analysis of the results

Tetrazolium viability results obtained by the different laboratories

1. Data exploration with side-by-side box plots

Statistical analysis was performed by ISTA Statistical Committee. Data exploration with side-by-side box plots showed a clear quality difference in seeds lot's quality (Figure 17). Seed lot #3 evidence lower quality than the other two. That difference was also evident in the box plot of viability per lot and per laboratory (Figure 18).

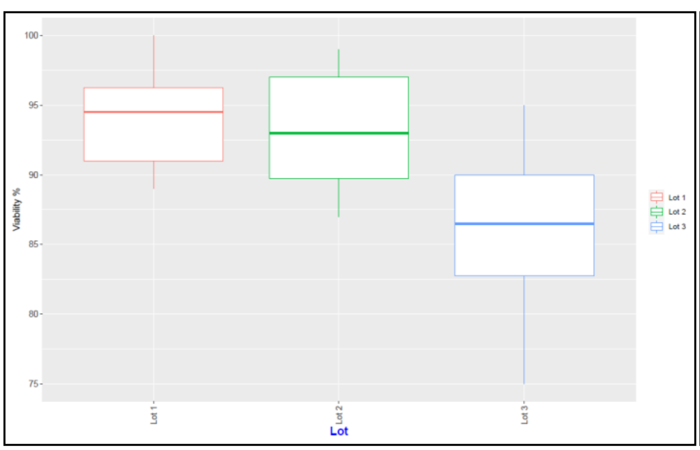

Figure 17. Box plot of Glycine max seed's viability results of all laboratories per seed lot.

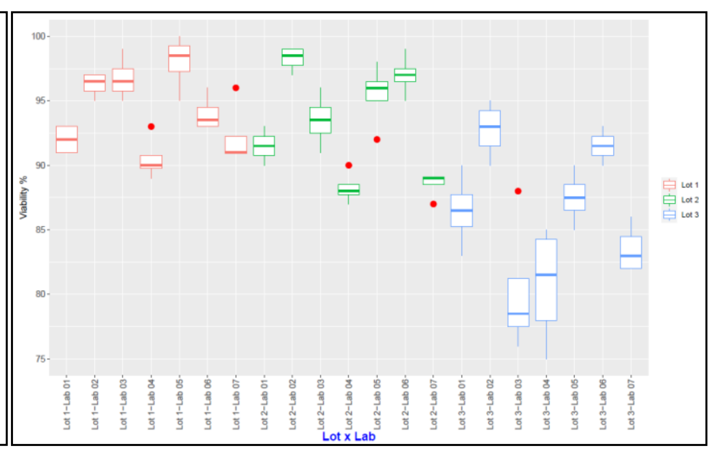

Figure 18. Box plot of viability results for all participating laboratories and seed lots of Glycine max.

Note: Figure references:

- Box (rectangle): Represents the interquartile range (IQR), that is, from Q1 (25%) to Q3 (75%). The middle 50% of the data is inside the box.
- Line inside the box: This is the median (Q2), the central value of the data.
- "Whiskers" (lines extending from the box): These extend to the minimum and maximum values within 1.5 × IQR (excluding outliers).
- Red points outside the whiskers: These are outliers, data points that are very far from the rest.

2. Data checking

Data checking has been performed according to Table 6B, ISTA Rules by computing tolerances for tetrazolium test replicates.
As shown in Table 1, all the results are within tolerance.

Table 1. Tolerances among tetrazolium test replicates of each seed lot and laboratory

Lot	Lab	Mean	# Reps	# seeds/rep		Range	Tol	Out of Tol
Lot 1	Lab 01	92	4	100		2	11	ok
Lot 1	Lab 02	96	4	100		2	8	ok
Lot 1	Lab 03	97	4	100		4	7	ok
Lot 1	Lab 04	90	4	100		4	12	ok
Lot 1	Lab 05	98	4	100		5	6	ok
Lot 1	Lab 06	94	4	100		3	10	ok
Lot 1	Lab 07	92	4	100		5	11	ok
Lot 2	Lab 01	92	4	100		3	11	ok
Lot 2	Lab 02	98	4	100		2	6	ok
Lot 2	Lab 03	94	4	100		5	10	ok
Lot 2	Lab 04	88	4	100		3	13	ok
Lot 2	Lab 05	96	4	100		6	8	ok
Lot 2	Lab 06	97	4	100		4	7	ok
Lot 2	Lab 07	88	4	100		2	13	ok
Lot 3	Lab 01	86	4	100		7	14	ok
Lot 3	Lab 02	93	4	100		5	10	ok
Lot 3	Lab 03	80	4	100		12	16	ok
Lot 3	Lab 04	81	4	100		10	15	ok
Lot 3	Lab 05	88	4	100		5	13	ok
Lot 3	Lab 06	92	4	100		3	11	ok
Lot 3	Lab 07	84	4	100		4	14	ok

3. Repeatability/Reproducibility

The following linear mixed model is fitted:

$$y_{ijk} = \mu + \alpha_i + L_j + (\alpha L)_{ij} + e_{ijk} \qquad (1)$$

in which:
. y_{ijk} is the viability % in Rep *k* of Lot *i* and Lab *j*.
. μ is the intercept.
. α_i is the fixed effect of Lot *i*.
. L_j is the random effect of Lab *j*. L_j ~ i.i.d. $N(0, \sigma^2_{Lab})$.
. $(\alpha L)_{ij}$ is the random interaction effect between Lot *i* and Lab *j*.
$(\alpha L)_{ij}$ ~ i.i.d. $N(0, \sigma^2_{Lot\times Lab})$.

. e_{ijk} are the residuals. e_{ijk} ~ i.i.d. $N(0, \sigma^2)$.

Repeatability standard-deviation is then given by $S_r = \sqrt{\hat{\sigma}^2}$ and reproducibility standard-deviation by $S_R = \sqrt{\hat{\sigma}^2_{Lab} + \hat{\sigma}^2_{Lot\times Lab} + \hat{\sigma}^2/K}$ where *K* is the number of reps.

- *Repeatability.*

The repeatability dispersion factor is calculated as $f_r = \sqrt{\frac{n\hat{\sigma}^2}{\bar{p}_{...}(100-\bar{p}_{...})}}$ where $\bar{p}_{...}$ is the overall average percentage of the trait analyzed, and *n* is the number of seeds per Rep.

The results are summarized in Table 2 below.

Table 2. Repeatability data for viability results.

$\bar{p}_{...}$	$\hat{\sigma}_{Lab}$	$\hat{\sigma}_{Lot\times Lab}$	S_r	f_r	S_R
91.00	3.08	2.27	2.31	0.82	4.00

If f_r > 1 one speaks of overdispersion because the data has larger variance than expected under the assumption of a binomial distribution. As f_r is 0.82 (< 1, Table 2), repeatability is acceptable

- *Reproducibility*

The *reproducibility* dispersion factor is calculated as $f_R = \sqrt{\frac{KnS_R^2}{\bar{p}_{...}(100-\bar{p}_{...})}}$ where S_R^2 is estimated from model (1) after excluding laboratory results for which their absolute deviation from the lot mean (computed from all the laboratories) divided by $\frac{\bar{p}_{...}(100-\bar{p}_{...})}{Kn}$ exceeds 4 (Miles, 1963); the reference reproducibility dispersion factor, $f_{Reference}$, is taken to be equal to 2.82 (TEZ Committee Report, 1998-2001). These results are displayed in Table 3.

Table 3. Reproducibility data for viability results.

$\bar{p}_{...}$	**Excluded samples %**	f_R	$f_{Reference}$
91.00	4.80	2.73	2.82

The method proved to be reproducible in different laboratories, considering that the f_R obtained (2.73) by the laboratories is lower than the reference reproducibility dispersion factor (Table 3, $f_{Reference}$).

4. Comparing Lot means

To compare the means of the seeds lots an ANOVA for the fixed effects was performed (Table 4).
The F value (21.24) is quite high. The p value = 0.000114 is very small (less than 0.05), which indicates that there are statistically significant differences between the lots. So, it means that the quality among seed lots is different and confirms quality differences observed in Figure 17.

Table 4. ANOVA for fixed effects

Source of variation	**Sum of Squares**	**Mean Square**	**Num DF**	**Den DF**	**F value**	**Pr(>F)**
Lot	227.20	113.60	2	12	21.24	0.000114

A Least Squares (LS) Means with standard-errors and 95% confidence intervals (Table 5) was run.

Table 5. Least Squares (LS) Means Table with standard-errors and 95% confidence intervals

Lot	Estimate	Standard Error	Lower CI	Upper CI
Lot 1	94.25	1.51	90.91	97.59
Lot 2	93.21	1.51	89.87	96.56
Lot 3	86.11	1.51	82.77	89.45

The differences among LS Means (Table 6) showed that Lot 3 behaves differently from Lots 1 and 2. Also, Lots 1 and 2 are like each other. This indicates that Lot 3 has a different quality.

Table 6. The difference of LS Means Table with p-values and standard-errors.

Comparison	Estimate	Standard Error	p-value
Lot 1 - Lot 2	1.036	1.36	0.461119
Lot 1 - Lot 3	8.143	1.36	0.000064
Lot 2 - Lot 3	7.107	1.36	0.000213

The LS Means are displayed in the bar plot (Figure 19) with error bars added corresponding to their standard errors. It shows that:

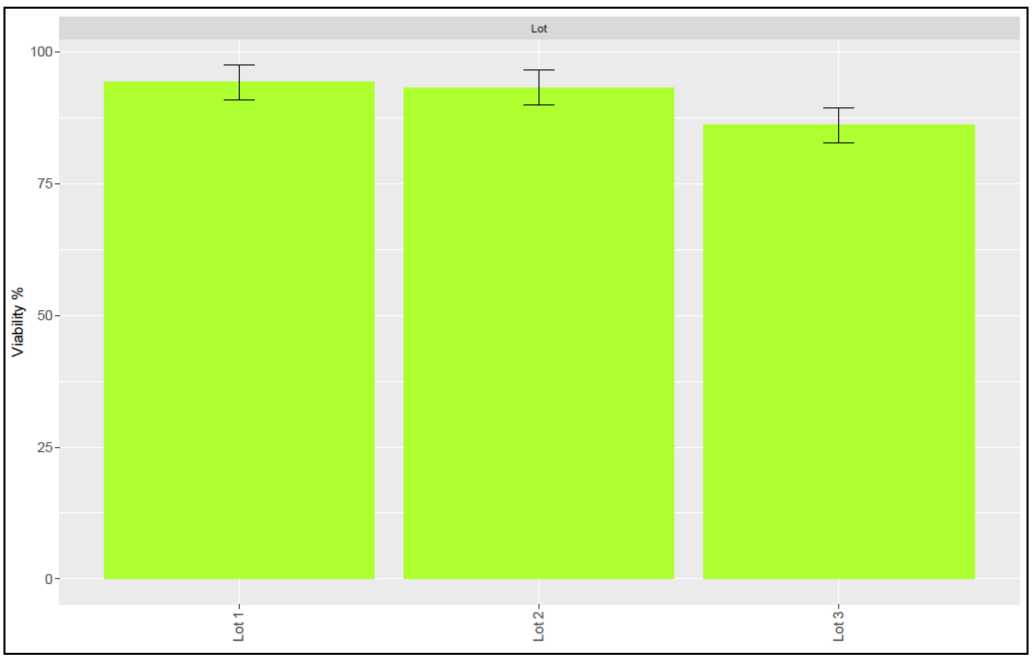

Figure 19. Bar plot for LS Means with error bars (standard errors).

Consistency between laboratories: Figure 19 shows the average from the 7 laboratories. The differences reflect real variability between lots, not between laboratories. The confidence intervals are narrow; it means the laboratories were consistent in their measurements.

Conclusion

After statistical analysis of the results, it was observed that the method meets the ISTA performance criteria since:

- The repeatability dispersion factor is well below 1.
- The reproducibility dispersion factor is below the reference value.

The viability test method proposed to be included in ISTA Rules for *Glycine max* seeds is as follows:

Pre-treatment: Soak the seeds 18 hours between moist rolled filter paper / paper towels at 20 °C ± 2 °C within sealed plastic bags to avoid evaporation.

Preparation before staining: Leave the seed intact.

Staining solution (%): 1.

Optimum staining time (h): 6 h.

Preparation for evaluation: Remove the seed coat to expose the embryo and cut down through the middle of the cotyledons and the hypocotyl-radicle axis.

Evaluation (maximum area of unstained, flaccid and/or necrotic tissue permitted): ⅓ radicle-hypocotyl axis measured from radicle tip, ½ of distal end of cotyledons if superficial, and damaged areas are not near the points of attachment to the seedling axis, ⅓ of distal end of cotyledons if pervading.

Remarks: If the viability of hard seeds is to be determined, the seed coat can be incised at distal end of cotyledons and soaked in water (4 hours).

Acknowledgements

We offer our thanks to all the laboratories that participated in this validation study, to the reviewers from ISTA Technical Committees and to Jean-Louis Laffont of the Statistics Committee that gave support on how to interpret the outcome of the results study.

References

- ISTA Working sheets on Tetrazolium Testing, Vol. I.
- Chapter 6, ISTA Rules, 2025.
- Miles S.R. (1963) Handbook of Tolerances and Measures of Precision for Seed Testing. *Proceedings of the International Seed Testing Association* 28/3.
- TEZ Committee Report, 1998-2001.

3. Validation of a new method for a "Microsatellite marker analysis for barley variety verification"

a. Authors

Verena Peterseil – Austrian Agency for Health and Food Safety, Vienna, Austria
Doris Kaiser – Austrian Agency for Health and Food Safety, Vienna, Austria
Marie-Claude Gagnon – Canadian Food Inspection Agency, Ottawa, Canada
Ana Laura Vicario - Instituto Nacional de Semillas INASE, Buenos Aires, Argentina

b. Summary

A new DNA-based method for barley variety verification by means of microsatellites markers was validated. To achieve this, two comparative tests (CTs) were organized that involved the participation of laboratories from several countries from around the world over a period of three years. The objectives of the CTs were to determine if it was possible to obtain comparable results among laboratories, as well to test the reproducibility of the markers and the repeatability of the method. Statistical analysis done by STACOM concluded that there was enough evidence for validating the method. As such, the Variety Committee is presenting this validation report for considering the inclusion of the DNA-based method for barley variety verification by means of microsatellites markers in Chapter 8 of the ISTA Rules.

Introduction

Traditionally, ISTA's standardized procedures for the determination of variety verification have been based on the examination of seeds, seedlings or plants in a laboratory, glasshouse, growth chamber or field plot, to assess morphology (grow-out tests), specific substances (biochemical methods) or protein characteristics (protein-based methods). Chapter 8 of the ISTA Rules is dedicated to species and variety testing. Conventional methods for cereals, Fabaceae and Poaceae are included in section 8.8 of the document, while section 8.9 relates to protein-based methods available for several crops including barley.

DNA-based approaches are very useful tools for variety verification and for assessment of purity. In comparison to traditional variety verification methods, DNA-based techniques may reveal more polymorphism thus allowing greater resolution among varieties. DNA-based techniques are also independent of environmental conditions or developmental stages. In 2017, DNA-based methods were included for the first time in Chapter 8 of the ISTA Rules. The first method to be included in this chapter was a microsatellite DNA-based test for wheat. Later, microsatellite DNA-based methods for maize, peas and oat were also included. DNA-based methods that were included in Chapter 8 of the ISTA Rules follow a semi-performance-based approach. This means that a core set of markers are prescribed for each of the crops, and that the other parts of the analyses can be carried out using any suitable procedure so long as the procedures have been validated as fit for purpose and that the end results meet acceptable standards as set by ISTA.

Now we propose the inclusion of a new DNA-based method for barley variety verification by means of microsatellites markers. To initiate this process, two comparative tests (CTs) for barley were organized by Verena Peterseil, with the participation of laboratories from several countries from around the world over a period of three years. The objectives of the CTs carried out are summarized below:

- The aim of CT1 was to compare results among participant laboratories and evaluate the possibility of obtaining the same SSR profiles and same allele sizes using different reagents, equipment and working protocols. Varieties and SSR markers were the same for all participant laboratories.

The expected result of CT1 was to obtain comparable results among laboratories.

- The aim of CT2 was also to compare results among participant laboratories and evaluate the possibility of obtaining the same SSR profile and same allele sizes when using different reagents, equipment and working protocols. Varieties and SSR markers were the same for all participant laboratories.

However, during CT2, the applicability of the method was evaluated by extending the range of varieties tested compared to CT1. In addition, reproducibility of the markers was tested by comparing the results of different laboratories with those of CT1. Repeatability was tested by using variety Semper which was also used in the first CT. Two laboratories participated in both CTs.

c. Materials and methods

i. *Samples:*

For CT1, 8 varieties (authorized in Austria) were analysed using 10 SSR markers obtained from Perry et al. (2013) and 4 SSR markers developed by AGES in-house (2010). Each participant received 6 individual crushed seeds per variety and two subsamples of a pool of 30-40 seeds. Samples were provided in sealed tubes labelled with the name or code of the variety.

For CT2, 9 varieties were analysed (4 from Iran, 2 from Estonia, 2 from Lithuania and 1 from Austria) using the same 14 SSR markers tested in CT1. The markers were tested as three different marker systems/panels, one had four markers, and the other two each had five markers. Each participant received 10 tubes with subsamples and two tubes of pools of 40 crushed seeds per variety. Samples were provided in sealed tubes labelled with the name or code of the variety. Duplicates were provided to all participants as a backup. However, if any participating laboratory ran out of a sample, they were able to contact CT leader to request more material.

Additional documents provided to participating laboratories:

- List of the varieties and the marker sequences to be used in both CTs and additional technical information: see files PCR protocol – barley.xlsx, List of SSR – barley.xlsx
- Document for reporting the results: see file CT results – barley.xls

ii. *Equipment, chemicals and procedure:*

Inclusion of DNA-based methods into the Rules is semi-performance based. Laboratories were provided with guidelines for running the SSR prescribed, but finally the specific procedure was up to the participating laboratories.

d. *Evaluation and reporting of results:*

Results were reported in an Excel sheet (CT results – barley.xls) indicating laboratory number, variety name, SSR name, and allele sizes.

The data analysis from CT1 aimed to evaluate if the marker panel was reproducible among laboratories and thus suitable for being kept for CT2 and eventually for the Rules proposal. This evaluation was carried out by the crop leader and consisted of verifying if markers gave the same allele's pattern across laboratories (even if different equipment and reagents were used). The evaluation of allelic profiles gave comparable results among laboratories; thus, the selected marker panel was deemed appropriate for CT2.

For CT2, the group leader compiled the results and prepared an Excel file with allele sizes and binary data. Binary data was sent to STACOM chair for their analysis.
For data analysis report prepared by STACOM chair, see file Var Com Barley CT2 – Stat analysis – 010522.docx.

e. *Participating Laboratories:*

i.

ii. CT1 Participating Laboratories

- Marie-Claude Gagnon; Canadian Food Inspection Agency (Canada)
- Heather Owen; SASA (United Kingdom)
- Anne Bernole; GEVES (France)
- Chiara Delogu; CREA (Italy)
- Doris Kaiser/Verena Peterseil; AGES (Austria)
- Jeffrey Prischmann; North Dakota State Seed Department (USA)

Five of the six laboratories that participated in CT1 sent a data package.

iii. CT2 Participating Laboratories

- Tertia Erasmus; SciCorp Laboratories (South Africa)
- Nicole Calliou; SGS BioVision (Canada)
- Aidin Hamidi; SPCRI (Iran)
- Doris Kaiser; AGES (Austria)
- Stephanie Guillet; Eurofins Biologie Moleculaire (France)
- Marie-Claude Gagnon; CFIA (Canada)
- Brigitte Roth; LTZ Augustenberg (Germany)
- Anne Bernole; GEVES (France)

All laboratories that participated in CT2 sent a data package.

f. Statistical analysis

Overall percentage agreements (p_a) and Cohen's kappas have been computed for all the possible laboratory pairs, considering as units either the marker alleles or the varieties. The computations have been performed with the R irr package (Gamer et al., 2012), which includes functions for computing various coefficients of reliability of agreement.

Within laboratory agreement has been assessed through *accordance* as described in Laffont (2021). The average *accordance* across varieties is high in all the laboratories (above 95%) except in laboratory G (92.3% when considering only individual seeds). Agreement of the marker results across laboratories has been assessed with Fleiss' kappa κ on all the varieties except varieties 3 and 4. The agreement is perfect ($\kappa = 1$) for all the varieties according to Landis and Koch (1977) table for the interpretation of κ.

The conclusion of the statistical analysis is that given these results, there is enough evidence for validating the method. For the statistical report details see: Var Com barley CT2 - Stat analysis – 010522.

g. Final comments and conclusions

After running two comparative tests for barley varietal identification using a panel of 14 SSR markers and with the participation of 10 laboratories from around the world, the statistical analysis done by STACOM concluded that there was enough evidence for validating the method.

Given the work carried out and the STACOM conclusion, the Variety Committee presents this validation report for considering the inclusion of the barley SSR marker panel in Rules Chapter 8.

i. References

- Perry et al. (2013): Simple sequence repeat-based identification of Canadian malting barley varieties.
- AGES (Peterseil, Kaiser): not published, but available on request.
- ISTA International Rules for Seed Testing.
- Gamer et al. (2012) Package 'IRR'. Various Coefficients of Interrater Reliability and Agreement.
- *Laffont, J-L. (2021). Within laboratory agreement for marker data: proposal. ISTA Statistics Committee report.*
- *Landis, J.R. and G.G. Koch (1977). The measurement of observer agreement for categorical data.*

ii.

iii. Reference documents

- CT1 protocol – barley
- CT2 protocol – barley
- PCR protocol – barley
- List of SSR - barley
- CT1 - barley – group result summary final
- CT2 - barley group result summary final
- CT2 - barley - group result binary data
- Var Com barley CT2 - Stat analysis – 010522

4. Method validation report for application of the radicle emergence test to *Allium cepa*.

Hulya Ilbi[1], G. V. Jagadish[2] and Alison A Powell[3]
[1] Department of Horticulture, Ege University, Bornova, Izmir, Turkiye
[2] IndoAmerican Hybrid Seeds, Bengaluru, India
[3] Aberdeen University, Aberdeen, Scotland.

Summary
The radicle emergence (RE) test was carried out on seed lots of *Allium cepa* by five laboratories from India and six laboratories within Europe. Problems with seed supply resulted in the Indian laboratories testing seven seed lots, while the European laboratories tested only three of the same lots. The test was carried out at 20ºC with RE assessed as a radicle ≥2mm after 72 hours. Data was analysed for the seven lots tested by five Indian laboratories, and for the three lots tested by both Indian and European laboratories. Low RE results compared to all other laboratories resulted in one laboratory from Europe being omitted from the analysis. The Indian laboratories consistently identified the same lots as having high RE (high vigour) and low RE (low vigour) and both the repeatability and reproducibility data were acceptable. Comparison of the RE for three seed lots tested by ten laboratories revealed similar RE values to those obtained by the Indian laboratories alone and both the repeatability and reproducibility data were acceptable. These analyses support the inclusion of onion as a species to which the radicle emergence test can be applied.

Introduction
The radicle emergence (RE) test is an ISTA vigour test currently validated for five crop species (*Zea mays*, *Brassica napus*, *Raphanus sativus, Triticum aestivum* subsp. *aestivum* and *Glycine max*). Many papers have reported correlations between an RE count and vigour (Powell, 2022) including two *Allium* species, *Allium cepa* and *Allium porrum*. RE counts of *A. cepa* predicted both seed storage potential (Demir *et al*., 2022) and field emergence (Ilbi, unpublished data, Appendix 1), while counts for *A. porrum* predicted early and final emergence and seedling fresh and dry weight (Ermis *et al*., 2015). The aim of this comparative test was to determine if RE counts of *A. cepa* are repeatable and reproducible for potential inclusion of this species in the ISTA Rules as one to which the RE test can be applied.

Materials and Methods
Seed Material
The aim of this comparative test was initially to have laboratories in India and Europe test seed lots that originated from seed companies in both India and Europe. Seed lots of commercially available onion seed were obtained from Nunhems Seeds and IndoAmerican Hybrid Seeds. Four seed lots were initially obtained from Nunhems and sent to the test organisers in Turkey (Dr Hulya Ilbi) and India (Dr G. V. Jagadish). However, tests of germination on receipt revealed that the germination of one lot had fallen to below commercially acceptable standards and was therefore not used. IndoAmerican Hybrid seeds provided a further four lots which were available in India and were also sent to Turkey to make seven lots available for use. Unfortunately, these seed lots were held by the Turkish customs and could not be released without substantial payment and phytosanaitary analysis in the Quarantine Department of the Turkish Ministry of Agriculture. As a result the comparative test was completed using three lots from Nunhems in laboratories in Europe and seven lots in Indian laboratories (three lots in common with Europe, four lots supplied by IndoAmerican Hybrid Seeds).

Participating laboratories
Coded samples were sent to the following laboratories (tables 1 and 2)
Table 1. Laboratories in India who tested seven seed lots of onion in the RE comparative test viz. three seed lots from Nunhems and four seed lots from IndoAmerican Hybrid Seeds.

Laboratory	Contact person
Sakata Seeds, Bengaluru, India	Ms Sunitha
Namdhari Seed, Bengaluru, India	Dr.Prashanth
East West Seeds, Hyderabad, India	Ms.Mohini
IndoAmerican Hybrid Seeds, Bengaluru, India	Ms Veena
Nunhems Seeds, Hyderabad, India	Ms.Muktha Pathre

Table 2. Laboratories in Europe who tested three seed lots of onion from Nunhems in the RE comparative test.

Laboratory	Contact person
Department of Horticulture, Ankara University, Turkey	Dr Ibrahim Demir
Official Seed Testing Station for Scotland, Edinburgh, UK	Gillian Musgrove
Official Seed Testing Station for England and Wales, Cambridge, UK	Gillian Durrant
Finnish Food Authority, Finland	Jaana Laurila
Department of Horticulture, Ege University, Turkey	Dr Hulya Ilbi
LaRAS, University of Bologna, Italy	Dr Enrico Noli
SNES, GEVES, France	Valerie Blouin

Testing procedure

Participants placed seed on receipt in a moisture proof bag at <10ºC until use and completed the test within 2 weeks of receiving the seed.

They were advised to consult the general guidelines for completion of the RE test as found in the ISTA Rules 15.8.4.

To complete the test for onion:

1. Each of four replicates of 50 seeds for each seed lot were placed on two germination papers (Whatman No: 5), that were held in 90 mm-diameter Petri dishes and had previously been moistened with 4 ml distilled water.

   In Europe:

   One laboratory in Europe used an alternative filter paper and care was taken to ensure that all the water was absorbed into the germination paper, and there was no free water on the surface.

   Laboratories used a 12-hour light/12-hour dark regime, and the test was set up at the beginning of the light period of that regime. In one laboratory, where an automated 12-hour/12-hour light regime was not possible, an 8-hour / 16-hour regime was used.

   In India:

   Tests were all on top of paper using an 8-hour light/16-hour dark regime. The tests were set up during the light period.

2. The Petri dishes were then placed into plastic bags in order to prevent water loss during the test and held at 20 ±1ºC. Stacking the Petri dishes on top of each other was avoided as this can generate anaerobic conditions in the lower dishes. If stacking could not be avoided, it was limited to two dishes. Care was taken to ensure that the temperature was accurate, and the Petri dishes were not crowded in the incubator.

3. Radicle emergence for each replicate was recorded after 72 hours ±15 minutes. The criterion of radicle emergence was ≥2 mm radicle protrusion. The data was recorded on the data sheets provided.

4. After 12 days, for each replicate, a final germination count of normal and abnormal seedlings was recorded (ISTA Rules, 2023), and dead seeds.

Information received from all laboratories confirmed that the test was completed following the guidelines. The only differences between laboratories were in light regime and, for one laboratory, germination paper, as noted above.

The data were analysed using R function ISTAgermMV() (2023) developed by ISTA Statistics Committee. Analysis was completed for:

1) the seven seed lots tested by the five Indian laboratories (i.e. three seed lots from Nunhems plus four lots from IndoAmerican Hybrid Seeds) and

2) the three seed lots from Nunhems tested by both the European and Indian laboratories (total 11 laboratories)

Results

1.Analysis of data for seven seed lots tested by five laboratories.

The mean laboratory germination for each lot in all laboratories was above 85% for all lots except lot 4 which had a mean laboratory germination of 51% (data not presented).

Radicle emergence counts after 72 hours at 20ºC revealed differences between seed lots in each laboratory (table 3). Comparing the overall means for seven seed lots in each laboratory, laboratory 3 had the lowest results for RE (table 3, figure 1), suggesting that radicles equal to or greater than 2mm may not have been clearly identified.

Table 3. Mean RE values for seven seed lots tested in five laboratories. Each value is the mean of four replicates of 50 seeds.

Lab	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6	Lot 7	Lab mean
1	80	56	64	50	66	87	54	**65**
2	88	93	82	38	78	84	84	**78**
3	58	68	62	30	30	61	30	**48**
4	89	86	90	74	82	90	82	**85**
5	86	83	79	48	58	79	56	**70**
Lot mean	**80**	**77**	**75**	**48**	**63**	**80**	**61**	**69**

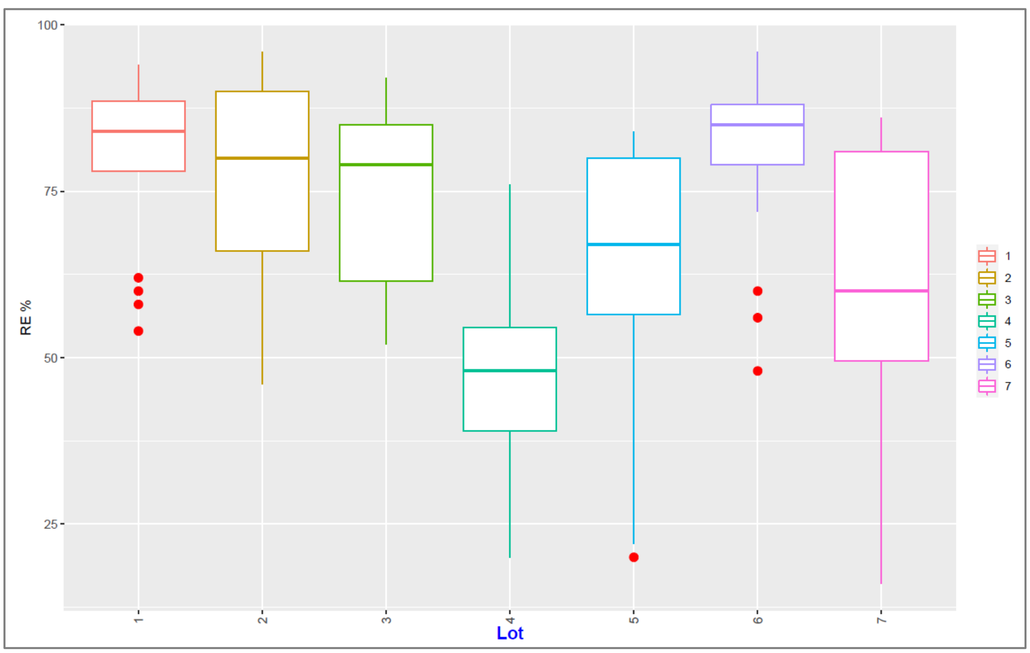

Figure 1. Radicle emergence after 72 hours at 20ºC represented as boxplots per laboratory for seven onion seed lots.

The seed lot mean from all laboratories identified two seed lots (1 and 6) with the highest RE value and hence the highest vigour, with lot 4 having the lowest vigour (table 3, figure 1). The ranking of seed lots was similar within each of the five laboratories (figure 2), clearly showing the highest vigour (highest RE) for lots 1 and 6 with lower vigour in lots 2 and lot 3 > lots 5 and 7. The low RE that suggested the lowest vigour for lot 4 was however, largely attributable to its low final normal germination. The greater variability in the RE data for lots 7 and 4 (figure 2) is what would be expected for seed lots with low vigour.

<u>Tolerances</u>: Considering 4 replicates of 50 seeds, five results out of 35 were out of tolerance four of which were from laboratory 3 (lots 3, 4, 5 and 6) and one from laboratory 2 (lot 4) (Appendix 2)

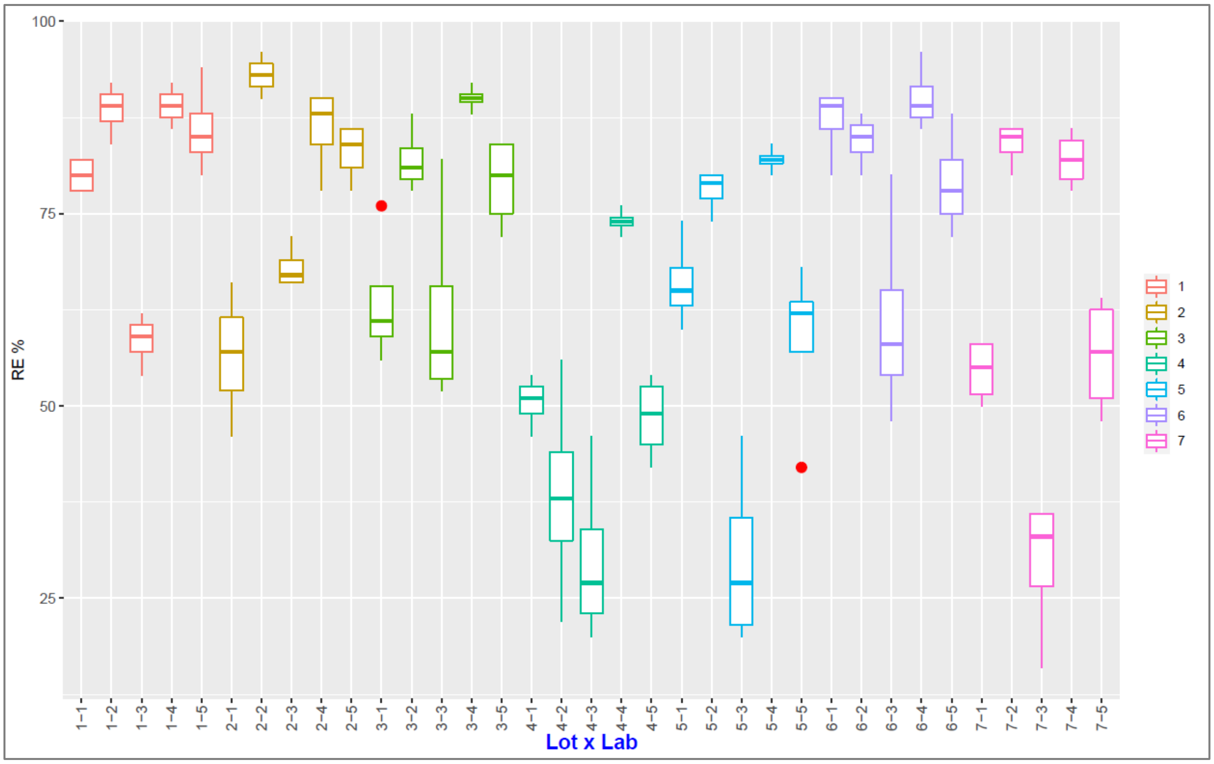

Figure 2: Ranking of the seven seed lots of onion (4 x 50 seeds).

Mixed model for comparing test mean seed lot values.

Results from fitting the mixed model are displayed in table 4 indicating a significant effect of seed lots on radicle emergence results.

Table 4: Results from fitting the mixed model

Source of variation	Sum of Squares	Mean Square	Num DF	Den DF	F value	Pr (>F)
Lot	2478.16	413.03	6	24	8.58	0.000049

Repeatability and reproducibility

The following linear mixed model was fitted:

$$y_{ijk} = \mu + \alpha_i + L_j + (\alpha L)_{ij} + e_{ijk} \qquad (1)$$

in which:

. y_{ijk} is the RE % in Rep *k* of Lot *i* and Lab *j*.

. μ is the intercept.

. α_i is the fixed effect of Lot *i*.

. L_j is the random effect of Lab *j*. L_j ~ i.i.d. $N(0, \sigma^2_{Lab})$.

. $(\alpha L)_{ij}$ is the random interaction effect between Lot *i* and Lab *j*.

$(\alpha L)_{ij}$ ~ i.i.d. $N(0, \sigma^2_{Lot\times Lab})$.

. e_{ijk} are the residuals. e_{ijk} ~ i.i.d. $N(0, \sigma^2)$.

Repeatability standard-deviation is given by $S_r = \sqrt{\hat{\sigma}^2}$ and reproducibility standard-deviation by $S_R = \sqrt{\hat{\sigma}^2_{Lab} + \hat{\sigma}^2_{Lot\times Lab} + \hat{\sigma}^2/K}$ where K is the number of reps.

The repeatability dispersion factor is calculated as $f_r = \sqrt{\frac{n\hat{\sigma}^2}{\bar{p}_{...}(100-\bar{p}_{...})}}$ where $\bar{p}_{...}$ is the overall average percentage of the trait analysed and n is the number of seeds per replicate. If $f_r > 1$ there is overdispersion because the data have larger variance than expected under the assumption of a binomial distribution.

The reproducibility dispersion factor is calculated as $f_R = \sqrt{\frac{KnS_R^2}{\bar{p}_{...}(100-\bar{p}_{...})}}$ where S_R^2 is estimated from model (1) after excluding laboratory results for which their absolute deviation from the lot mean (computed from all the laboratories) divided by $\frac{\bar{p}_{...}(100-\bar{p}_{...})}{Kn}$ exceeds 4 (Miles, 1963).
Repeatability (f_r) and reproducibility (f_R) values are presented in tables 5 and 6. The value for f_r (1.06, table 5) is close to 1.0 and is therefore acceptable. The value of 1.04 for f_R, (table 6) is close to that theoretically derived using the over-dispersion factor between labs established by Miles (1963).

Table 5: Repeatability estimates for seven lots of *Allium cepa* using Linear mixed model.

$\bar{p}_{...}$	$\hat{\sigma}_{Lab}$	$\hat{\sigma}_{Lot\times Lab}$	S_r (Repeatability Standard deviation)	f_r	S_R (Reproducibility Standard deviation)
69	13.43	8.63	6.94	1.06	16.33

Table 6: Reproducibility estimates for seven lots of *Allium cepa* using Linear mixed model.

$\bar{p}_{...}$	**Excluded samples %**	f_R	f_{Miles}
72	40	2.09	1.78

2. Analysis of data for three seed lots tested by eleven laboratories.
Comparison of box plots revealed that the data from one laboratory (lab 4, figure 3) was markedly lower than for other labs. This did not appear to be related to the test conditions used and therefore suggests that there was a problem in evaluation of radicle emergence. Lab 4 was therefore excluded from further analysis. Seven laboratories (lab 5, labs 7 to 11) used a light regime of 8 hours light /16 hours dark compared to 12 hour/12 hours for all other laboratories. However the mean RE values of and 80% (labs 5, 7-11) and 75% (labs 1,2,3,6) suggest that there was no effect of the different light regimes. All laboratories had set the test up at the beginning of (Europe) or during (Indian labs) the light period.

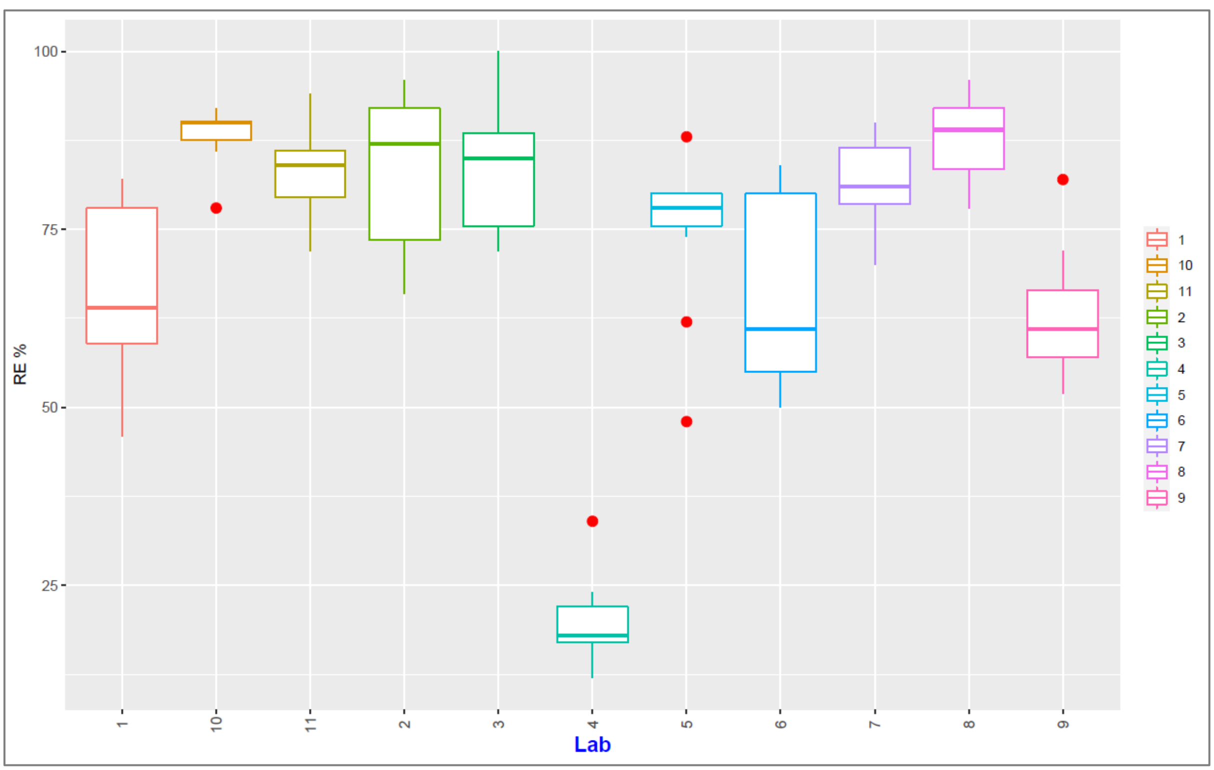

Figure 3 Box plots of mean RE of three seed lots tested in 11 laboratories

Radicle emergence counts after 72 hours at 20°C revealed small differences between in the mean data for the three seed lots (table 7, figure 4), as also seen when the means were compared to the data from the five labs in India (table 3). There were however differences in the lab means for the three lots (table 7, figure 5) with labs 1, 6 and 9 having lower overall means (table 7).
Tolerances: Only three tests out of 30 (10 labs x 3 lots) were out of tolerance, with outliers in laboratories 3 (lot 1), 5 (lot 2) and 9 (lot 3) (Appendix 3).

Table 7. Mean RE values for three seed lots tested in ten laboratories. Each value is the mean of four replicates of 50 seeds.

Lab	**Lot 1**	**Lot 2**	**Lot 3**	**Lab mean**
1	80	56	64	**67**
2	90	71	90	**84**
3	84	80	87	**84**
5	78	66	81	**75**
6	82	52	62	**65**
7	86	83	74	**81**
8	88	93	82	**88**
9	58	68	62	**63**
10	89	86	90	**88**
11	86	83	79	**83**
Lot mean	**82**	**74**	**77**	**78**

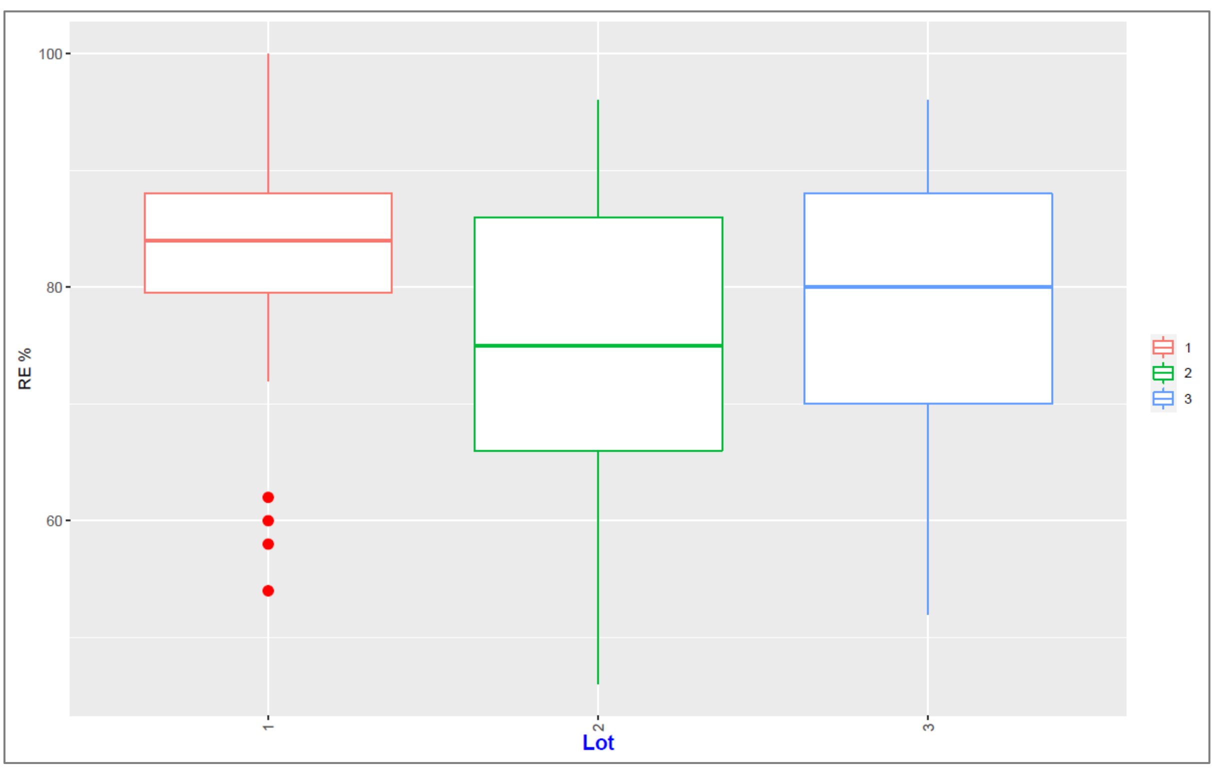

Figure 4. Radicle emergence after 72 hours at 20°C represented as boxplots per laboratory for three onion seed lots.

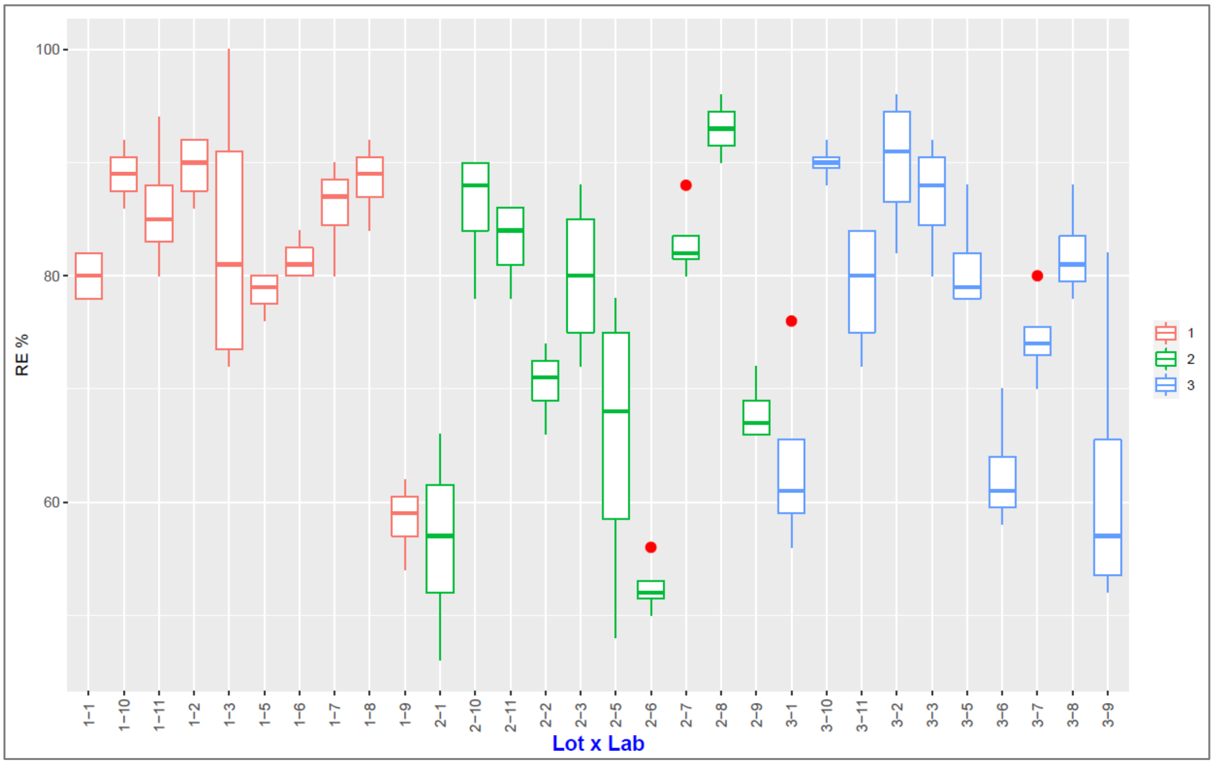

Figure 5: Ranking of the three seed lots of onion in ten laboratories.

<u>Repeatability and reproducibility</u>
The following linear mixed model was fitted:

$$y_{ijk} = \mu + \alpha_i + L_j + (\alpha L)_{ij} + e_{ijk} \qquad (1)$$

in which:
. y_{ijk} is the RE % in Rep *k* of Lot *i* and Lab *j*.
. μ is the intercept.
. α_i is the fixed effect of Lot *i*.
. L_j is the random effect of Lab *j*. L_j ~ i.i.d. $N(0, \sigma^2_{Lab})$.
. $(\alpha L)_{ij}$ is the random interaction effect between Lot *i* and Lab *j*.
$(\alpha L)_{ij}$ ~ i.i.d. $N(0, \sigma^2_{Lot\times Lab})$.
. e_{ijk} are the residuals. e_{ijk} ~ i.i.d. $N(0, \sigma^2)$.
Repeatability standard-deviation is given by $S_r = \sqrt{\hat{\sigma}^2}$ and reproducibility standard-deviation by $S_R = \sqrt{\hat{\sigma}^2_{Lab} + \hat{\sigma}^2_{Lot\times Lab} + \hat{\sigma}^2/K}$ where *K* is the number of reps.

The repeatability dispersion factor is calculated as $f_r = \sqrt{\frac{n\hat{\sigma}^2}{\bar{p}_{...}(100-\bar{p}_{...})}}$ where $\bar{p}_{...}$ is the overall average percentage of the trait analysed and *n* is the number of seeds per replicate. If f_r > 1 there is overdispersion because the data have larger variance than expected under the assumption of a binomial distribution.

The reproducibility dispersion factor is calculated as $f_R = \sqrt{\frac{KnS_R^2}{\bar{p}_{...}(100-\bar{p}_{...})}}$ where S_R^2 is estimated from model (1) after excluding laboratory results for which their absolute deviation from the lot mean (computed from all the laboratories) divided by $\frac{\bar{p}_{...}(100-\bar{p}_{...})}{Kn}$ exceeds 4 (Miles, 1963).
Repeatability (f_r) and reproducibility (f_R) values are presented in tables 8 and 9. The value for f_r (1.04 table 5) is close to 1.0 and is therefore acceptable. The value of 2.14 for f_R, (table 6) is close to that theoretically derived using the over-dispersion factor between labs established by Miles (1963).

Table 8 Repeatability estimates for three lots of *Allium cepa* using Linear mixed model.

$\bar{p}_{...}$	$\hat{\sigma}_{Lab}$	$\hat{\sigma}_{Lot\times Lab}$	S_r	f_r	S_R
78	8.50	6.78	6.15	1.04	11.30

Table 9 Reproducibility estimates for three lots of *Allium cepa* using Linear mixed model

$\bar{p}_{...}$	Excluded samples %	f_R	f_{Miles}
81	30	2.14	1.71

Conclusion

Seven seed lots tested within five laboratories in India were ranked similarly and achieved acceptable repeatability and reproducibility. Analysis of the data for three of these lots when tested in five Indian and six European laboratories again revealed acceptable repeatability and reproducibility. This provides support for the inclusion of onion as a species to which the radicle emergence test can be applied.

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Powell. A.A. (2022). Seed vigour in the 21st century. *Seed Science and Technology*, **50**, 1, **Suppl.**, 45-73 https://doi.org/10.15258/sst.2022.50.1.s.04

Acknowledgements

We are grateful to Jean-Louis Laffont for completing the statistical analysis and his advice on interpretation of the data. We thank the participants in the comparative test in both India and Europe, particularly for their patience as we encountered problem with the availability of seeds.

Appendix 1

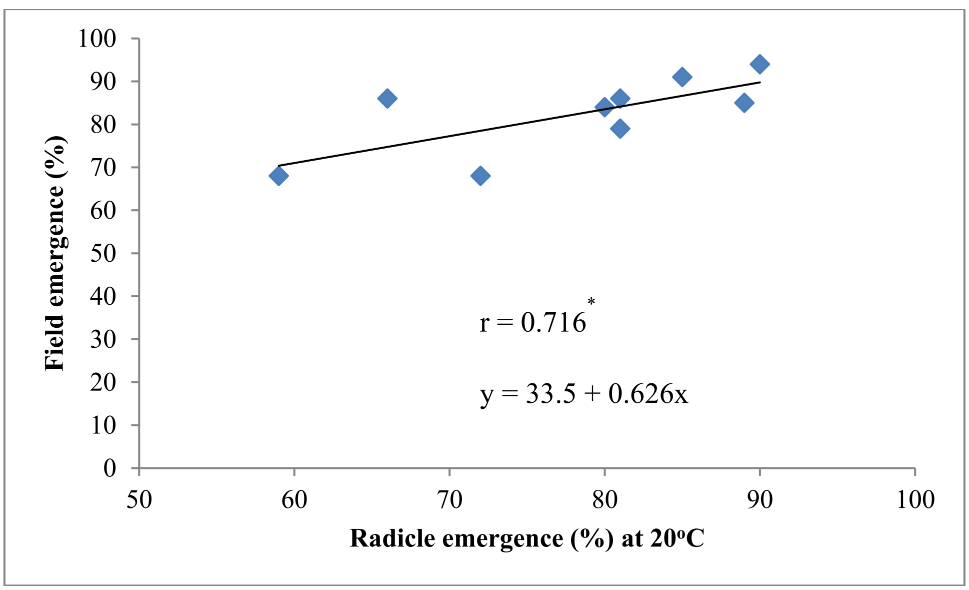

Figure 1 relationship between radicle emergence and field emergence in *Allium cepa* (unpublished data)

Appendix 2
Data checking for seven seed lots tested in five laboratories

Data checking has been performed according to ISTA rules by computing tolerances for germination test replicates.

Results:

Lot	Lab	Mean	# Reps	# seeds/rep	Range	Tol	Out of Tol
1	1	80	4	50	4	22	ok
1	2	88	4	50	8	18	ok
1	3	58	4	50	8	28	ok
1	4	89	4	50	6	18	ok
1	5	86	4	50	14	20	ok
2	1	56	4	50	20	28	ok
2	2	93	4	50	6	15	ok
2	3	68	4	50	6	26	ok
2	4	86	4	50	12	20	ok
2	5	83	4	50	8	21	ok
3	1	64	4	50	20	27	ok
3	2	82	4	50	10	22	ok
3	3	62	4	50	30	27	OUT
3	4	90	4	50	4	17	ok
3	5	79	4	50	12	23	ok
4	1	50	4	50	8	28	ok
4	2	38	4	50	34	27	OUT
4	3	30	4	50	26	25	OUT
4	4	74	4	50	4	25	ok
4	5	48	4	50	12	28	ok
5	1	66	4	50	14	26	ok
5	2	78	4	50	6	23	ok
5	3	30	4	50	26	25	OUT
5	4	82	4	50	4	22	ok
5	5	58	4	50	26	28	ok
6	1	87	4	50	10	19	ok
6	2	84	4	50	8	21	ok
6	3	61	4	50	32	27	OUT
6	4	90	4	50	10	17	ok
6	5	79	4	50	16	23	ok
7	1	54	4	50	8	28	ok
7	2	84	4	50	6	21	ok
7	3	30	4	50	20	25	ok
7	4	82	4	50	8	22	ok
7	5	56	4	50	16	28	ok

Five results out of 35 are out of tolerance

Appendix 3
Data checking for three seed lots tested in 10 laboratories

Data checking has been performed according to ISTA rules by computing tolerances for germination test replicates.

Lot	Lab	Mean	# Reps	# seeds/rep	Range	Tol	Out of Tol
1	1	80	4	50	4	22	ok
1	10	89	4	50	6	18	ok
1	11	86	4	50	14	20	ok
1	2	90	4	50	6	17	ok
1	3	84	4	50	28	21	OUT
1	5	78	4	50	4	23	ok
1	6	82	4	50	4	22	ok
1	7	86	4	50	10	20	ok
1	8	88	4	50	8	18	ok
1	9	58	4	50	8	28	ok
2	1	56	4	50	20	28	ok
2	10	86	4	50	12	20	ok
2	11	83	4	50	8	21	ok
2	2	70	4	50	8	26	ok
2	3	80	4	50	16	22	ok
2	5	66	4	50	30	26	OUT
2	6	52	4	50	6	28	ok
2	7	83	4	50	8	21	ok
2	8	93	4	50	6	15	ok
2	9	68	4	50	6	26	ok
3	1	64	4	50	20	27	ok
3	10	90	4	50	4	17	ok
3	11	79	4	50	12	23	ok
3	2	90	4	50	14	17	ok
3	3	87	4	50	12	19	ok
3	5	81	4	50	10	22	ok
3	6	62	4	50	12	27	ok
3	7	74	4	50	10	25	ok
3	8	82	4	50	10	22	ok
3	9	62	4	50	30	27	OUT

Three results out of 30 are out of tolerance:

5. Cold test development to identify differences in vigour of *Zea mays*.

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Summary

The cold test identified differences between six seed lots from two cultivars of maize (*Zea mays* L.) having a standard germination above 90%. The seed lots were tested for seven days at 10°C followed by five days at 25°C by five laboratories comparing two highly moistened substrates (paper or sand), one laboratory using only paper and one laboratory using only sand for a total of six cold tests per substrate. All laboratories consistently identified the same significant differences in seed vigour, and the results were repeatable within each laboratory and reproducible between laboratories. Sand used as a substrate resulted in higher correlations to field emergence in four locations, providing evidence in support of the introduction of a cold test method in ISTA Rules as a vigour test for maize.

Introduction

The cold test is widely used worldwide for maize, but many labs use in-house methods which may differ leading to differences in results between them. Some seed companies have asked the French Seed Testing Laboratory of GEVES to develop a common method they could use as a reference when they have to exchange results with overseas stakeholders, allowing results to be compared based on standardised methods. It does not stop laboratories using their in-house method when necessary.

Many factors cause variability in cold test results, one of which is sowing temperature which depends on the world region. Thus, standardisation towards one worldwide acceptable cold test appears difficult and the idea was abandoned by the VIG committee some years ago. Based on recommended methods from ISTA vigour testing handbook (ISTA, 1995), inert substrates were retained to increase reproducibility.

After reviewing scientific literature to analyse the main factors influencing cold test, three laboratories compared their results on 6 different samples comparing two substrates and several combinations of cold duration, moistening of the substrate, and growth temperature after cold treatment (appendix 1).

In 2023, seven laboratories compared their in-house method to a single combination of cold period and growth temperature using sand or paper which are the most substrates used worldwide for maize germination (ISTA, 2021). Some consistent ranking of seed lots has been obtained in controlled conditions (appendix 2), but no relationship was found with field emergence as no cold stressful conditions were present when sowing in spring 2023.

The present report concerns the international comparative test held in 2024 with 7 participants from four countries: Canada, Italy, The Netherlands, France which compared two cold test methods to determine whether a cold test could be proposed for introduction into the ISTA Rules as a vigour test for maize.

Material and Methods

Seed material

Six samples of *Zea mays* were provided among which three seed lots were from one variety and three from another variety. All samples were above 90% of germination when tested in 2023. Their germinations were tested by five laboratories during the comparative trials and their germination was still above 90% in 2024 (table 1).

Table 1: Details of six lots of Zea mays L.: mean of standard germination (SG %) measured in five laboratories

	M1	M2	M3	M4	M5	M6
Mean SG (%)	98	95	95	95	91	96
Standard deviation	0.3	1.2	1.1	1.6	3.9	2.1

Conditions for setting up the tests

5 laboratories compared cold tests in both BP and sand, and two laboratories used their familiar substrate: rolled paper for lab 1 and sand for lab 3, so that, in total, six laboratories could be compared for each method. Seeds were sown with high levels of water in each substrate.

- BP moistened at 292% of blotter weight (94% of WHC): 4 replicates of 50 seeds.
- Sand moistened at 14% of its weight: 4 replicates of 50 seeds.

The use of 200 seeds is current practice for vigour tests and in the cold test should be enough to rank seed lots according to their vigour. The germination temperature was 10°C for 7 days and 25°C for 5 days.

In addition, 5 participants performed a standard germination (SG) test according to Chapter 5 of the ISTA Rules. Note that 3 laboratories used BP and 2 sand for their SG test. It is representative of international spread of uses germination media if we compared to the PT21-1 (ISTA, 2021) where 52% of the participants used BP and 41% of the laboratories used sand for maize germination.

Table 2 : Mean standard germination results (± SD) according to the substrate

SEED LOT	BP1	BP2	BP3	SAND 1	SAND 2
M1	98 ± 0.0	97.5 ± 1.0	97.5 ± 2.5	97.5 ± 1.0	98.0 ± 1.6
M2	94.5 ± 3.4	93.5 ± 1.9	95.0 ± 3.5	96.0 ± 0.0	96.5 ± 3.0
M3	93.0 ± 1.2	95.5 ± 1.0	95.5 ± 1.9	94.0 ± 1.6	95.0 ± 3.8
M4	94.0 ± 2.3	94.5 ± 1.0	93.5 ± 1.9	94.5 ± 5.5	97.5 ± 1.0
M5	90.5 ± 3.4	88.0 ± 2.3	86.5 ± 4.1	92.0 ± 1.6	96.5 ± 1.0
M6	93.5 ± 3.8	98.0 ± 0.0	95.0 ± 2.6	96.0 ± 1.6	98.5 ± 1.0
OVERALL MEAN	**93.9 ± 2.4**	**94.5 ± 3.6**	**93.8 ± 3.8**	**95.0 ± 1.9**	**97.0 ± 1.3**

Seed lot M5 has the highest susceptibility to substrate with the largest range of standard germination from 86.5% in BP to 96.5% in sand.

Field trials

In three locations, contrasted in soil and climatic conditions (Arras, St Mathurin s/ Loire, Chappes, figure 1), 3 seed lots from the same variety were sown at an early sowing in experimental plots using 4 blocks of 100 seeds. Due to shipment delay, the three other seed lots could not be tested in these sub-optimal field conditions. A first count of field emergence was done 8-10 days after sowing and then regularly every 3 days until May for these trials. Due to the wet spring, the last field in Southwest of France (Haut-Mauco) was sown on the 19 of April with all seed lots (figure1).

Figure 9: Map of the four locations for field emergence trials in France, with early sowings in spring 2024

Statistical analyses

All replicates were computed for their tolerance in germination test using 4 x 50 seeds in ISTA website tolerance tool. Possible outliers were assessed using side by side boxplots. The performance of the method was assessed through the estimation of repeatability and reproducibility parameters in the context of binomial data. A Linear Mixed Model (LMM) was fitted using a R function developed by the ISTA Statistics Committee, statistical analyses were carried out using 4 replicates of 50 and could reveal means out of tolerance.

As seedling evaluation could need training with an unusual substrate, a second comparison has been done taken in account the routine substrate used by each laboratory and pairs comparison involved this time four cold tests carried out in sand and three in paper.

The meaningfulness of the cold test has also been evaluated by correlation analysis between cold test, germination test and field emergence results.

Results

Establishment of a relationship between field emergence and CT

The spread of field establishment depended on the location, being over two weeks in the Southwest (Haut-Mauco), three weeks (Chappes and Saint Mathurin sur Loire) or 45 days (Arras) (figure 2). There was ten days delay for first emergence between the North (Arras) and the Southwest (Haut-Mauco). Seed lot M3 had the lowest emergence in three conditions, M5 was the worst one in the warmest field and M1 was the fastest to emerge everywhere.

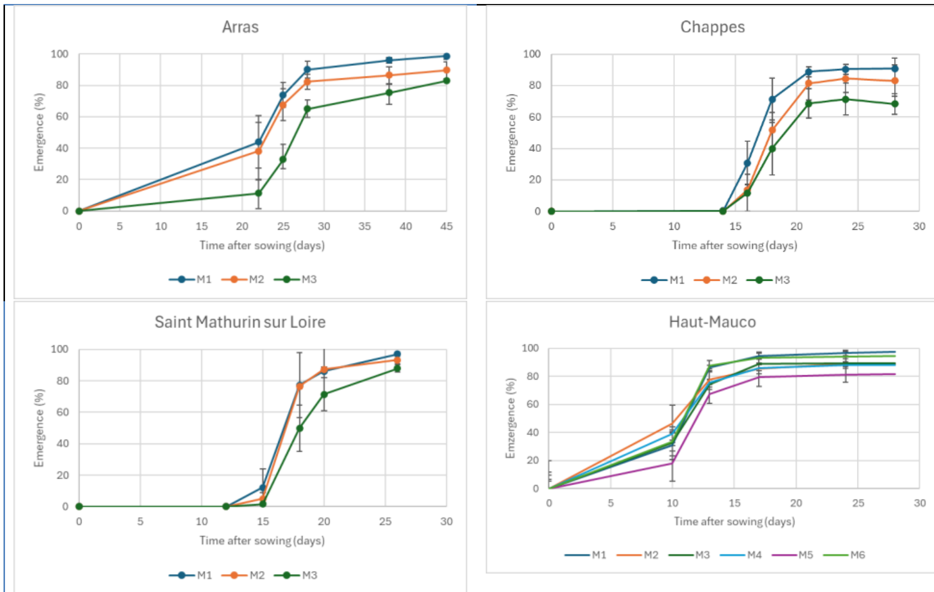

Figure 10: Field emergence time progress curves (%) of the samples M1 to M3 of Zea mays in three experimental plots (4x100 seeds) in France and lots M1 to M6 in one field. Lots M4 to M6 arrived too late to be sown in the first three plots. From the left top to the bottom right: Arras in the North of France, Chappes in the Centre, Saint Mathurin sur Loire in the West and Haut-Mauco in the Southwest.

Means of daily temperature were recorded in the four locations. Sowing temperature varied between 7.8°C in Chappes (earliest date of sowing) and 10.1°C in Haut-Mauco sown 25 days later (figure 3). The highest variability of temperature was recorded in Chappes and the monthly temperature corresponding to the final field emergence was 11.1°C for Arras, 11.5°C for Chappes, 12.3°C for Saint Mathurin sur Loire and 13.6°C for Haut-Mauco.

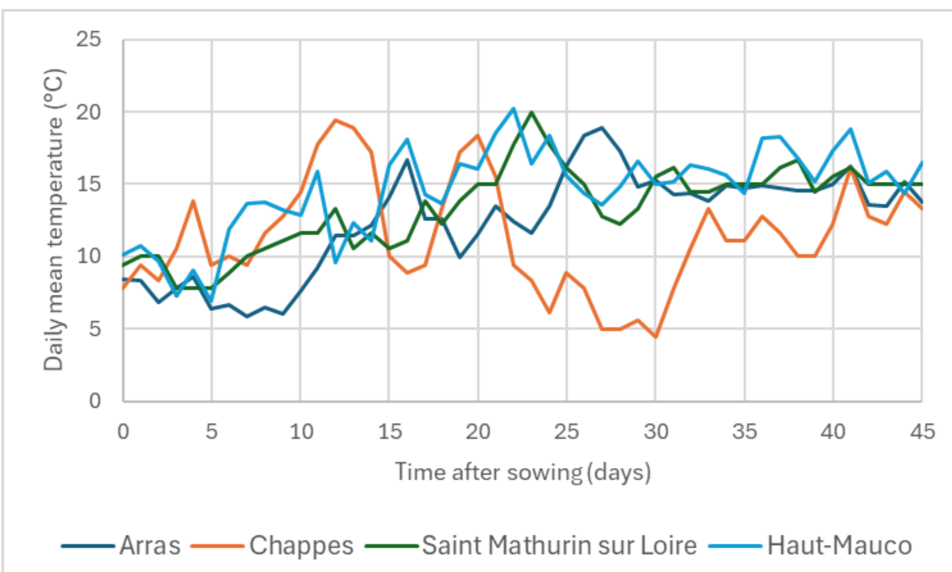

Figure 11 : Thermal profile of the four fields during 45 days after an early sowing (see dates on figure 1)

There was a significant relationship between the cold test and field emergence (r ≥ 0.95) for the 3 seed lots sown early (table 3), especially in the Northern field (Arras with r=1 in sand). However, there was also a positive correlation (r = 0.96) between SG and the Southwest field emergence (Haut-Mauco) where the six seed lots used for the comparative test were considered (figure 4).

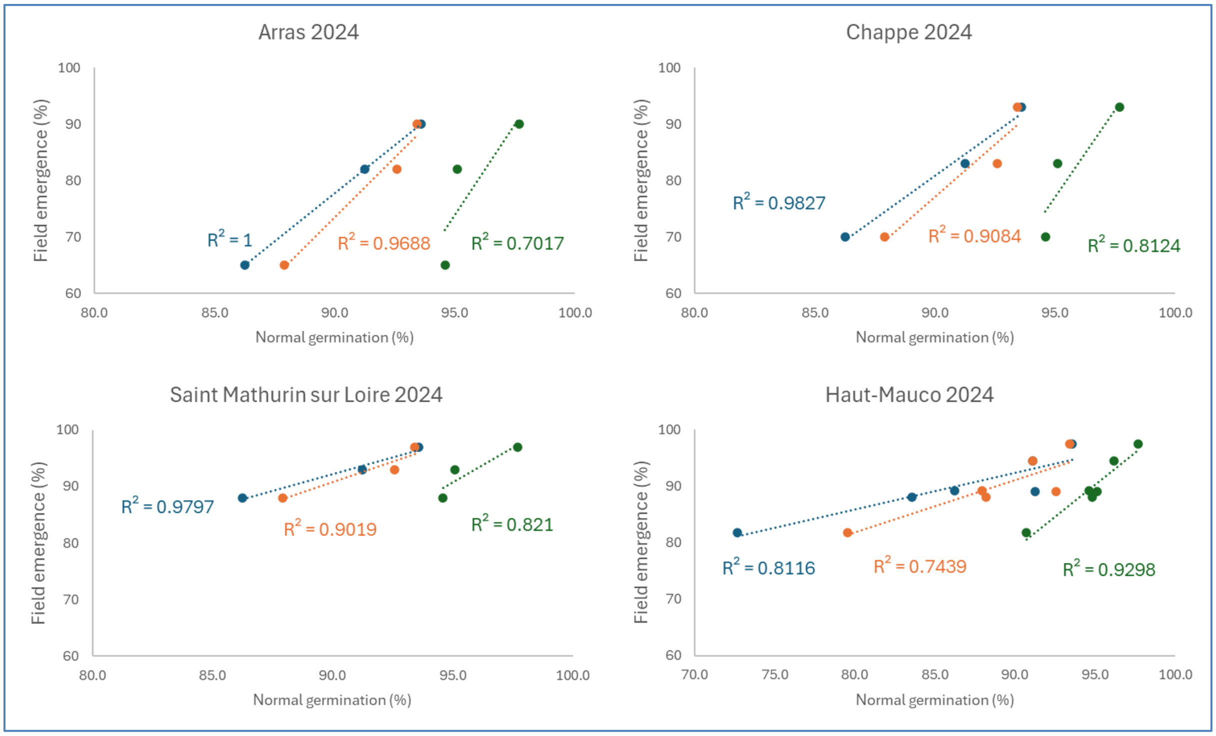

Figure 12: Relationships between field emergence in four locations and mean CT results (blue dots using sand and orange for BP) or SG (green dots) in controlled conditions.

Table 3: Coefficients of correlation between cold test in sand, cold test between paper or standard germination test and final field emergence in the four locations. Significant relationships are indicated with * p>0.05, ** p>0.01.

Field location	Cold test in sand	Cold test BP	Standard germination test
Arras	1.00**	0.98	0.84
Chappes	0.99*	0.95	0.90
St Mathurin sur Loire	0.99*	0.95	0.91
Haut-Mauco	0.90	0.86	0.96

It was interesting to note the higher correlation of field emergence with cold test results obtained in sand and the different levels of field emergence for seed lots with very similar germination levels. For example, lots M1 and M3 with above 90% normal germination gave field emergences of 90% and 60% respectively in the North of France (Arras) and also had 9% of difference in favourable field (Haut-Mauco; figure 5). In this maize traditional crop area (Haut-Mauco), the lowest emergence was 82% for lot M5; M1 and M6 having FE close to their standard germination (figure 5).

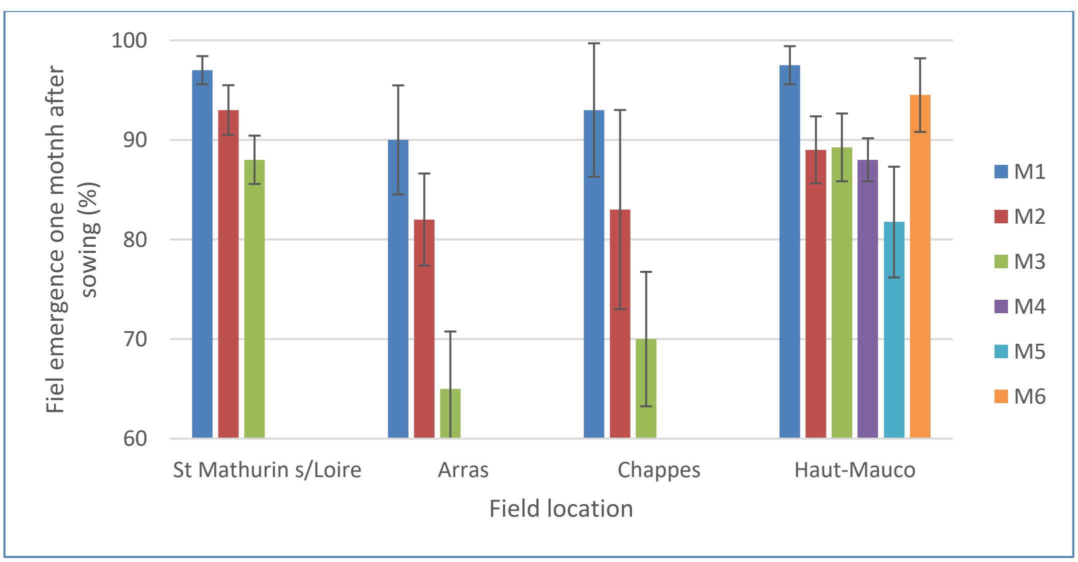

Figure 13: Final mean field emergence (%) of the samples of Zea mays in the four experimental plots (4x100 seeds). Lots M4 to M6 arrived too late to be sown in the three first places.

Nevertheless, seed lots ranking was very similar between laboratory and field (table 4) with M1 and M6 having the highest vigour and M5, the lowest vigour.

Table 4: Mean results (± SD) for final field emergence (28 days after sowing) in four locations and mean cold test results (± SD) obtained in six laboratories with two different substrates.

	M1	M2	M3	M4	M5	M6
Lab cold test (%)	93.5 ± 4.2	91.9 ± 3.8	87.1 ± 7.0	85.9 ± 5.5	76.0 ± 8.5	91.1 ± 4.7
Haut-Mauco (%)	98 ± 1.9	89 ± 3.4	89 ± 3.4	88 ± 2.2	82 ± 5.6	95 ± 3.7
Arras (%)	90 ± 5.5	82 ± 4.6	65 ± 5.8	nd	nd	nd
Chappes (%)	93 ± 6.7	83 ± 10	70 ± 6.8	nd	nd	nd
St Mathurin (%)	97 ± 1.4	93 ± 2.5	88 ± 2.4	nd	nd	nd

Comparative cold tests

Normal germination counts after cold testing revealed differences between seed lots in each laboratory whatever the substrate (Figure 6). Both substrates were significantly correlated (r=0.99).

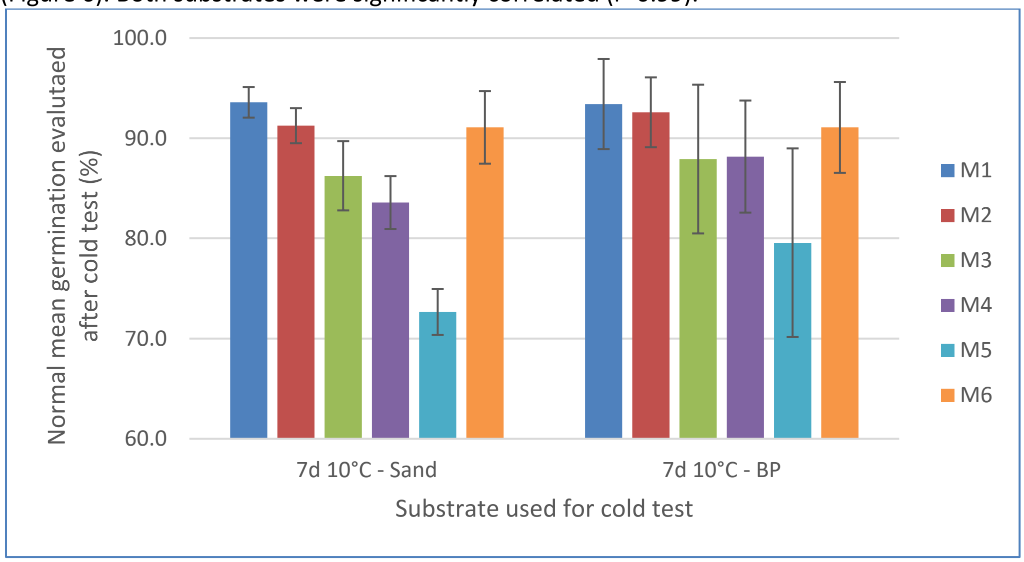

Figure 6: Normal mean germination after cold test (%) of the 6 samples of Zea mays in six different laboratories for each substrate (4x50 seeds).

The two substrates gave similar results without any significant difference (table 5). The total germination after 7d at 10°C and 5d at 25°C was over 90% for all samples and in most laboratories so that data are not reported here. Results obtained by each laboratory are presented on figure 7.

Table 5: Cold germination mean results (%) assessed after 7d at 10°C and 5d at 25°C in six laboratories for 6 seed lots of maize according to the substrate; each mean is derived from 4 replicates of 50 seeds per laboratory. For each method, means with different letters in the same raw are significantly different according to Tukey test (α=0.05).

	M1	M2	M3	M4	M5	M6
7d 10°C - Sand	93.6a	91.3a	86.3b	83.6b	72.7c	91.1a
7d 10°C - BP	93.4a	92.6ab	87.9b	88.2b	79.6c	91.1ab

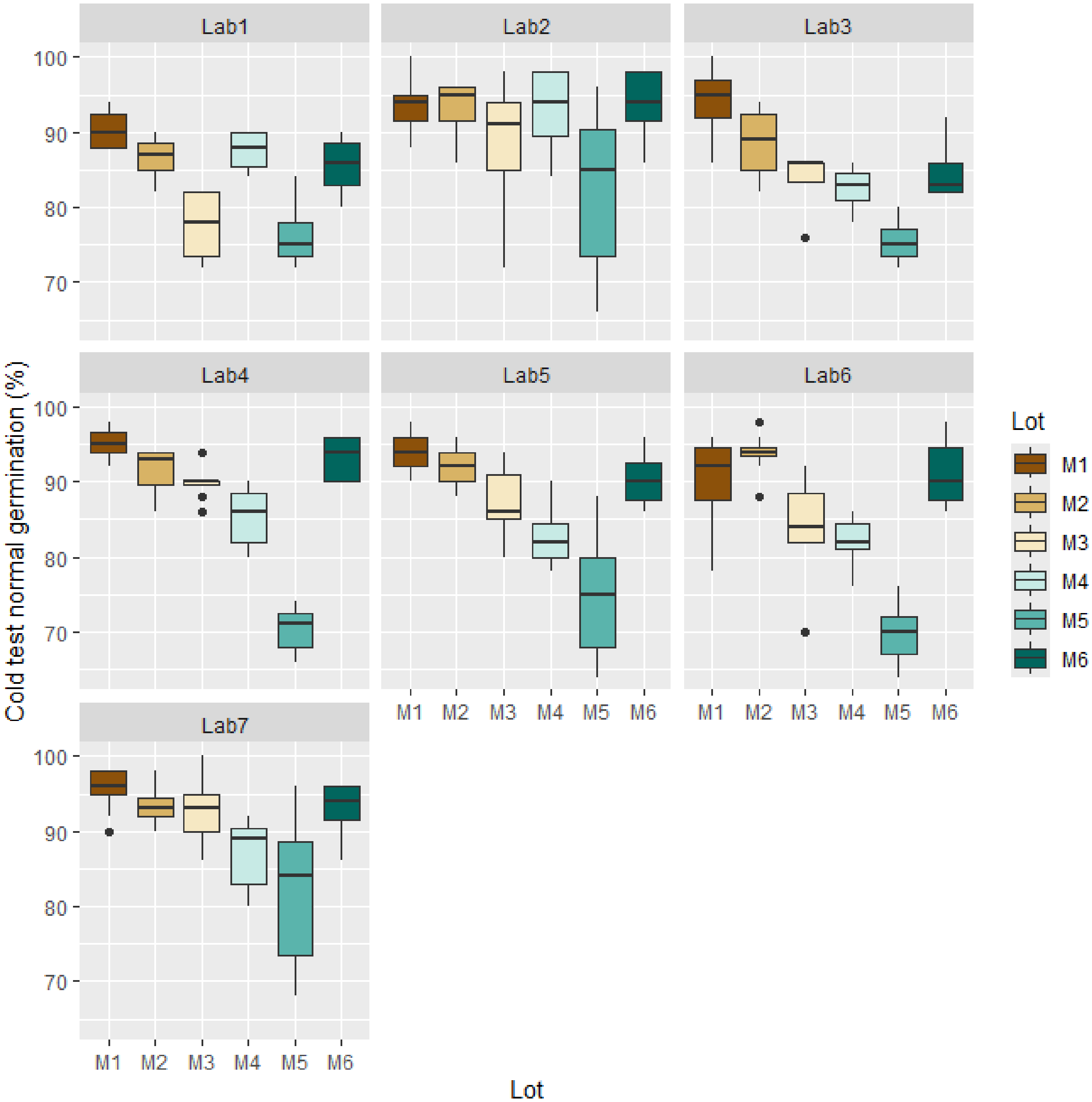

Figure 7: Mean cold tests results per laboratory for six maize seed lots. Note that lab 1 and 3 used only one substrate (BP and sand respectively) leading to a total of 6 laboratories by substrate.

The interaction between lot and substrate was also significant, mainly due to the low vigour seed lot M5 as shown on figure 7 for laboratories 2, 5 and 7 and summarised on figure 8.

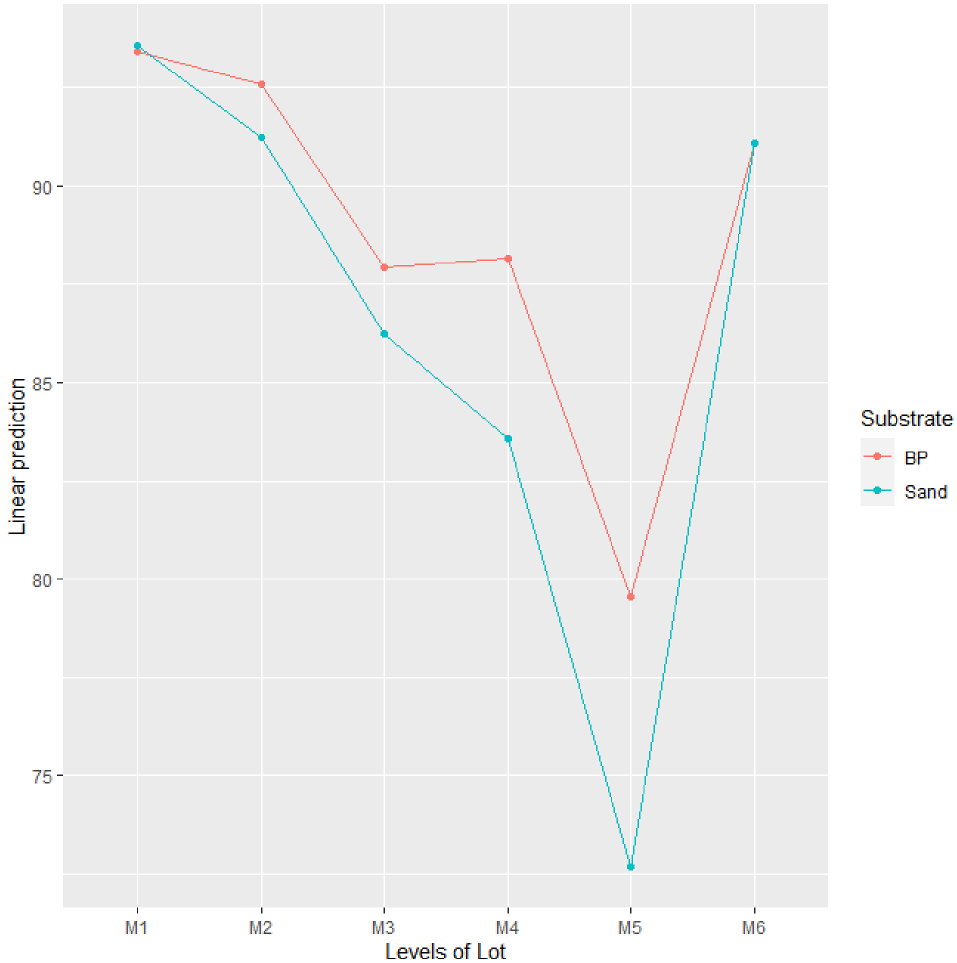

Figure 8: Interaction graph comparing cold test in sand or between paper for six seed lots of maize analysed in six different laboratories.

Tolerances*:* Considering 4 replicates of 50 seeds, only one sample M5 was out of tolerance in Lab 6 for BP substrate which represented only one test out of tolerance from a total of 72 (1.4% of outliers).

Comparing the overall means for 6 laboratories having compared the two substrates, lot M5 presented the highest difference between sand and paper but in both cases, it was ranked as low vigour (figure 7). It is also clear that vigorous samples gave the same results whatever the substrate.

Nevertheless, as means were quite different between the two substrates for M4 and M5, laboratories were asked about their routine substrate for a cold test and a second analysis was done keeping only this substrate for each participant. This second variance analysis maintained no significant effect of the substrate and cancelled the previous significant interaction with the seed lot (table 6).

Table 6: Analysis of variance results using four CT carried out in sand to three CT carried out in paper according to the routine substrate of each participant combined in a linear model.

	Sum Sq	Df	F value	Pr(>F)
(Intercept)	106032	1	4232.3270	< 2.2e-16 ***
Substrate	1	1	0.0385	0.8447
Lot	2517	5	20.0895	1.945e-15 ***
Substrate: Lot	63	5	0.5008	0.7753
Residuals	3908	156		

Significant codes : *** 0.001, ** 0.01, * 0.05

The mean results per seed lot were close for both substrate (figure 9).

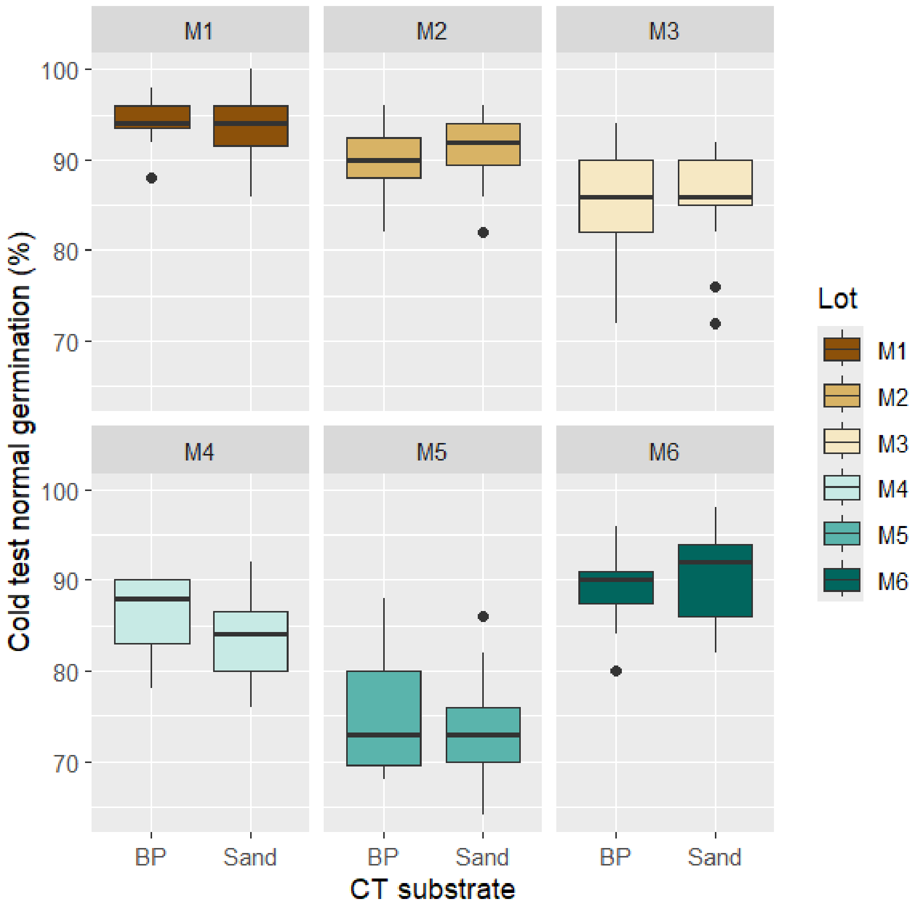

Figure 9: Mean cold tests results per seed lot for seven laboratories using only one substrate leading to a total of 3 CT in BP and 4 CT in sand.

Repeatability and reproducibility estimates are presented in table 7 for all data (six laboratories and two substrates = 12 cold tests carried out for six samples). Dispersion factors were close and less than 1 for cold test indicating that there is no evidence of overdispersion within laboratories (f_r). Between laboratories, results in sand were more reproducible (f_R).

Table 7: Repeatability/Reproducibility estimates using Linear Mixed model

Method	p_avg	s_Lab	s_LotxLab	s_r	f_r	s_R	Excluded samples %	f_R	f_Miles
BP	89	4.48	3.3	4.09	0.92	5.93	8.3	2.4	1.64
Sand	86	0	1.57	4.32	0.89	2.67	0	1.1	1.66

Discussion and conclusion

The cold test consisting of 7 days at 10°C and 5 days at 25°C clearly identified the same seed lots as high or low vigour in six laboratories for both substrates. The two methods are repeatable and results in sand were more reproducible. Variability among laboratories might be explained by substrate use in routine tests. When staff are used to evaluating seedlings grown in sand or organic media, seedling evaluation between paper needs time for training. This hypothesis was confirmed when analysing cold tests data obtained in usual substrate for each laboratory (seven cold tests): mean results were closer between the two substrates compared to the overall data when five laboratories used both substrates and two their own routine one (12 cold tests). Sand and paper represent together 93% of the substrates used in routine for maize germination worldwide (ISTA, 2021).

Both substrates were consistent with field emergence results, but with a higher correlation when sand was used. Previous work in Italy using more seed lots also obtained significant correlations with the same method of 7d at 10°C in sand (Noli *et al*., 2008). The method in rolled paper has been developed for a long time in the USA for field maize vigour using soil or not (Loeffler *et al*., 1985), the method tested here used only paper and is one day shorter.

Factors influencing cold test results have been described in literature and former comparative tests have pointed that CT in sand presented fewer outliers than CT in soil and that a method was less variable between different regions in the world than between different labs from the same region especially for in-house methods (Nijenstein and Kruse, 2000). The present work also revealed that CT in sand was more reproducible than paper and without any outlier.

Even though it would have been preferable to use more than three seed lots in some field trials, the differences between three lots were very clear in both their CT result and their field emergence percentage in all three locations (Arras, Chappe and Saint Mathurin sur Loire). In addition, the same lots emerged well, or poorly in each location. The CT also clearly identified differences in seed lot performance in the better conditions of Haut-Mauco. Thus, the test can identify possible differences in emergence in all field conditions. The CT revealed these differences more clearly than the standard germination test, even though the SG results correlated with field emergence. Therefore, given the limitations that inevitably arise when sourcing seed lots and achieving a wide range of field conditions, we believe that the CT can identify vigour differences in a wide range of environments.

The repeatability and reproducibility of the cold test for maize shown in this comparative test indicate that a cold test carried out at 10°C for 7 days followed by 5 days at 25°C using sand can be applied as a vigour test for this species. Germination tolerance tables could be used to check outliers.

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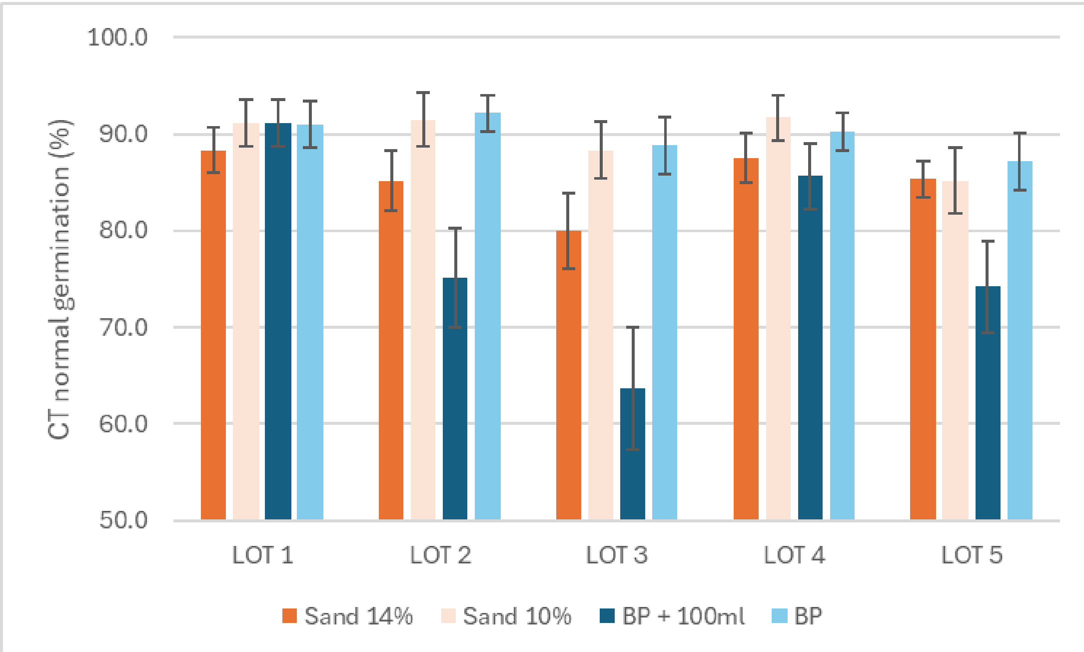
Appendix 1: Cold test mean results (4x50 seeds) illustrating importance of test conditions

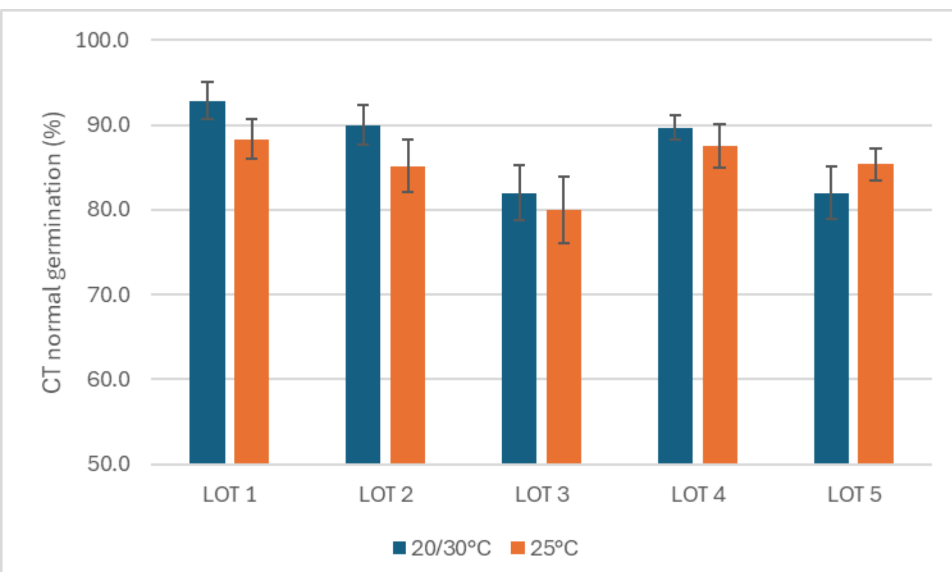
a) Substrate moisture

Cold test of 7d at 10°C + 5d at 25°C in sand or paper moistened with two rates of water

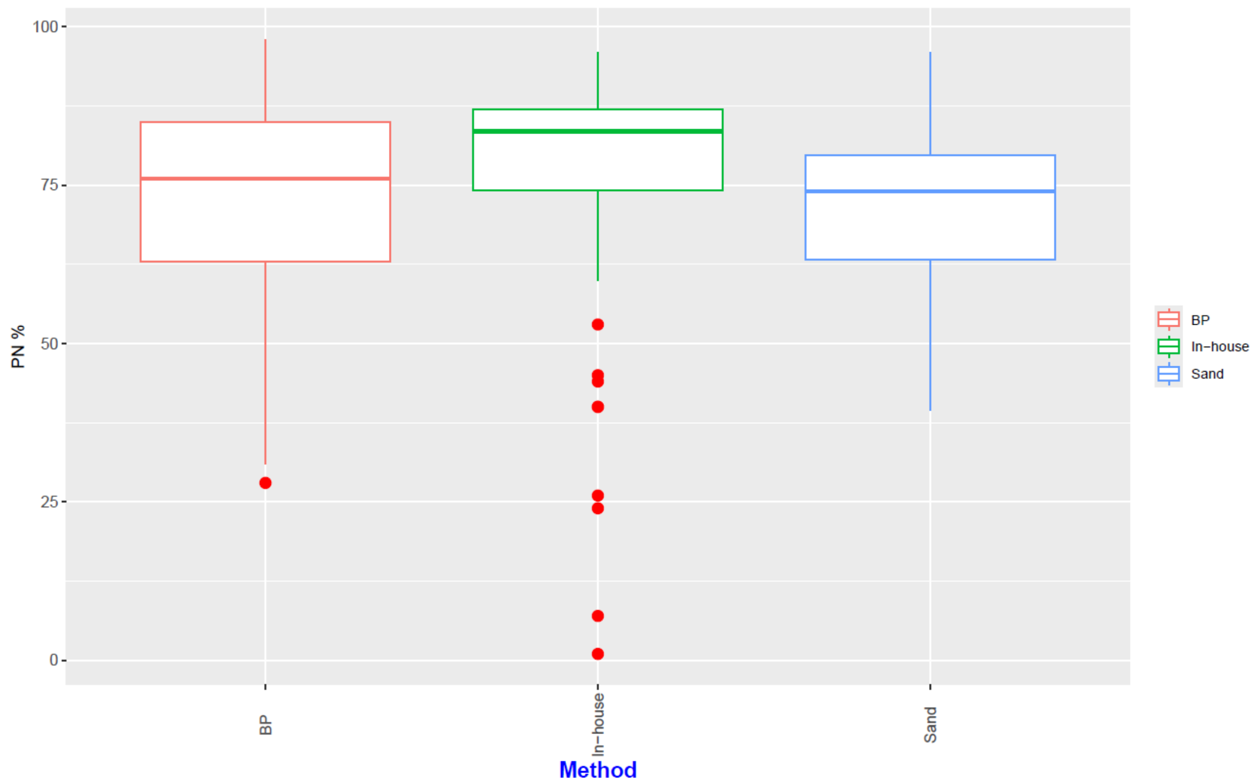


b) Growth temperature

Cold test in sand moistened at 14% during 7d at 10°C and 7d at 20/30°C or 25°C



Appendix 2: Cold test mean results obtained in 2023 in a comparative trial with seven laboratories and six seed lots. CT in-house methods were less discriminant than the suggested method of 7d at 10°C and 5d at 25°C whatever the neutral substrate, sand or between paper, both decreasing variability between laboratories.



Method	Mean	s_repeatability	disp	s_Reproducibility	s_Lab	s_LotxLab
BP	73.00	4.29	0.96	12.52	9.66	6.72
In-house	77.00	3.26	0.77	18.64	15.37	10.02
Sand	71.00	5.61	1.24	11.40	7.45	6.56

6. ISTA validation report of the detection of Fusarium species on cereal seeds.

1. Organization and design

1.1. Authors

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1.2. Participating laboratories and timeline

Required criteria: Experienced laboratory on detection of *Fusarium* on media.
Performance criteria have been divided between participating laboratories, each lab responsible of a set of isolates and species, by using Potato Dextrose Agar (PDA) or Malt Agar (MA), and subculturing on Carnation Leaf Agar (CLA) or Synthetic Nutrient-Poor Agar (SNA).
The details are described in Table 10 and schedule in Table 11.

Table 10. Participating laboratories

Laboratories	Performance criteria on media (PDA, MA, CLA, SNA)			CT
	Analytical specificity	Analytical sensitivity	Robustness	
Kimen/NIBIO	1/3 of isolates on PDA and SNA	On wheat	Incubation on PDA	X
CREA	1/3 of isolates on PDA and SNA	On barley	/	X
SASA	1/3 of isolates on PDA and all on CLA	On oat	/	X
GEVES	All isolates on MA, 1/3 of isolates on SNA	On wheat, barley, oat	Plating design on MA Light on MA and PDA	X
Additional laboratories	/	/	/	X

Table 11. Timeline

Time	Action	Person
June 2019-October 2020	Test plan redaction	All organisers
October 2020	Test plan submitted to ISTA	All organisers
Jan 2019-June 2020	Isolate collection	All organisers
September 2020-2021	Study of performance criteria	All organisers
March-April 2023	Comparative Test	All participants
2024	Analysis of CT results	Test organiser
June 2025	Report	CT organiser
September 2025	Validation report	CT organiser

2. Introduction and objectives of the method

2.1. Background

Fusarium Head Blight (FHB) complex besides *Microdochium majus* and *Microdochium nivale* is also caused by several *Fusarium* species and is considered one of the most important diseases of cereals worldwide. The disease may result in significant yield losses and poor seed quality. Several of these *Fusarium* species can produce toxic secondary metabolites (mycotoxins) (Desjardins 2006) that can reduce the use of the grain for human and/or animal consumption. For example, *Fusarium graminearum*, one of the most aggressive pathogens of FHB and producer of the important mycotoxin deoxynivalenol (DON), was ranked number four in an international nomination of the top ten most economically important fungal pathogens (Dean et al 2012).

At the ISTA seed health workshop in Angers, France, 2010, participants commented that the ISTA method 7-022 is suitable for detection of *Fusarium* spp. It was also stated in the validation report for the ISTA 7-022 method for detecting *Microdochium* spp. that the method was suitable for the generic detection of *Fusarium* species, with the addition of further steps to aid species identification.

PDA and MA are suitable media to detect *Fusarium* species on cereal seed (Leslie and Summerell 2006) and have been used for the validation. Incubation at 20 ±2 °C will either be with NUV light, or in darkness. However, transfer of colonies to special low nutrient media, e.g. SNA or CLA, would facilitate species identification. This low nutrient SNA media and CLA induce sporulation and reliably produce uniform conidia more representative of the species than those produced on PDA or MA. If plated on different media, Fusarium species can present notoriously different phenotypes, so for identification it is important to standardize the media used within the laboratory and ensure it is consistent make-up.

The SHC has decided to carry out a validation study for detection of *Fusarium* spp. on cereal seeds, based on the ISTA 7-022 method for detection of *Microdochium* spp. on wheat (ISTA, 2020).

2.2. Pathogens

Nirenberg 1982, Nelson et al 1983, cited and discussed by Summerell and Leslie (2011). The 'modern' *Fusarium* taxonomy was established by Wollenweber and Reinking (1935) who distinguished between 65 species and 55 varieties and 22 forms, in 16 sections. The sections contained species that were united by morphological characters, e.g. macroconidial morphology, pigment, and occurrence of chlamydospores. In the 1940-ies and 1950-ies Snyder and Hansen tried to simplify the system and reduced the number of species to nine. Among other things, they merged the species in the sections *Roseum*, *Discolor*, *Arthrosporoides* and *Gibbosum* to one species: *Fusarium roseum*. However, this simplification was not widely accepted. Leslie and Summerell (2006) comment that the name *F. roseum* include so many different taxa that it was of little value and that it is no longer used. Moreover, Neergaard (1977) referred to *F. avenaceum*, *F. culmorum* and *F. graminearum* in the index for *F. roseum* (i.e. *F. roseum* was not used by Neergaard 1977).

The *Fusarium* species listed below were included in this validation study. The names are based on the taxonomy from the Species Fungorum database (indexfungorum.org), which in most cases is the same as in Leslie and Summerell (2006), except for *F. roseum*. Also, two *Microdochium* species are included. Note that the current name and taxonomy may change over time.

- *Fusarium avenaceum* (Fries) Saccardo
- *Fusarium graminearum* Schwabe
- *Fusarium culmorum* (W.G. Smith) Saccardo
- *Fusarium crookwellense* Burgess, Nelson & Toussoun
- *Fusarium langsethiae* Torp & Nirenberg
- *Fusarium poae* (Peck) Wollenweber
- *Fusarium tricinctum* (Corda) Saccardo

- *Fusarium sporotrichioides* Sherbakoff
- *Fusarium pseudograminearum* Aoki & O'Donnell

2.3. Objectives

The objective of this validation plan is to provide an ISTA internationally accepted seed testing method for the detection of *Fusarium* species (targets) infecting the cereals wheat, barley and oats. This method will include a detection on PDA and MA media based on the ISTA 7-022 method for detecting *Microdochium* spp. on wheat seed, then morphological identification and an optional species identification on CLA or SNA depending on expertise of laboratories (Figure 14).

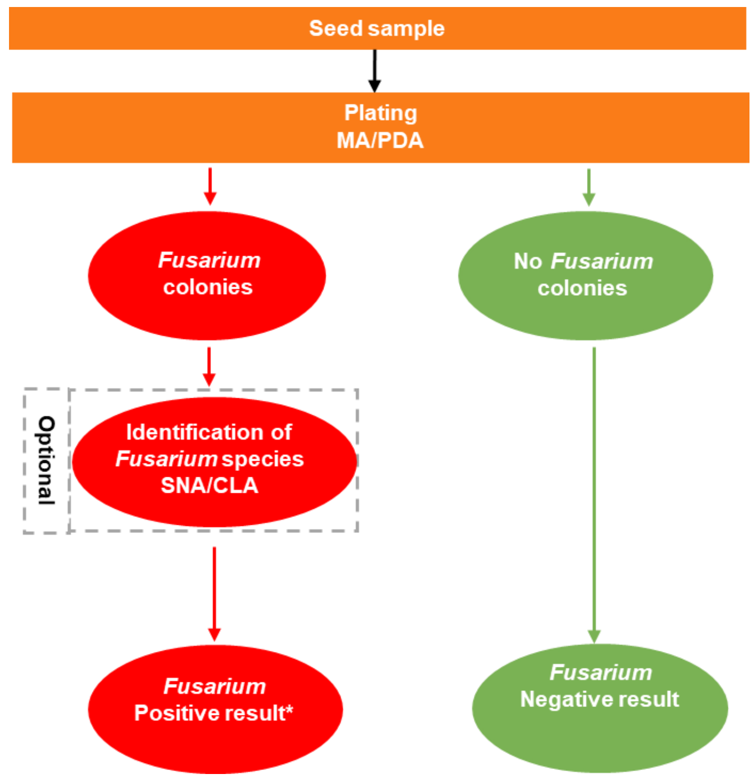


Figure 14. Workflow describing the steps and decisions for detection of *Fusarium* spp. by plating on media and morphological identification.
**The result expression is detailed in the method*

3. Materials and methods

3.1. Materials needed to perform the validation

3.1.1. Man

- Validation of performance criteria: See Table 10
- Reproducibility of the method: See Table 10

3.1.2. Machine

- Autoclave
- Sterilizer
- Stereomicroscope (x 6.5 – 50 magnification)
- Compound microscope (x100-400 magnification)

3.1.3. Material

- Sodium hypochlorite 1%

- Sterile distilled/deionized water
- Sterile blotter paper
- Tweezers
- Plates: 90 mm sterile Petri dishes
- Malt-agar (MA) media (Annex 1)
- Potato Dextrose Agar (PDA) media (Annex 2)
- Synthetic Nutrient-Poor Agar (SNA) + Filter Paper media (Annex 3)
- Carnation Leaf Agar (CLA) media (Annex 4)
- Reference strains of *Fusarium*

3.1.4.**Medium**

- Incubator: 20 ± 2 °C darkness (incubation of samples)
- Incubator: 20 ± 2 °C UV (confirmation and subculture)
- Storage room: 10 ± 2 °C for the storage of the samples

3.2. Method used

The main steps of the method used for the validation are described below.
The method was tested on untreated cereal seeds.
Pretreatment is necessary on cereal seeds to avoid the presence of surface saprophytes that may interfere during reading.

Pretreatment: Immerse seeds in a solution of sodium hypochlorite (NaOCl) (1% available chlorine) for 10 mins, then drain and rinse well in sterile water and drain.
Plating: Plate a maximum of 10 seeds per Petri dish (90 mm Ø) containing the medium (MA or PDA).
Positive Process Control (PPC): Plate a reference isolate on media, then incubate in the same conditions as the samples to control that all the conditions allow the growth of *Fusarium.*
Sterility Control: Place a non-inoculated Petri-dish containing the media in a climate chamber T=20 ± 2°C, to control the sterility of the medium.
Air control: Open a Petri dish containing the medium for 2 min and incubate it with the samples to control the absence of contaminant in the air.
Incubation: 7 days at 20 ± 2˚C,darkness.
Examination: Description of the morphological criteria of *Fusarium* in Annex 5.

4. Results

4.1. Analytical specificity

The analytical specificity is the ability to detect target pests while not detecting closely related and other organisms or samples which do not contain the target.
For methods whose result is based on morphological criteria, the analytical specificity is validated if the target isolates criteria correspond to those described and the non-targets do not.

The performance of the analytical specificity was based on the morphological criteria (described in Annex 5) and performed on 2 media, MA and PDA and on a collection of 44 targets and 19 non-targets isolates.
MA and PDA are both non-selective media and allow the isolates to produce sporulation with the same morphological criteria.
SNA and CLA both enhance the sporulation and are used only for confirmation if necessary.
The analytical specificity tests were shared between the laboratories as indicated in Table 12.

Table 12. Distribution of isolates

Laboratories	Analytical specificity
Kimen/NIBIO	20 target isolates and 19 non-target isolates on PDA and SNA
CREA	19 target isolates and 19 non-target isolates on PDA and SNA
SASA	19 target isolates and 19 non-target isolates on PDA and CLA
GEVES	44 target isolates and 19 non-targets isolates on MA and SNA

The results of targets and non-targets are shown in Table 13. Raw data are available in Annex 6.

Table 13. Analytical specificity results

Targets	Nb of isolates	*Fusarium* detected	Species identified
F. avenaceum	5	5/5	5/5
F. crookwellense	5	5/5	4/5
F. culmorum	5	5/5	5/5
F. graminearum	6	6/6	4/6
F. langsethiae	5	5/5	5/5
F. poae	5	5/5	5/5
F. pseudograminearum	3	3/3	3/3
F. sporotrichioides	5	5/5	5/5
F. tricinctum	5	5/5	4/5

Non targets	Nb of isolates	Different from criteria
Helminthosporium gramineum	1	Yes
Helminthosporium teres	1	Yes
Bipolaris sorokiniana	1	Yes
Helminthosporium avenae	1	Yes
Parastagonospora nodorum	1	Yes
Parastagonospora avenae	1	Yes
F.equiseti	1	Yes
F.acuminatum	1	Yes
F.fujikuroi	1	Yes
F.proliferatum	1	Yes
F.oxysporum	1	Yes
F.solani	1	Yes
F. lateritium	1	Yes
Epicoccum sp.	1	Yes
F. venenatum	1	Yes
F. caeruleum	1	Yes
Alternaria tenuissima	1	Yes
Cladosporium sp.	1	Yes
Penicilium sp.	1	Yes

Conclusion

The analytical specificity has been validated for all non-target isolates with a result of 100% (19/19)

As the objective of the method is to detect the Fusarium species responsible for seedling blight and head-blight in cereals (identified as target isolates, table 4) and not to identify all the Fusarium species, during the analysis of the data we only considered the detection of the target Fusariums to genus level and not the Fusarium species identification. This assessment was carried out using the morphological data as described in Annex 5. The analytical specificity has been successfully validated for all target isolates on both MA and PDA as all target Fusariums were identified, and there was no confusion observed with non-target Fusariums, with a result of 100% (44/44) for the detection.

4.2. Analytical sensitivity

The analytical sensitivity is the lowest quantity or concentration of a pest that can be reliably detected with a given analytical method.

The performance of the analytical sensitivity was based on the ability to detect 1 infected seed in a sample of 399 healthy seeds (0.25% of contamination).

Artificial contamination

To obtain contaminated seeds, artificial contamination using gum arabic was performed on barley, oat and wheat seeds with reference material.

5 seeds of each artificial contaminated lot have been tested to check if all seeds were contaminated, as well as the stability of the level of contamination after two months. Results are shown in Table 14.

Table 14. Results of artificial contamination

	Barley				
1st test	**Replicate**	***F.tricinctum***	***F. avenaceum***	***F.culmorum***	***F. langsethiae***
	1	5/5	5/5	5/5	0/5
	2	5/5	4/5	5/5	2/5
	3	5/5	5/5	5/5	0/5
	4	5/5	4/5	5/5	0/5
	5	5/5	5/5	5/5	0/5
	Contamination	**100%**	**92%**	**100%**	**8%**
	Decision	**Chosen**	**Not chosen**	**Chosen**	**Not chosen**
2nd test after 2 months	1	5/5		5/5	
	2	5/5		5/5	
	3	5/5		5/5	
	4	5/5		5/5	
	5	5/5		5/5	
	Contamination	**100%**		**100%**	
	Oat				
1st test	**Replicate**	***F.tricinctum***	***F. avenaceum***	***F.culmorum***	***F. langsethiae***
	1	5/5	5/5	5/5	5/5
	2	5/5	3/5	5/5	4/5
	3	5/5	5/5	5/5	5/5
	4	5/5	5/5	5/5	5/5
	5	5/5	5/5	5/5	5/5
	Contamination	**100%**	**92%**	**100%**	**96%**
	Decision	**Chosen**	**Not chosen**	**Chosen**	**Not chosen**
2nd test after 2 months	1	5/5		5/5	
	2	5/5		5/5	
	3	5/5		5/5	
	4	5/5		5/5	
	5	5/5		5/5	

	Contamination	**100%**		**100%**	
	Wheat				
	Replicate	***F.tricinctum***	***F. avenaceum***	***F.culmorum***	***F. langsethiae***
1st test	1	5/5	5/5	5/5	5/5
	2	5/5	5/5	5/5	5/5
	3	5/5	5/5	5/5	5/5
	4	5/5	5/5	5/5	5/5
	5	5/5	5/5	5/5	5/5
	contamination	**100%**	**100%**	**100%**	**100%**
	Decision	**Chosen**	**Not chosen**	**Chosen**	**Not chosen**
2nd test after 2 months	1	5/5		5/5	
	2	5/5		5/5	
	3	5/5		5/5	
	4	5/5		5/5	
	5	5/5		5/5	
	contamination	**100%**		**100%**	

Conclusion

The artificial contamination was validated for *F.culmorum* and *F.tricinctum* on barley, oat and wheat.

To test the analytical sensitivity, 3 samples free from *F.culmorum* and *F.tricinctum* of each crop have been spiked with 1 contaminated seed in 399 healthy seeds. The tests have been shared between laboratories as indicated in Table 15.

Table 15. Distribution of the tests

Laboratories	**Analytical sensitivity**
Kimen/NIBIO	On wheat
CREA	On barley
SASA	On oat
GEVES	On wheat, barley and oat

As the healthy samples used for the spiking are free from *F.culmorum* and *F.tricinctum* but not for other *Fusarium* species, we considered only the results for both spiked pathogens.

Like for the analytical specificity we focused on detection, not on the identification.

We considered whether the laboratory was able to detect the *Fusarium*, even if the species was not correctly identified, and comforted by the results of all positive and negative controls conform as expected, we assumed the sample results conform.

Results are recorded in Table 16 and raw results in Annex 7.

Table 16. Analytical sensitivity results

		Samples			
Crop	***Fusarium***	**GEVES**	**KIMEN/NIBIO**	**CREA**	**SASA**
Wheat	*culmorum*	3+/3	3+/3[1]		
	tricinctum	3+/3	3+/3[2]		
Barley	*culmorum*	3+/3		3+/3	
	tricinctum	3+/3		3+/3[2]	
Oat	*culmorum*	3+/3			3+/3
	tricinctum	3+/3			3+/3[3]

[1] Confusion with *F. crookwellense*

[2] species not identified

[3] Confusion with *F. acuminatum*

Conclusion

The method was able to detect 1 contaminated seed on all samples in 400 seeds.

Analytical sensitivity of the detection method is validated.

4.3. Robustness

Robustness is the ability to not vary according to small variations of parameters in the method.

Three parameters have been studied

1. Light: effect of sporulation in darkness compared to NUV.
2. Duration of incubation.
3. Plating design: 5 compared to 10 seeds per plate.

4.3.1. Light

The effect of sporulation in darkness versus NUV has been tested on a selection of isolates from the collection. Based on the isolate performance results on Potatoes Dextrose Agar (PDA) and Malt-Agar (MA) in darkness observed in the analytical specificity, 1 isolate that sporulated well, and 1-2 isolates that did not sporulate well, have been selected. The isolates have been plated on PDA and MA (2 plates per isolate), incubated at 20 ± 2 °C in darkness (1 plate) or NUV (1 plate), and sporulation has been registered after 7 days.

The results are shown in Table 17.

Table 17. Robustness for light parameter results

		PDA		MA	
Pathogen	**Conidia**	**Darkness**	**NUV**	**Darkness**	**NUV**
F. tricinctum *Isolate 1*	Microconidia	-	-	+++	+
	Macroconidia	+	+	+	+++
F. tricinctum *Isolate 2*	Microconidia	+	+	+++	+++
	Macroconidia	-	+	-	+
F.graminearum *Isolate 1*	Macroconidia	-	+++	-	++
F. graminearum *Isolate 2*	Macroconidia	-	+	-	-

- : Absence of conidia

+ : Very few conidia

++ : Some conidia

+++ : Lots of conidia

Conclusion

Sporulation is most of the time better with NUV light, but results vary depending on the media and isolates.

As the method includes a subculture on CLA or SNA to ensure sporulation, the robustness for the light parameter is validated.

4.3.2. Incubation

This performance criteria was tested on PDA, medium used by the laboratory in charge of this test. Both media, MA and PDA allow to detect *Fusarium* as validated in the analytical specificity.
Notations after 6, 7 and 9 days of incubation have been compared on three replicates of wheat naturally infected seed samples of 400 seeds. Results are shown in Table 18.

Table 18. Robustness for incubation duration parameter results

Sample n°	Media	Nb days of incubation	% *Fusarium sp.*
1	PDA	6	11.25%
	PDA	7	11.25%
	PDA	9	11.25%
2	PDA	6	14.75%
	PDA	7	14.75%
	PDA	9	14.75%
3	PDA	6	18.0%
	PDA	7	18.0%
	PDA	9	18.5%

Conclusion

There is no difference between 6, 7 or 9 days of incubation, the robustness for the incubation duration parameter is validated. The extra 0.5% found in the sample 3 after 9 days was identified by the laboratory in charge as a cross contamination.
This result led us to propose the notation after 7 days of incubation.

4.3.3. Plating design

This performance criteria was tested on MA, medium used by the laboratory in charge of this test. Both media, MA and PDA allow to detect Fusarium as validated in the analytical specificity.
Comparison between 5 and 10 seeds per plate has been performed on two samples of 400 seeds, infected with *F.culmorum* and *F.graminearum* after 7 days of incubation. Results are shown in Table 19.
Allowed tolerance was provided by the Miles (1963) tolerance table. Results are shown in Table 20.

Table 19. Robustness for plating design parameter results

Sample n°	Crop	Media	Nb of seeds/plate	% *Fusarium culmorum*	% *Fusarium graminearum*
1	Wheat	MA	10	12.25	8.25
2	Wheat	MA	5	10.5	12.75

Table 20. Statistical results of robustness for plating design parameter

F.culmorum	
Mean	11.375
Rounded	11
Allowed tolerance	5
Difference	1.75

F.gramineraum	
Mean	10.5
Rounded	11
Allowed tolerance	5
Difference	4.5

Conformity	OK		Conformity	OK

Conclusion
Based on the statistical analysis there is no difference between 5 and 10 seeds per plate. The robustness for the plating design parameter is validated.

4.4. Accuracy (sensitivity and specificity diagnostic) - Repeatability and reproducibility

These criteria have been validated through a comparative test (CT) between seven laboratories.
For identification test on isolates, the laboratories indicated the identified *Fusarium* species.
For detection method, the laboratories indicated: quantitative results for each sample and information about the method used.

4.4.1. Isolates identification

All laboratories received 8 coded *Fusarium* species isolates *in vivo* on agar plates (Table 21).
The morphological identification was done according to the morphological criteria (Annex 5) after culturing them on one of the two media validated (MA or PDA) and by subculture on CLA or SNA if needed (Table 22)
Results are presented in Table 23.

Table 21. Collection of isolates

***Fusarium* species**	**Code**
Fusarium avenaceum	PAS 2714
Fusarium graminearum	PAS 2674
Fusarium culmorum	PAS 227
Fusarium crookwellense	PAS 2721
Fusarium langsethiae	PAS 2720
Fusarium poae	PAS 2719
Fusarium tricinctum	PAS 2522
Fusarium sporotrichioides	PAS 2718

Table 22 Media used

Lab code	**Media**	**Subculture (optionnal)**
01	PDA	CLA
02	Malt agar	SNA
05	PDA	SNA
07	PDA	SNA
08	PDA	SNA
09	PDA	Not done
10	PDA	CLA

Table 23. Results of isolates identification

Lab code	**Identification conformity**
01	8 conform/8
02	8 conform/8
05	8 conform/8
07	8 conform/8
08	8 conform/8
09	8 conform/8

10	8 conform/8

Conclusion

The media, the incubation conditions and morphological criteria allowed all laboratories to identify the different species of *Fusarium*, they are therefore validated.

4.4.2. **Detection on seed samples**

4.4.2.1. Validation of samples

12 naturally infected seed lots were available with different levels of contamination, 5 wheat lots, 4 barley lots and 3 oat lots. All the 12 lots were tested on 400 seeds to evaluate their level of contamination and the *Fusarium* species present. As it was not possible to test all the *Fusarium* species, it was decided to focus on *Fusarium* species easier to identify by morphological criteria. Two species are chosen, *F. avenaceum* and *F. poae* on two crops, Oat and Wheat. Barley lots were not kept because they did not have sufficient level of contamination for *F. avenaceum* and *F. poae.*
3 levels of infection have been determined: high, medium and healthy (Table 24)

Table 24. Samples characterisitcs

Code	Level of contamination	Number of samples	Expected values
A	Oat - High *F.poae*	3	Positive for *F.poae*
B	Wheat - mix of many *Fusarium*	4	Positive for *F.poae* and *F.avenaceum*
D	Wheat - Healthy	3	Negative for *F.poae* and *F.avenaceum*

Samples for the detection method have been validated through homogeneity and stability tests. The results from participating laboratories were compared to the results obtained of these tests.

4.4.2.1.1. Homogeneity test

Homogeneity has been tested for each level of contamination on 10 replicates of 400 seeds on MA, with a notation after 7 days of incubation. Qualitative results, minimum, maximum and average values are given in Table 25. Results in percentage are recorded in Table 26. Quantitative results were analyzed by Hampel's method (Table 27) and by distribution against the mean (Figure 15 and Figure 16) for lot A and B.

Table 25. Homogeneity test results

Seed lot Code	Level of infection	Expected result	Quantitative results		Conformity
			Min.-Max. (%)	Average (%)	
A	Oat - High *F.poae*	Positive *F.poae*	23.0-31.5	28.23	Conform
B	Wheat – Medium mix 2 *Fusarium*	Positive *F.poae*	0.25-3.25	2.00	Conform
		Positive *F.avenaceum*	1.75-5.50	3.50	Conform
D	Wheat - Healthy	Negative	0	0	Conform

Table 26. Homogeneity results in %

Healthy (Wheat)

Replicate	*F.poae* %	*F.avenaceum* %
1	0.00	0.00
2	0.00	0.00
3	0.00	0.00
4	0.00	0.00
5	0.00	0.00

High (Oat)

Replicate	*F.poae* %
1	31.00
2	31.50
3	29.50
4	23.00
5	26.50

Medium (Wheat)

Replicate	*F.poae* %	*F.avenaceum* %
1	3.25	2.75
2	2.00	1.75
3	2.00	3.00
4	0.25	3.50
5	2.00	3.50

6	0.00	0.00
7	0.00	0.00
8	0.00	0.00
9	0.00	0.00
10	0.00	0.00

6	28.25
7	31.00
8	31.25
9	24.75
10	25.50

6	2.00	5.00
7	2.00	3.25
8	3.25	5.50
9	2.75	4.25
10	2.75	5.50

Table 27. Hampel analysis results

High (Oat / *F.poae*)				Medium (Wheat / *F. poae*)				Medium (Wheat / *F. avenaceum*)			
Rep	Values (Xi)	| Xi - M|	Status	Rep	Values (Xi)	| Xi - M|	Status	Rep	Values (Xi)	| Xi - M|	Status
1	31	2.125	OK	1	3.25	1.250	OK	1	2.75	0.750	OK
2	31.5	2.625	OK	2	2	0.000	OK	2	1.75	1.750	OK
3	29.5	0.625	OK	3	2	0.000	OK	3	3	0.500	OK
4	23	5.875	OK	4	0.25	1.750	OK	4	3.5	0.000	OK
5	26.5	2.375	OK	5	2	0.000	OK	5	3.5	0.000	OK
6	28.25	0.625	OK	6	2	0.000	OK	6	5	1.500	OK
7	31	2.125	OK	7	2	0.000	OK	7	3.25	0.250	OK
8	31.25	2.375	OK	8	3.25	1.250	OK	8	5.5	2.000	OK
9	24.75	4.125	OK	9	2.75	0.750	OK	9	4.25	0.750	OK
10	25.5	3.375	OK	10	2.75	0.750	OK	10	5.5	2.000	OK

Conclusion

Based on the Hampel analysis of the homogeneity test results all seed lots (Healthy, Low and Medium) were homogeneous.

Figure 15. Homogeneity test results, distributed against the mean. High level (lot A) and Medium level (lot B *F.poae*)

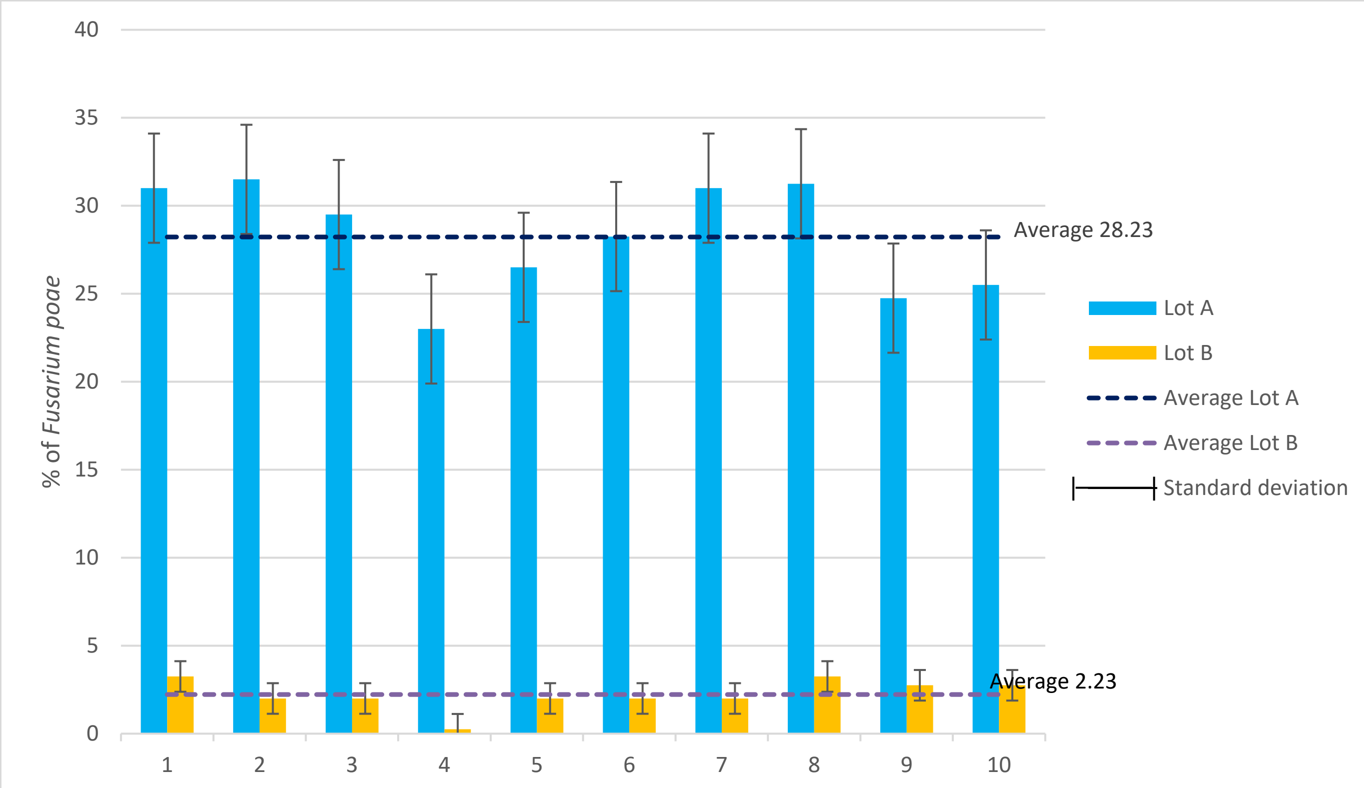
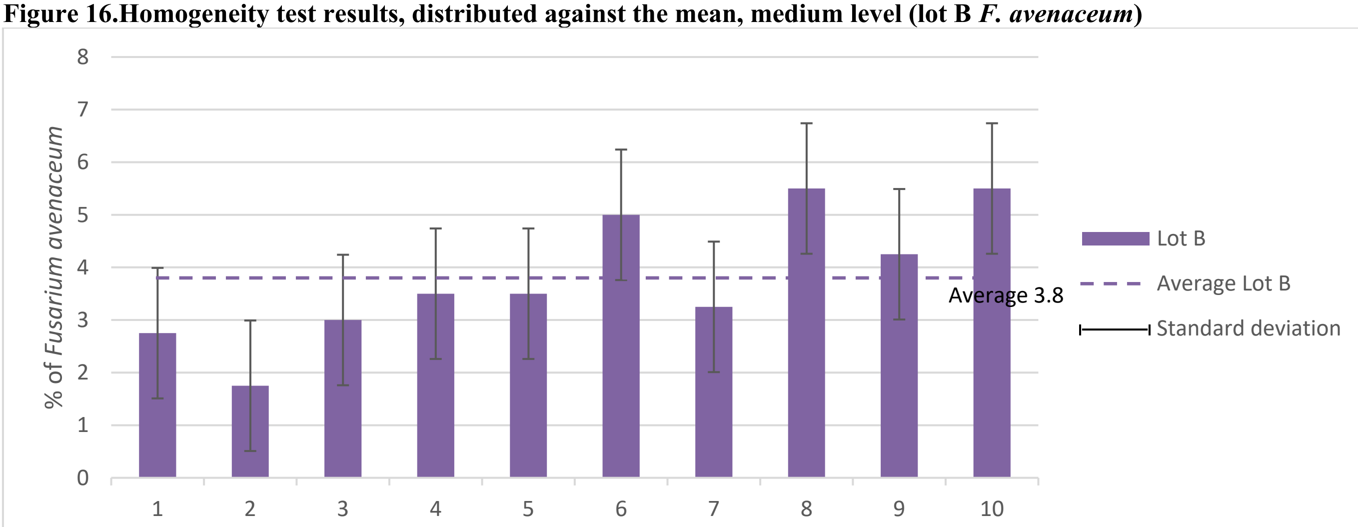


Figure 16.Homogeneity test results, distributed against the mean, medium level (lot B *F. avenaceum*)



Conclusion

Medium level

Samples are homogeneous, there was no outlier nor false negative for both *Fusarium.*

High level

Samples are homogeneous, there was no outlier nor false negative.

Healthy level

Samples are homogeneous, all were negative, there were no false positive results.

Based on the results, homogeneity of samples is therefore validated.

4.4.2.1.2. Stability tests

Stability test was done after the last confirmation of starting of analysis by laboratories. It started on June 26th, 2023, based on the same method. It was tested on 4 samples of 400 seeds for each level of infection.

Quantitative results minimum, maximum and average values are given in Table 28.

Quantitative results were analyzed by Hampel's method (Table 29) and by distribution against the mean (

Figure **17** and Figure 18) for lot A and B.

Table 28. Stability test results

Seed lot Code	Level of infection	Expected result	Quantitative results		Conformity
			Min.-Max. (%)	Average (%)	
A	Oat - High *F.poae*	Positive *F.poae*	25.75-36.00	32.00	Conform
B	Wheat – Medium mix 2 *Fusarium*	Positive *F.poae*	2.00-4.25	3.13	Conform
		Positive *F.avenaceum*	2.25-4.50	3.13	Conform
D	Wheat - Healthy	Negative	0	0	Conform

Table 29. Stability tests results for Lot A and B using the Hampel's method

Lot A *F.poae*			
Rep	Values (Xi)	| Xi - M|	Status
1	33.75	0.625	OK
2	36	2.875	OK
3	32.5	0.625	OK
4	25.75	7.375	OK

Lot B *F.avenaceum*			
Rep	Values (Xi)	| Xi - M|	Status
1	4.5	1.625	OK
2	3.25	0.375	OK
3	2.5	0.375	OK
4	2.25	0.625	OK

Lot B *F.poae*			
Rep	Values (Xi)	| Xi - M|	Status
1	2.75	0.375	OK
2	2	1.125	OK
3	4.25	1.125	OK
4	3.5	0.375	OK

Figure 17. Stability test results, distributed against the mean. High level (lot A) and Medium level (lot B) for *F.poae*.

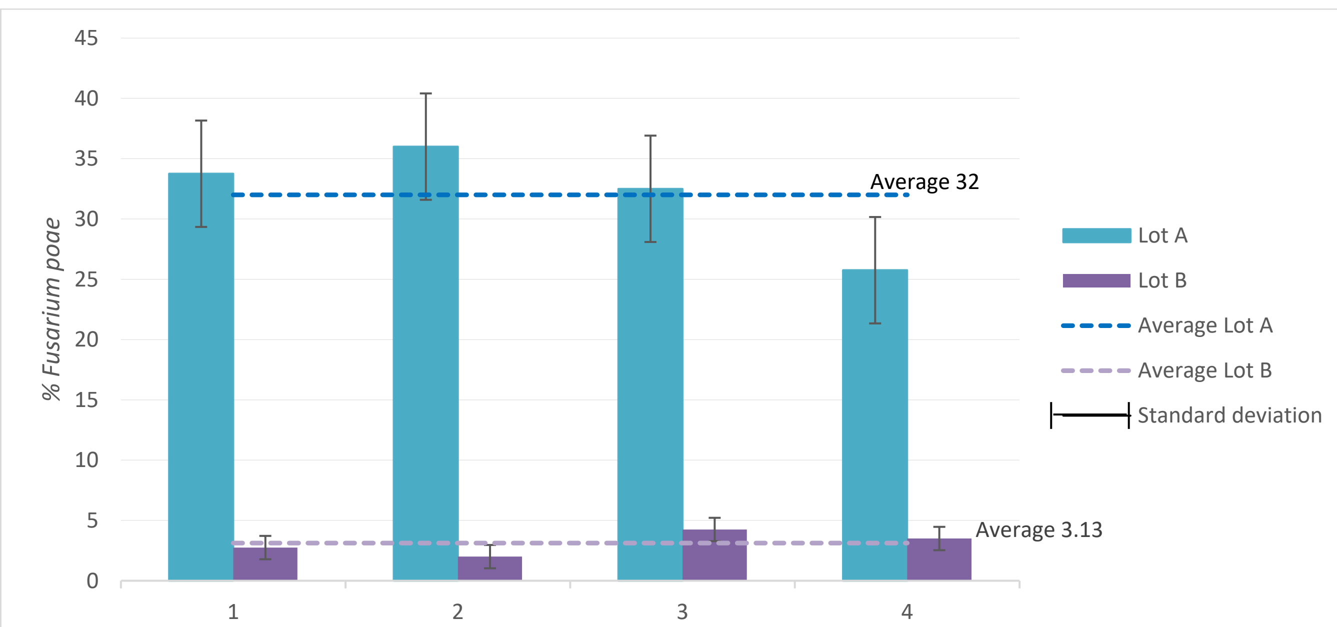
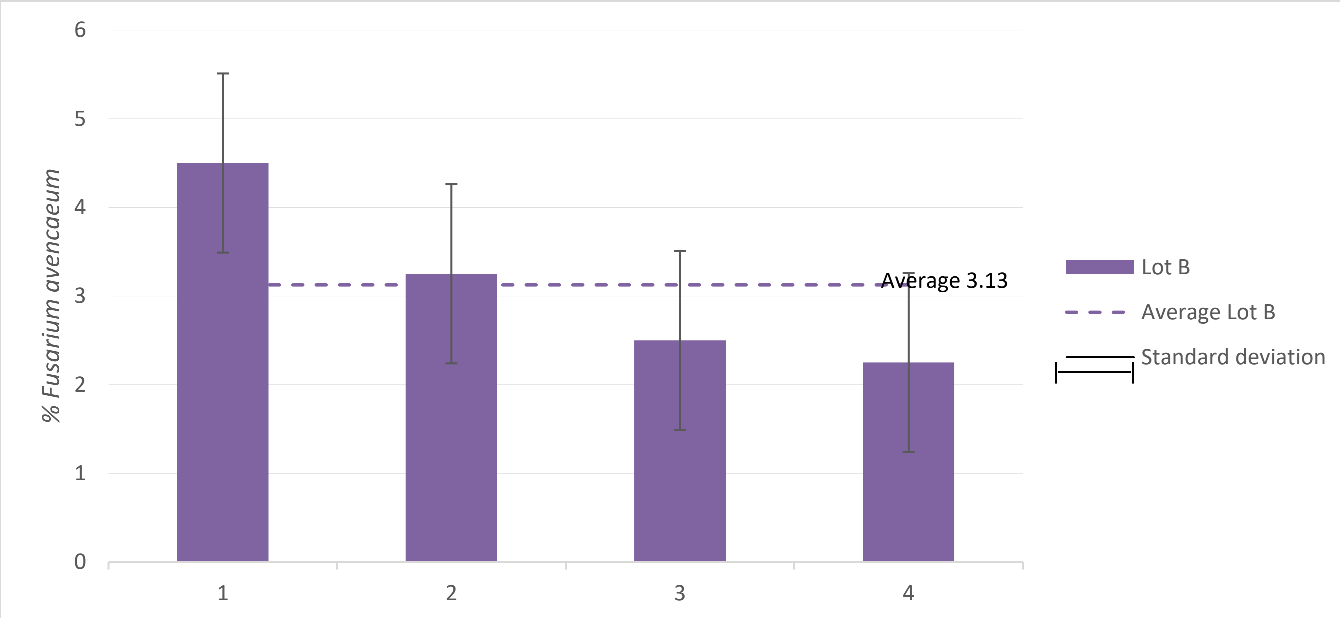


Figure 18. Stability test results, distributed against the mean, medium level (lot B *F. avenaceum*)

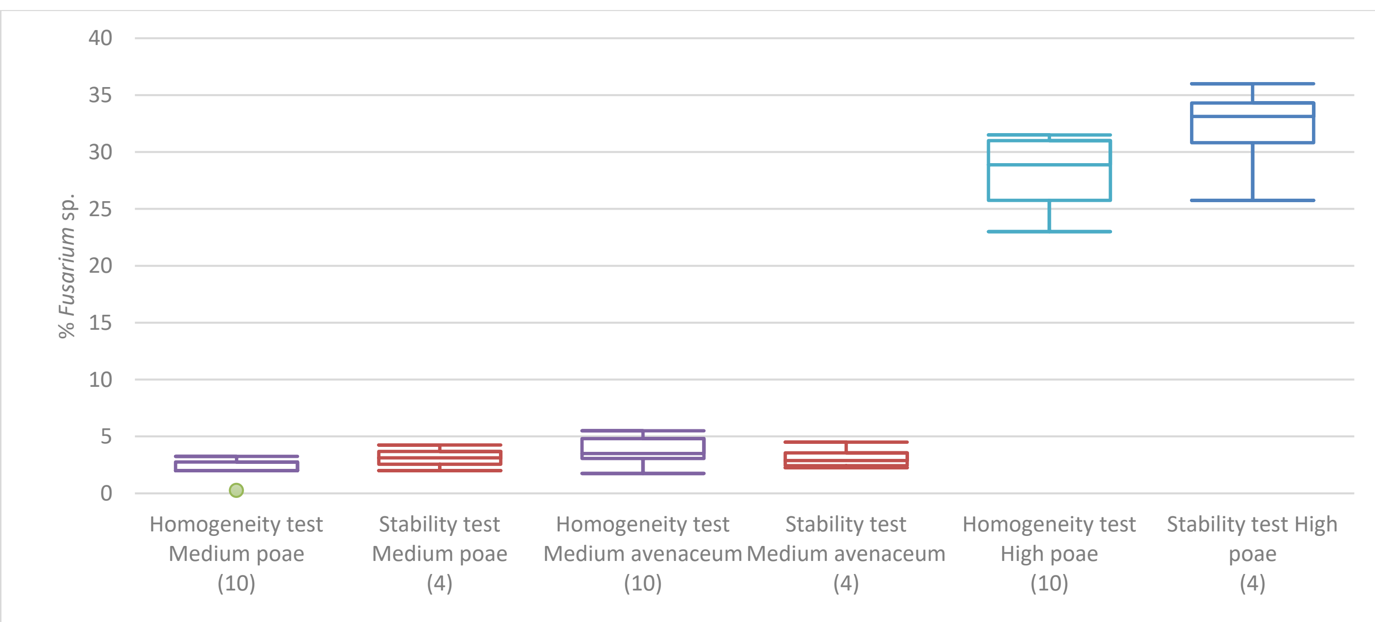


Comparison between homogeneity and stability results for healthy and medium level is given in Table 30 and Figure 19.

Table 30. Comparison between homogeneity and stability results

Seed lot Code	Level of infection	Homogeneity results	Stability results	Deviation
A	Oat - High *F.poae*	28.90	32.00	+ 3.1
B	Wheat – Medium mix 2 *Fusarium*	2.00	3.13	+ 1.13
		3.50	3.13	- 0.37
D	Wheat - Healthy	0	0	0

Figure 19. Box Plot comparison between homogeneity and stability tests



Conclusion

Medium and high level

The percentage of contamination was stable over time and did not decrease within the timeframe tested.

Healthy level

All samples were negative, there was no cross-contamination over time.

Stability is validated.

4.4.2.2. Results

The analysis of the qualitative results for a participating laboratory led to a declaration of conformity or non-conformity of the results.

Samples used for CT have been selected from naturally infected seed lots, presenting several *Fusarium* species. In order to obtain data that can be analyzed it has been decided to focus on the main *Fusarium* species occurring in each seed lot (Lot A : *Fusarium poae* / Lot B : *Fusarium poae* and *Fusarium avenaceum).*

The diversity of *Fusarium* species in the seed lots (up to 5 different *Fusarium* species in Lot B) might have caused difficulties for some laboratories to identify the species *avenaceum* and resulted in having undetermined species in the final results or a confusion between *F.poa*e and *F. sporotrichoides*. We considered these undetermined species in the final result when *F.avenaceum* was not identified and *F. sporotrichoides* as *F. poae*. This decision was taken because the method allowed to detect *Fusarium* even if the species was not identified by the laboratories. From this point, data with results where undetermined species and non-conforming Fusarium species have been reclassified is referred to as 'processed data'.

Analysis of qualitative and quantitative results are given for both use of the data, raw data and processed data.

Processed data were constructed based on the detection level and not the identification level, when the percentage of *Fusarium* spp. or undetermined *Fusarium* matched to the expected *Fusarium* species, we classified them in the expected species.

Analysis of results of each level has been carried out for each laboratory, at the qualitative level (detected/not detected), with the method developed by Langton et al. (2002) for repeatability (accordance) and reproducibility (concordance). They were also analyzed at the quantitative level (rate of seed infection) with the box plot tool (ISTA Seed Health Toolbox).

4.4.2.2.1. Qualitative results

Analysis of the qualitative results of raw data is given in Table 31 and qualitative results of processed data in Table 32.

Table 31. Results of detection method based on identification of the *Fusarium* species

Lab code	Obtained results			
	Medium Lot B *F.poae*	**Medium Lot B *F.avenaceum***	**High Lot A *F.poae***	**Healthy Lot D**
1	4+/4	4+/4	3+/3	0+/3
2	4+/4	4+/4	3+/3	0+/3
5	4+/4	0+/4	3+/3	0+/3
7	3+/4	2+/4	3+/3	0+/3
8	4+/4	4+/4	3+/3	0+/3
9	4+/4	4+/4	3+/3	0+/3
10	4+/4	4+/4	3+/3	0+/3
Repeatability (accordance)	**92.9%**	**90.5%**	**100%**	**100%**
Reproducibility (concordance)	**92.9%**	**61.9%**	**100%**	**100%**

Table 32. Results of detection method based on detection of the *Fusarium* species (including undetermined *Fusarium* species and *F. sporotrichiodes*)

Lab code	Obtained results			
	Medium Lot B *F.poae*	**Medium Lot B *F.avenaceum***	**High Lot A *F.poae***	**Healthy Lot D**
1	4+/4	4+/4	3+/3	0+/3
2	4+/4	4+/4	3+/3	0+/3
5	4+/4	4+/4	3+/3	0+/3
7	4+/4	4+/4	3+/3	0+/3
8	4+/4	4+/4	3+/3	0+/3
9	4+/4	4+/4	3+/3	0+/3
10	4+/4	4+/4	3+/3	0+/3
Repeatability (accordance)	**100%**	**100%**	**100%**	**100%**
Reproducibility (concordance)	**100%**	**100%**	**100%**	**100%**

For each of the seven participating laboratories, all negative (healthy) samples were detected as negative (0+/3) as expected, as well as all positive samples for the high level were obtained positive (3+/3) as expected.
For medium levels, when undetermined species and *F. sporotrichioides* are included, all positive samples are obtained positive (4+/4) as expected. Therefore, the accordance and concordance for the negative and positive seed lots, calculated by Langton et al., obtained a result of 100%.

Conclusion:
Repeatability (accordance) and reproducibility (concordance) are then validated according to the qualitative results.

4.4.2.2.2. Quantitative results

Analysis by Hampel's method of the quantitative results of raw data is given in Table 33.

Table 33. Laboratory results for medium and high level using the Hampel's method.

Medium - *F.avenaceum*					**Medium - *F.poae***					**High – *F.poae***				
Lab	**Rep**	**Values (Xi)**	**| Xi - M|**	**Status**	**Lab**	**Rep**	**Values (Xi)**	**| Xi - M|**	**Status**	**Lab**	**Rep**	**Values (Xi)**	**| Xi - M|**	**Status**
1	1	3.50	1.375	OK	1	1	1.50	0.875	OK	1	1	37.75	1.750	OK
1	2	3.25	1.125	OK	1	2	3.50	1.125	OK	1	2	32.00	4.000	OK
1	3	2.50	0.375	OK	1	3	2.50	0.125	OK	1	3	36.00	0.000	OK
1	4	4.00	1.875	OK	1	4	2.75	0.375	OK	2	1	32.50	3.500	OK
2	1	1.25	0.875	OK	2	1	3.25	0.875	OK	2	2	37.00	1.000	OK
2	2	1.50	0.625	OK	2	2	3.75	1.375	OK	2	3	28.00	8.000	OK
2	3	2.50	0.375	OK	2	3	2.25	0.125	OK	5	1	41.50	5.500	OK
2	4	2.75	0.625	OK	2	4	1.50	0.875	OK	5	2	43.50	7.500	OK
5	1	0.00	2.125	OK	5	1	4.00	1.625	OK	5	3	37.00	1.000	OK
5	2	0.00	2.125	OK	5	2	3.00	0.625	OK	7	1	37.00	1.000	OK
5	3	0.00	2.125	OK	5	3	2.00	0.375	OK	7	2	29.25	6.750	OK
5	4	0.00	2.125	OK	5	4	3.00	0.625	OK	7	3	31.75	4.250	OK
7	1	0.25	1.875	OK	7	1	0.00	2.375	OK	8	1	40.50	4.500	OK
7	2	0.00	2.125	OK	7	2	0.75	1.625	OK	8	2	40.00	4.000	OK
7	3	1.25	0.875	OK	7	3	0.75	1.625	OK	8	3	37.00	1.000	OK
7	4	0.00	2.125	OK	7	4	2.00	0.375	OK	9	1	32.25	3.750	OK
8	1	0.75	1.375	OK	8	1	2.25	0.125	OK	9	2	37.50	1.500	OK
8	2	4.25	2.125	OK	8	2	1.50	0.875	OK	9	3	32.75	3.250	OK
8	3	2.25	0.125	OK	8	3	2.75	0.375	OK	10	1	34.25	1.750	OK
8	4	3.25	1.125	OK	8	4	3.00	0.625	OK	10	2	29.75	6.250	OK
9	1	3.75	1.625	OK	9	1	2.50	0.125	OK	10	3	24.50	11.500	OK
9	2	3.75	1.625	OK	9	2	1.50	0.875	OK					
9	3	3.75	1.625	OK	9	3	3.75	1.375	OK					
9	4	2.00	0.125	OK	9	4	2.00	0.375	OK					
10	1	1.25	0.875	OK	10	1	1.50	0.875	OK					
10	2	2.78	0.660	OK	10	2	1.27	1.109	OK					
10	3	2.25	0.125	OK	10	3	4.25	1.875	OK					
10	4	1.75	0.375	OK	10	4	3.00	0.625	OK					

Box plot comparison of homogeneity, participating laboratories, and stability test of raw data is given in Figure 20, Figure 21, Figure 22 and of processed data in Figure 23 and Figure 24.

Figure 20. Box plot comparison of homogeneity, participating laboratories, and stability test on *F.avenaceum* on medium level on raw data

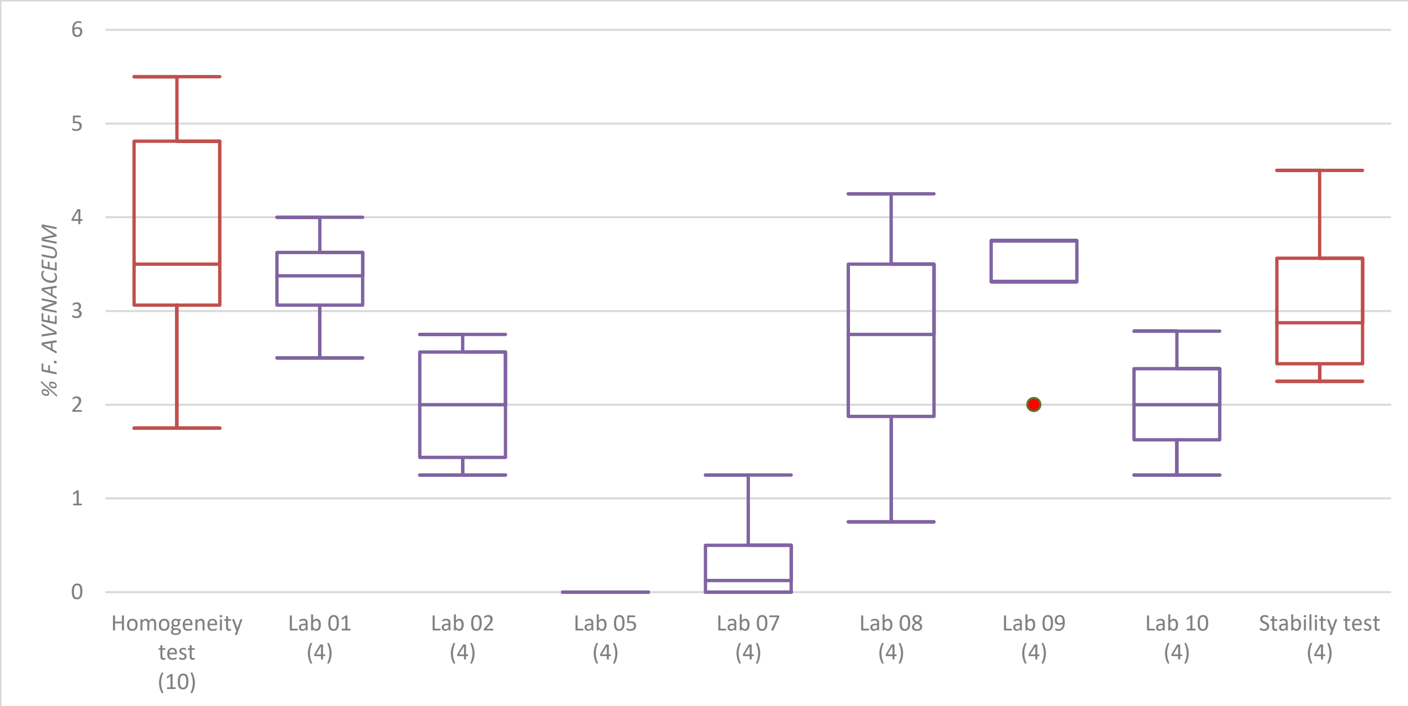


Figure 21. Box plot comparison of homogeneity, participating laboratories, and stability test on *F.poae* on medium level on raw data

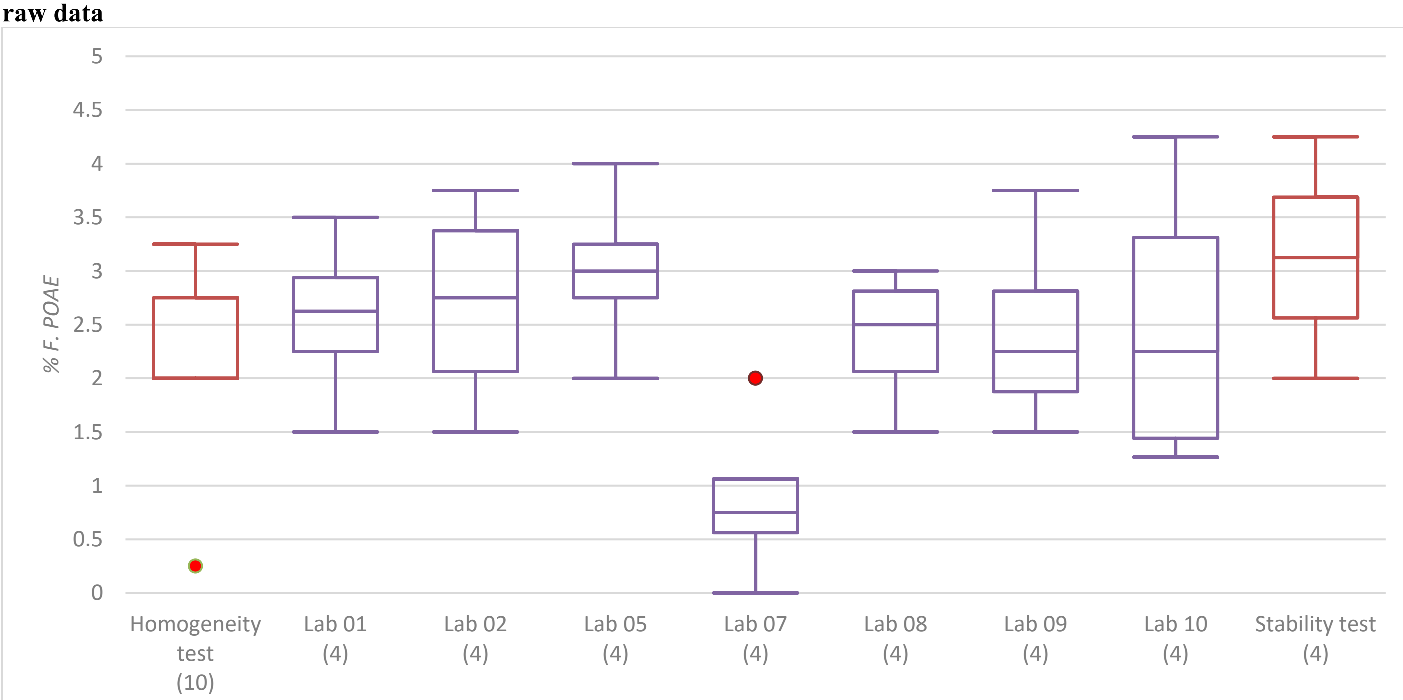


Figure 22. Box plot comparison of homogeneity, participating laboratories, and stability test on *F.poae* on high level on raw data

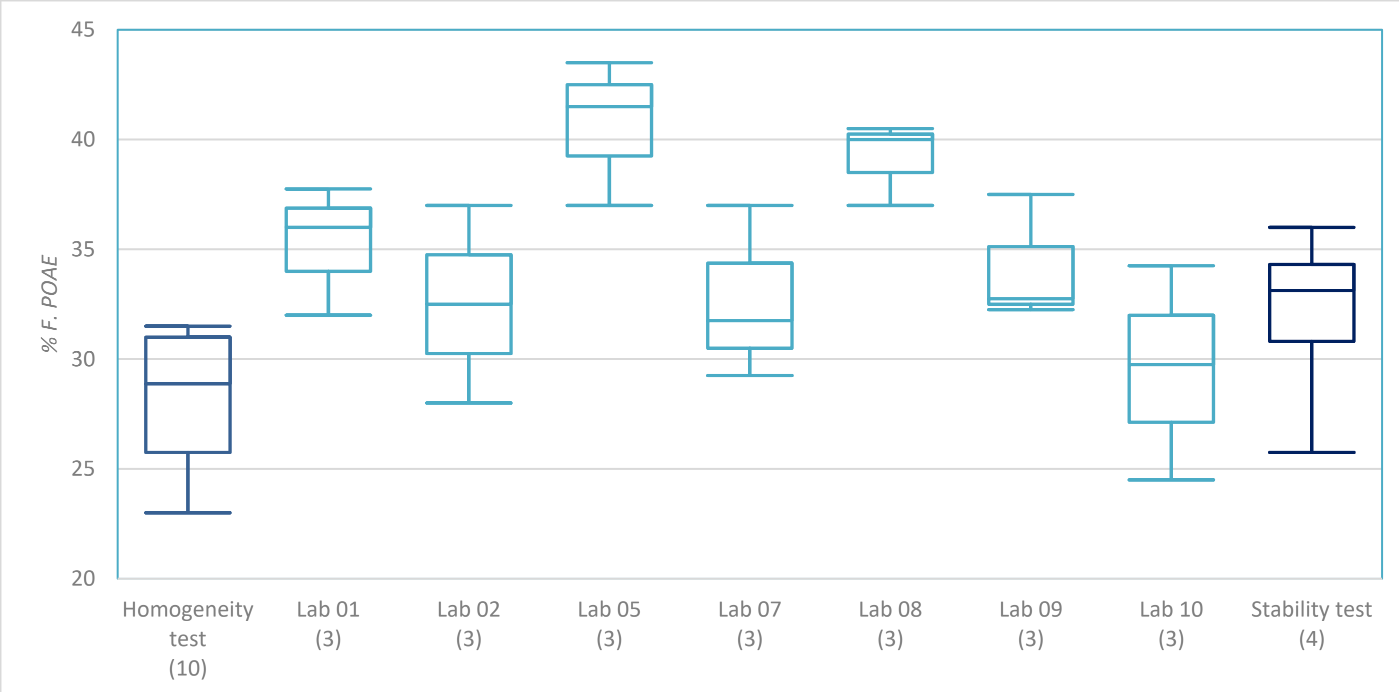
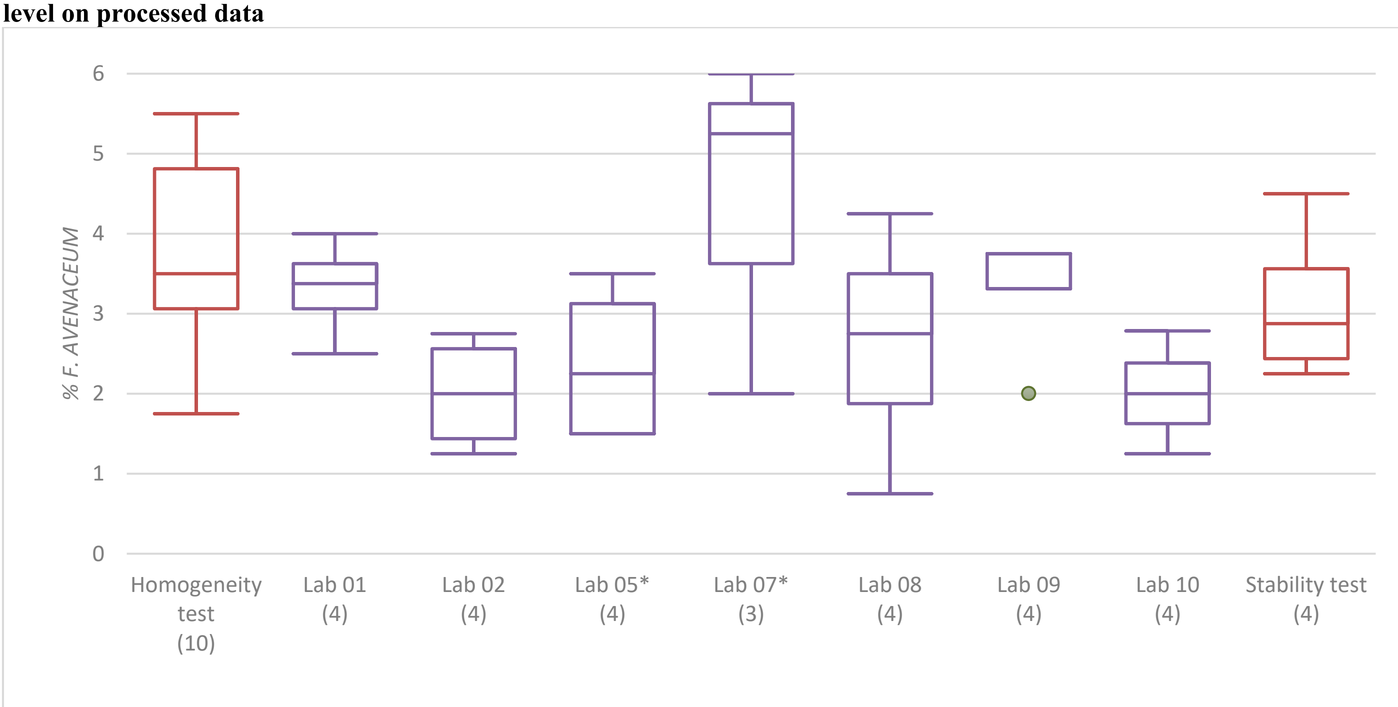
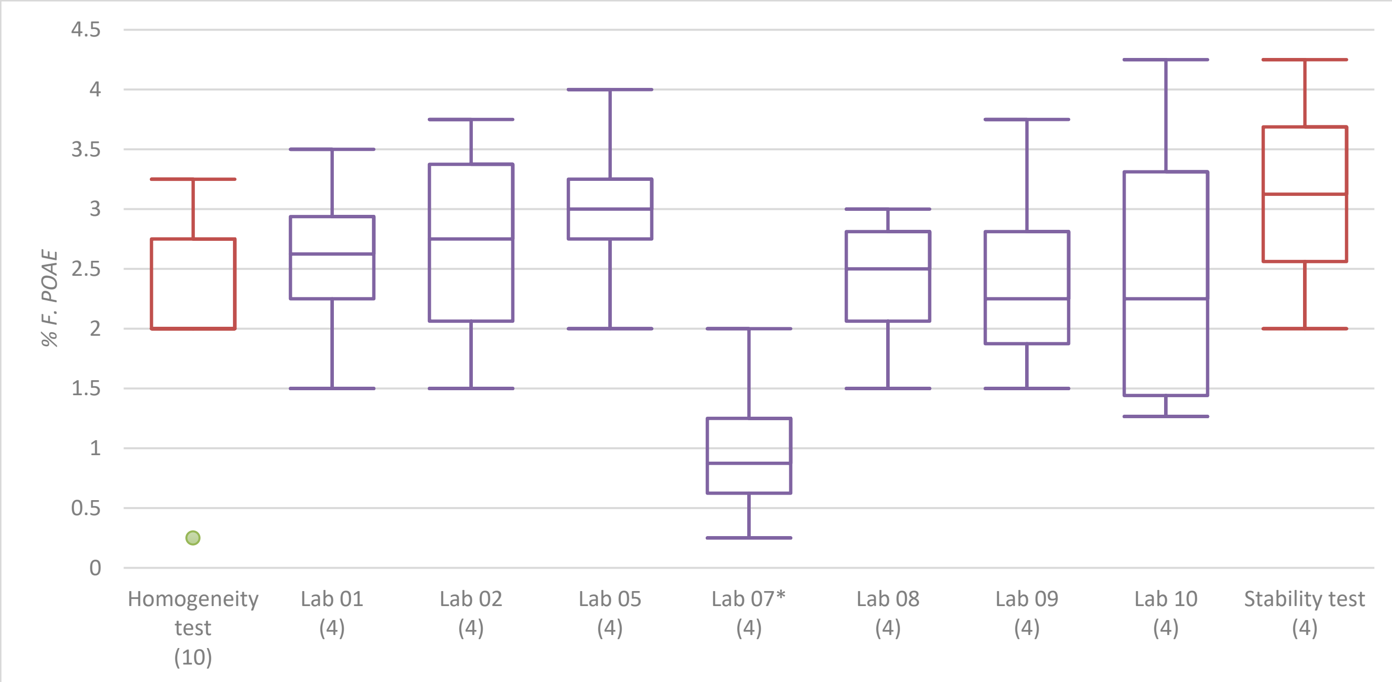


Figure 23. Box plot comparison of homogeneity, participating laboratories, and stability test on *F.avenaceum* on medium level on processed data



* Laboratories for which the data were reclassified as explained in 4.4.2.2

Figure 24. Box plot comparison of homogeneity, participating laboratories, and stability test on *F.poae* on medium level on processed data



* Laboratories for which the data were reclassified as explained in 4.4.2.2

According to the quantitative analysis of results by Hampel's method, no outliers were identified for the two levels of contamination (medium and high) for raw data.
The raw data analysis by Box Plot, highlights outliers on Lab 09 on medium level contaminated with *F.avenaceum*, Lab 07 on medium level contaminated with *F.poae* and homogeneity test on medium level contaminated with *F.poae*.
When looking at the processed data, only one outlier remains (Lab 09 medium level - *F.avenaceum*), this result was not considered as an outlier. Indeed, it has been detected by the method and the laboratory, and it is included in the value range of the homogeneity test.
The processed data analysis by Box Plot showed no differences between laboratories.

Conclusion
Repeatability and reproducibility are then validated according to the quantitative results.

4.4.3. CT Conclusion

Based on homogeneity test, each seed lot was characterized as homogeneous qualitatively and quantitatively.
Stability test showed that each seed lot was stable in time, both qualitatively and quantitatively.
This comparative test (CT) organized with 7 participants allowed the evaluation and validation of the accuracy, repeatability and reproducibility of the method, the media used, the incubation conditions and the morphological criteria. Validation of the other performance criteria of the method is presented priorly in this validation report.
The method allowed the detection and identification on isolates of the *Fusarium* targets as expected.

5. General conclusion

All performance criteria were validated, a summary is available Table 34.

Table 34. Summary of performance criteria

Performance criteria		Practical information	Validation
Analytical specificity		Identification based on morphological criteria of 63 isolates (44 target and 19 non-target) using MA, PDA, SNA and CLA	**Validated**
Analytical sensitivity		3 spiked samples/crop (wheat, barley and oat) on MA and PDA	**Validated**
Robustness	**Light**	Sporulation compared for 4 isolates on MA and PDA, incubation under darkness or NUV	**Validated** Sporulation is mostly better under NUV for both media.
	Incubation	Comparison of 6, 7 and 9 days of incubation on 3 replicates of naturally infected wheat on PDA.	**Validated for 6 and 7 days of incubation** Cross contamination could occur after 7 days.
	Plating design	2 samples of 400 seeds with 5 or 10 seeds/plate	**Validated**
Repeatability/Reproducibility		Whole method tested on 10 samples by 7 labs	**Validated**

Nevertheless, the CT results on naturally infected seeds showed that the identification of the species by morphological criteria is difficult. The difficulty is related to the presence of several different species and not due to the lack of expertise of the laboratories.
All laboratories identified the species by morphological criteria for the target isolates.

This conclusion highlights the decision that must be taken about the expression of the result in the future rule.

Different types of results were proposed in the test plan:

1. There is no need to identify at the species level, the result will be *Fusarium* and/or *Microdochium* spp.
2. There is a need to identify at the species level and the morphological criteria are sufficient to make identification, it will be done on PDA/MA, and if necessary, additionally on CLA/SNA, and the result will specify the *Fusarium* species
3. There is a need to identify at the species level, but the morphological criteria are difficult or overlapping for some species identification, the result will specify the identified *Fusarium* species if identified, possible options of *Fusarium* species and/or *Fusarium* spp

Conclusion regarding the rule:

1. *Fusarium* and *Microdochium* can be distinguished and there is an existing separate rule for *Microdochium*.
2. The morphological criteria are not sufficient to identify all the target species, so this proposition is not selected.
3. There is a need to identify at the species level, but the morphological criteria are difficult or overlapping for some species identification, so this proposition is the most fit for purpose:
   The expression of the result will:
   - **Specify the identified *Fusarium* species**

And/or

- **Be given in complex following Crous et al., 2021; O'Donnel et al., 2022**

And/or

- **Be given as *Fusarium* sp. when the identification is not possible.**
- **A mix of these expressions depending on the *Fusarium* species present.**

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7. Annexes

List of annexes provided with report:

- Annex 1: Malt-agar media (MA)
- Annex 2: Potato Dextrose Agar media (PDA)
- Annex 3: Spezieller Nahrustoffarmer Agar (Synthetic Nutrient-Poor Agar) (SNA)
- Annex 4: Carnation Leaf-piece Agar (CLA)
- Annex 5: Morphological criteria of main Fusarium on cereals
- Annex 6: Analytical specificity raw data
- Annex 7: Analytical sensitivity raw data

Annex 1: Malt-agar media (MA)

Agar: 20 g
Malt: 10 g
Distilled/deionised water: 1000 ml
Streptomycin sulphate*: may be used between 50 mg and 130 mg, depending on the level of saprophytic bacterial contamination commonly encountered.

* Added after autoclaving. Streptomycin sulphate can be dissolved in water. Filter sterilisation is required.

Preparation

1. Weigh out the malt into a suitable container.
2. Add 1000 mL of distilled/deionised water:
3. Add the agar and the remainder of the water, and dissolve completely before autoclaving.
4. Autoclave at 121 °C and 15 psi for 15 min.
5. Allow agar to cool to approximately 50 °C and add streptomycin sulphate dissolved in water.
6. Pour 18-20 ml of the molten agar into 90 mm Petri dishes and allow to solidify before use.

If using ready-to-use 1 % malt agar, prepare according to specifications of supplier.

Annex 2: Potato Dextrose Agar media (PDA)

Potato dextrose agar: make according to specification of supplier
Distilled/deionised water: 1000 ml
Streptomycin sulphate*: may be used between 50 mg and 130 mg, depending on the level of saprophytic bacterial contamination commonly encountered

* Added after autoclaving. Streptomycin sulphate can be dissolved in water. Filter sterilisation is required.

Preparation

1. Weigh out ingredients into a suitable autoclavable container.
2. Add 1000 ml of distilled/deionised water.

3. Dissolve powdered PDA in the water by stirring.
4. Autoclave at 121 °C and 15 psi for 15 min.
5. Adjust to pH 5.6 ±0.2 pH units.
6. Allow agar to cool to approximately 50 °C and add streptomycin sulphate dissolved in water.
7. Pour 20–22 ml of molten agar into 90 mm Petri dishes and allow to solidify before use.

Annex 3: Spezieller Nahrustoffarmer Agar (Synthetic Nutrient-Poor Agar) (SNA)

Agar: 15 g
K2HPO4: 1.0 g
KNO3: 1.0g
MgSO47H2O: 0.50 g
KCL: 0.50 g
Glucose: 0.20 g
Saccharose: 0.20 g
Distilled/deionised water: 1000 mL
Sterile filter paper (around $1cm^2$ each)

1. Weigh out agar and other compounds into a suitable autoclavable container.
2. Add 1000 ml of distilled/deionised water.
3. Dissolve completely the ingredients in water by stirring.
4. Autoclave at 15 psi and 121 °C for 15 min.
5. Allow to cool to approximately 50°C.
6. Pour 20 ml into 90 mm petri dishes and allow to solidify before use.
7. Aseptically place 3 filter papers onto the surface of each media

Annex 4: Carnation Leaf-piece Agar (CLA)

Agar: 20 g
Distilled/deionised water: 1000 mL
Carnation leaf pieces (5 - 8 mm^2, dried at 70°C for 3 to 4 hours, and gamma irradiated at 2.5 megarads) (CCP): up to 10 pieces are placed onto a 90 mm water agar plate

1. Weigh out agar into a suitable autoclavable container.
2. Add 1000 ml of distilled/deionised water.
3. Dissolve completely the ingredients in water by stirring.
4. Autoclave at 15 psi and 121 °C for 15 min.
5. Allow to cool to approximately 50°C
6. Pour 20 ml into 90 mm petri dishes and allow to solidify before use.
7. Aseptically place 10 pieces (spaced apart) of carnation leaves onto the surface of each plate.

Annex 5: Morphological criteria of main Fusarium on cereals

The morphological criteria listed below are based on descriptions from bibliography (see references) and from observations obtained during the performance study of the method in analytical specificity on a collection of target isolates. The media used, the components of the media, and incubation conditions (darkness/NUV light) can lead to slight differences in morphological appearance. Main criteria are described below and are exemplified in photos of different species.

The species which are described here are based on Index Fungorum (http://www.speciesfungorum.org/Names/Names.asp). When other species names are described, the reference is given in the text.

Table 1: *Fusarium* complex

Species	**Fusarium complex**
F. graminearum *Schwabe*	*F. sambucinum* complex
F. pseudograminearum *Aoki & O'Donnell*	
F. culmorum *(W.G. Smith) Saccardo*	
F. crookwellense *Burgess, Nelson & Toussoun*	
F. langsethiae *Torp & Nirenberg*	
F. poae *(Peck) Wollenweber*	
F. sporotrichioides *Sherbakoff*	
F. tricinctum *(Corda) Saccardo*	*F. tricinctum* complex
F. avenaceum*(Fries) Saccardo*	

Fusarium avenaceum (Fr.) Sacc.

See Figure 1

On Malt-agar the mycelium is abundant and aerial, yellow to pink coloured, sometimes cream white. Sporodochia are orange, concentrated in the centre of the colony.

On PDA, growth can vary from slow to somewhat rapid, the aerial mycelium is dense and white but frequently varies in colour from yellow/tan to reddish-brown. The under-surface colouration varies from tan to carmine red to dark brown. Orange sporodochia may form on colonies originating from seed, depending on growth conditions. Sporodochia form quite often in the centre of the colony on pure cultures.

Conidiophores: Unbranched and branched monophialides.

Macroconidia are very long, slender, thin walled with a basal cell foot-shaped and an apical cell elongated that may be bent.

Microconidia (cigar-shaped) are often rare. Mixed sizes of conidia, micro and macro are observed from sporodochia on isolates on PDA, but on CLA only macroconidia true to type are observed.

Chlamydospores are absent.

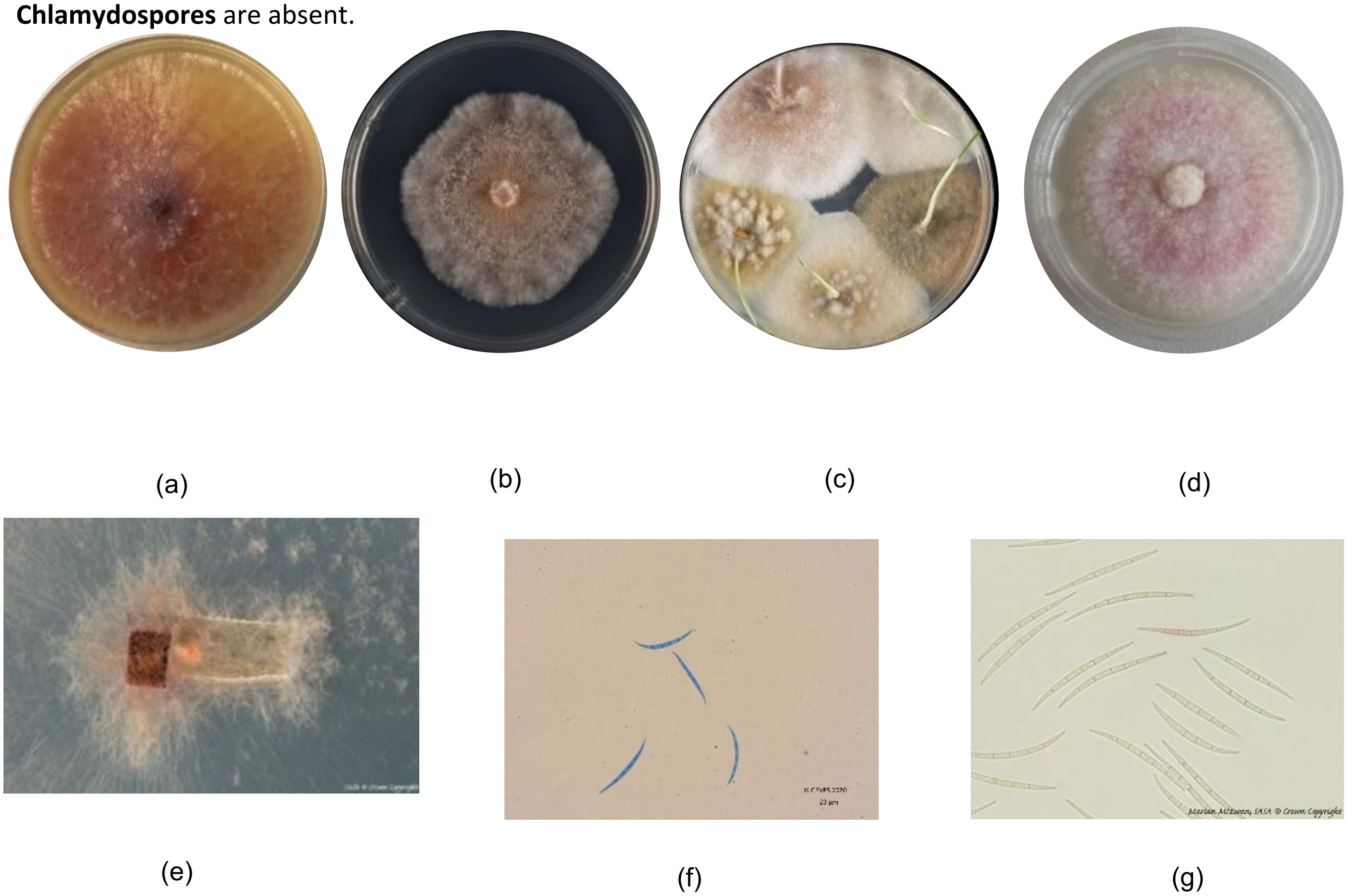


Fig 25 - *Fusarium avenaceum*

(a) MA darkness for 14 days
(b) PDA (Oxoid) with NUV light for 5 days
(c) PDA (Oxoid) after 7 days growth on seed (top left colony)
(d) PDA (Difco) in darkness for 7 days
(e) Formation of orange sporodochia containing macroconidia on CLA after incubation under near NUV light
(f) Macroconidia stained with methyl blue stain (x200)
(g) Macroconidia unstained from CLA (x400)

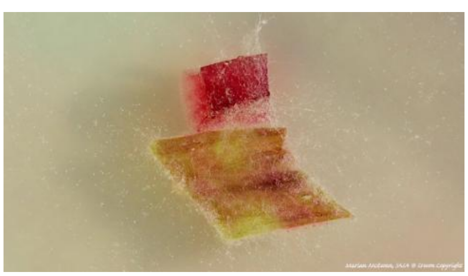
***Fusarium graminearum* Schwabe**
***Fusarium pseudograminearum* O'Donnell & T. Aoki**
See Figure 2

On Malt-agar the mycelium is abundant and aerial, pink coloured and white to yellow on the aerial part of the mycelium. Sporodochia are orange and concentrated in the centre of the colony.
On PDA, growth is usually rapid (e.g. fill in Petri dishes within 4 days on pure culture) but could be variable, with dense aerial mycelium and is frequently yellow to tan with the margins white to carmine red. The under-surface is usually carmine red. Red brown to orange sporodochia, if present, are sparse in the dark. Sporodochia often appears in less than a week when under NUV light.
Conidiophores: Unbranched and branched monophialides.
Macroconidia are often present distinctly septate, thick walled, straight to moderately sickle-shaped, the ventral surface is almost straight, and the dorsal surface smoothly arched. The basal cell is distinctly foot shaped. Apical cell curved; cone shaped as a beak.
Microconidia are absent.
Chlamydospores are rare, formed in the macroconidia or the mycelium.
As these both species share common morphological criteria they cannot be distinguished visually on plates.


(a)


(b)


(c)


(d)

(e)

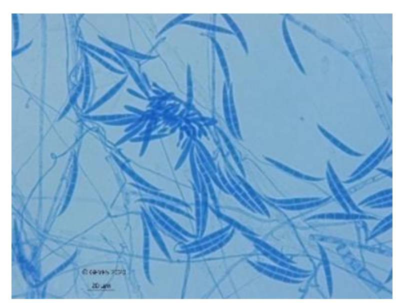

(f)

Fig 26- *Fusarium graminearum*

(a) MA in darkness for 14 days
(b) PDA (Oxoid) in darkness
(c) PDA (Difco) in darkness for 7 days
(d) *F. graminearum* on CLA
(e) Macroconidia with methyl blue stain (x200)
(f) Macroconidia unstained from CLA (x400)

Fusarium culmorum (Wm.G. Sm.) Sacc
See Figure 3

On Malt-agar the mycelium is short, pink to carmine red. Sporodochia form on older cultures and are orange to red brown in colour.

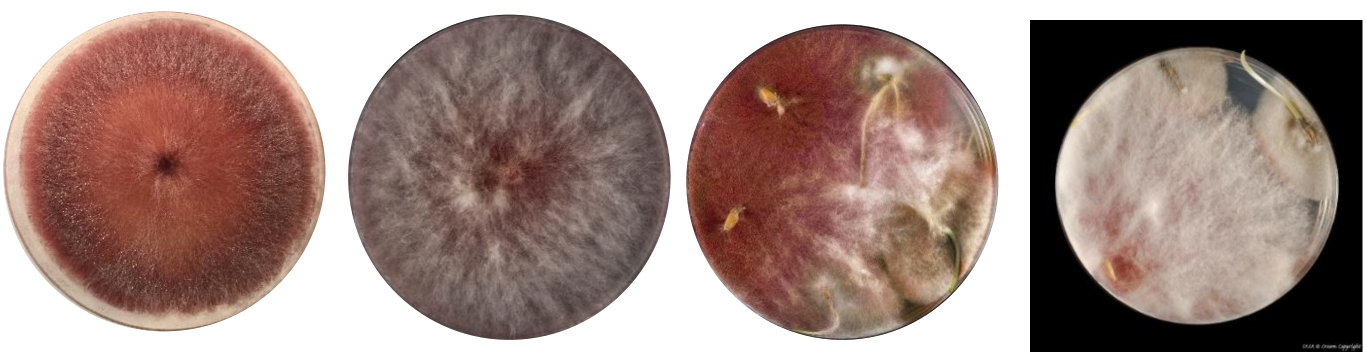
On PDA growth is rapid, with dense aerial mycelium, generally white but often yellow to tan on the base. The undersurface is carmine red. Orange to red brown sporodochia appear readily.

Conidiophores: Unbranched and branched monophialides.

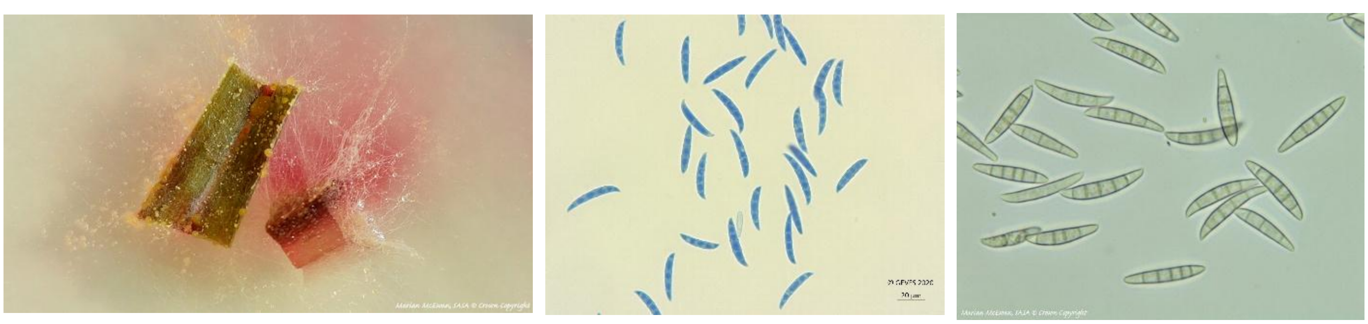
Macroconidia are stout, distinctly septate, thick-walled, and have straight to curved ventral and dorsal surfaces. The basal cell is usually distinctly round and can occasionally be foot-shaped, to slightly notched.

Microconidia are absent.

Chlamydospores generally form abundantly and quickly; they may occur singly, in chains, or in clumps.



(a) (b) (c) (d)



(e) (f) (g)

Fig 27 - *Fusarium culmorum*

(a) MA darkness for 14 days
(b) PDA (Oxoid) in darkness
(c) High sporulating isolate PDA (Oxoid) left of image, 7 days in darkness
(d) Low sporulating isolate PDA (Oxoid) left of image, 7 days in darkness
(e) *F. culmorum* on CLA with orange sporodochia
(f) Macroconidia with methyl blue stain (x200)
(g) Macroconidia unstained from CLA (x400)

Fusarium crookwellense L.W. Burgess, P.E. Nelson & Toussoun
Fusarium cerealis (ref)

See Figure 4

On Malt-agar the mycelium is abundant and aerial, white to pink, sometimes yellow. Sporodochia are orange to red brown.

On PDA growth is rapid, with dense aerial mycelium, white in colour and then tan. The under-surface is carmine red. Orange to red brown sporodochia generally appear early in the centre of the culture and later in other portions of the culture.

Conidiophores: Unbranched and branched monophialides.

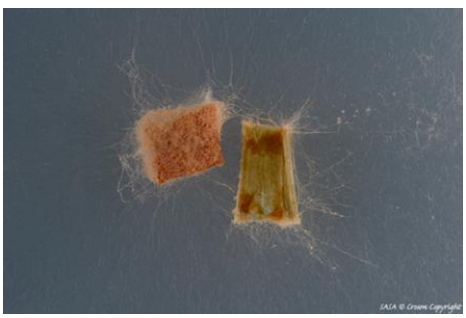
Macroconidia are strongly septate, thick-walled, and unequally curved, with the ventral surface less curved than the dorsal surface, which is strongly arched. The basal cell is distinctly foot shaped. The apical cell is distinctly curved and tapers to a narrow tip.

Microconidia are absent.

Chlamydospores are present and are formed in the hyphae and the macroconidia.

(a)

(b)



(c)

(d)

(e)

Fig 28 - *Fusarium crookwellense*

(a) MA in darkness for 14 days
(b) PDA (Oxoid) in darkness for 7 days
(c) *F. crookwellense* on CLA with dark orange sporodochia containing macroconidia forming readily
(d) Macroconidia unstained (x400) from sporodochia on CLA
(e) Macroconidia with methyl blue stain (x400)

Fusarium langsethiae Torp & Nirenberg

See Figure 5

On Malt-agar the mycelium is short, white to yellow or pale orange. Sporodochia are rare.

On PDA, growth is not very rapid. Usually white powdery mycelium. The under-surface is often white but can vary in several colours (yellow, pink, purple). Very little aerial mycelium, variation could occur. Globose microconidia in abundance are formed in the powdery mycelium.

Conidiophores: Unbranched and branched monophialides and polyphialides.

Macroconidia are absent.

Microconidia are abundant, globose shaped, in clumps.

Chlamydospores are absent.

(a) (b) (c) (d)

(e) (f) (g)

Fig 29 - *Fusarium langsethiae*

(a) MA darkness for 17 days
(b) PDA (Difco)
(c) PDA (Difco) in darkness for 7 days
(d) PDA (Oxoid) darkness for 7 days
(e) Microconidia with methyl blue stain (x200)
(f) Microconidia unstained (x400)
(g) Microconidia with methyl blue stain (x1000)

Fusarium poae (Peck) Wollenw.

See Figure 6

Colonies have a distinct smell of apples.

On Malt-agar the mycelium is dense, aerial, white to pink. Sporodochia are rare.

On PDA growth is rapid, with dense aerial, cotton like mycelium that is white, yellow to pink in colour. Spores are readily formed in the mycelium. The under-surface is usually carmine red. Sporodochia are rare.

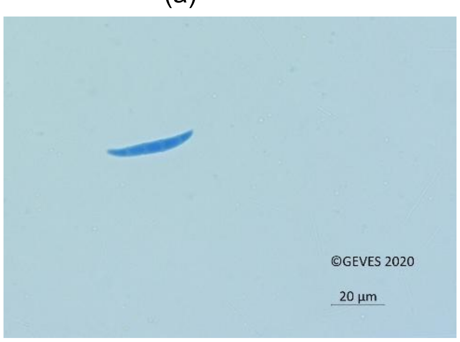
Conidiophores: Unbranched and branched monophialides. Microconidiophores are short and fat, almost globose, and quite distinctive.

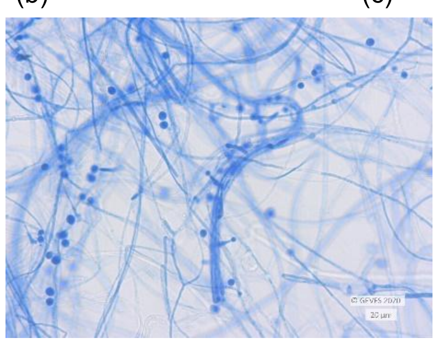
Macroconidia are generally rare, typically sickle-shaped, 3-5 septate, and have a foot-shaped basal cell.

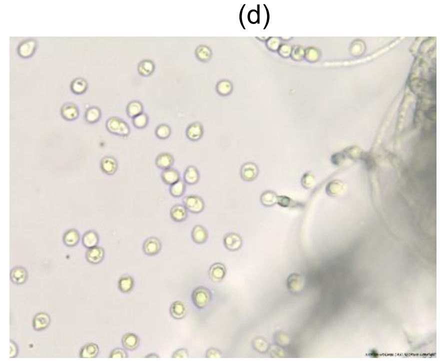
Microconidia are abundant, globose, or oval in shape (napiform), 0-1 septate, and often have a distinct papilla.

Chlamydospores occurs infrequently and may be in clumps or chains

(a) (b) (c) (d)







(e) (f) (g)

Fig 30 - *Fusarium poae*

(a) MA darkness for 14 days
(b) PDA (Oxoid) after 7 days in darkness
(c) PDA (Oxoid) on seed sample after 7 days in darkness, colony at top
(d) PDA (Difco) darkness for 7 days
(e) Macroconidia with methyl blue stain (x400)
(f) Microconidia with methyl blue stain (x200)
(g) Microconidia unstained (x400)

Fusarium tricinctum (Corda) Sacc.,

See Figure 7

On Malt-agar the mycelium is short, white to dark pink, sometimes orange. Sporodochia only appears on old cultures.

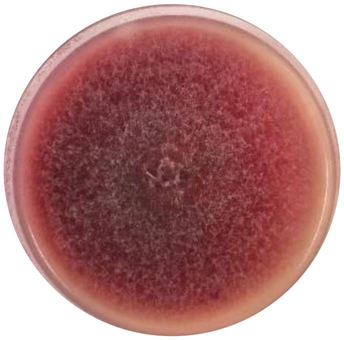
On PDA, the aerial mycelium is dense and white to yellow. The under-surface is carmine red. Sporodochia appearing as the culture ages.

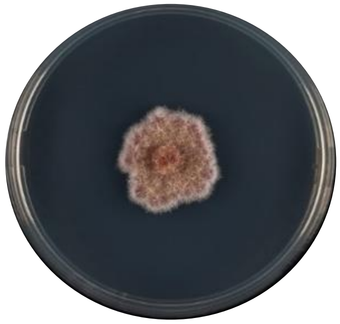
Conidiophores: Unbranched and branched monophialides.

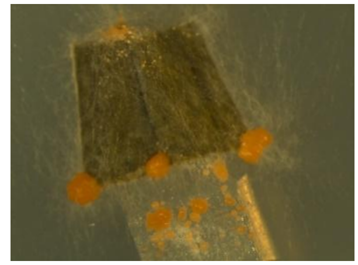
Macroconidia are abundant, sickle shaped. The basal cell is distinctly foot-shaped or notched.

Microconidia are abundant and are lemon- to pear-shaped or spindle shaped, 0-1 septate, and often have a papilla at the base. Often appears in false heads like a cluster of grapes in the arial mycelium.

Chlamydospores can be formed by some isolates, but forms very slowly. If present, they are formed singly or in chains.



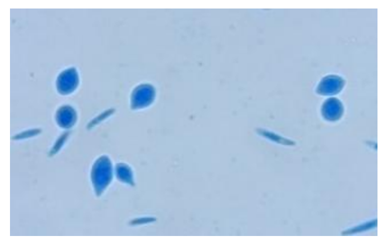




(c)

(a)

(b)



(d)

(e)

(f)

(g)

Fig 31 - *Fusarium tricinctum*

(a) MA darkness for 17 days
(b) PDA (Oxoid) darkness after 7 days
(c) Orange sporodochia on CLA
(d) Macroconidia with methyl blue stain (x400)
(e) Macroconidia unstained from CLA (x400)
(f) Juvenile macroconidia and microconidia (x400)
(g) Juvenile macroconidia and microconidia with methyl blue stain (x1000)

***Fusarium sporotrichioides* Sherb.,**

See Figure 8

On Malt-agar the mycelium is aerial, white to pink, sometimes orange. Sporodochia only appears on old cultures.

On PDA growth is rapid and the aerial mycelium is dense and white to yellow. The under-surface is carmine red. Sporodochia appearing as the culture ages.

Conidiophores: Unbranched and branched monophialides and polyphialides.

Macroconidia are abundant, sickle shaped, 3-5 septate. The basal cell is not distinctly foot-shaped or notched.

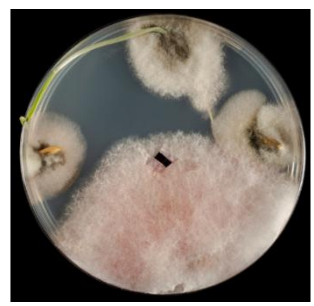
Microconidia are abundant and are oval to pear-shaped or spindle shaped, 0-1 septate, often with a papilla at the base.

Chlamydospores are present and abundant and formed singly, in chains, or in clumps.

(a)

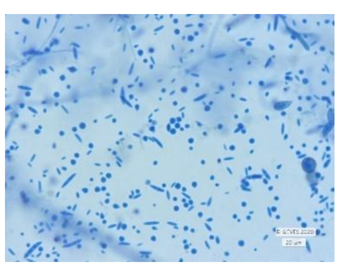
(b)

(c)



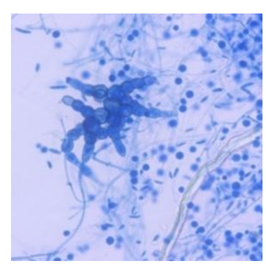
(d)

(e)



(f)

(g)



(h)

Fig 32 - *Fusarium sporotrichioides*

(a) MA darkness for 21 days
(b) PDA (Oxoid) after 7 days in darkness
(c) PDA (Oxoid) on seed sample (bottom left) at 7 days
(d) PDA (Oxoid) (bottom colony) on seed sample after 7 days

(e) isolate on CLA
(f) Microconidia with methyl blue stain (x200)
(g) Macroconidia unstained from CLA (x400)

(h) Chlamydospores with methyl blue stain (x400)

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Annex 6: Analytical specificity raw data

TARGET		Match with target's criteria							
Strain	**N° PAS**	**MA**	**PDA Difco**	**PDA Oxoid**	**SNA**	**CLA**	**General conclusion on the isolate**	**PCR Validation by CREA/SASA**	**Comments**
F. avenaceum	11	Yes	Yes		Yes		**Yes**		
F. avenaceum	2675	Yes		Doubt	Yes	Yes	**Yes**		PDA Oxoid : Absence of microconidia
F. avenaceum	2689	Yes	Yes		Yes		**Yes**		
F. avenaceum	2690	No	Yes		No		**Yes**	*F. avenaceum*	MA : Mycelium cream white. Lots of macro and microconidia, very confusing. PDA Difco and Oxoid : The mycelium is aerial, roseum and sterile.
F. avenaceum	2714	Yes		Yes	Yes	Yes	**Yes**		
F. crookwellense / F. cerealis	150	Yes	Yes		Yes		**Yes**		
F. crookwellense / F. cerealis	865	No	Yes		Yes		**Yes**	*F.cerealis*	MA : The mycelium is short white to pink. Macroconidia present but they are quit long and thin. Chlamydospores are absent.

TARGET		Match with target's criteria							
Strain	N° PAS	MA	PDA Difco	PDA Oxoid	SNA	CLA	General conclusion on the isolate	PCR Validation by CREA/SASA	Comments
F. crookwellense / F. cerealis	1015	Doubt	Doubt		Doubt		**Doubt**	*F.cerealis*	MA : Mycelium is abundant, aerial, pink but absence of sporulation PDA Difco :The mycelium is aerial, red, macroconidia with pronounced dorsal curvature, basal cell foot shaped.
F. crookwellense / F. cerealis	2706	Yes		Doubt	Yes	Doubt	**Yes**		PDA Oxoid : Mycelium fine, flat in centre but no sporulation on plate. Poor sporulation when F. crookwellense should sporulate readily. Macroconidia restricted, but do slightly resemble F. culmorum more than F. graminearum.
F. crookwellense / F. cerealis	2721	Yes	Yes		Yes		**Yes**		
F. culmorum	20	Yes	Yes		Yes		**Yes**		
F. culmorum	227	Yes	Yes		Yes		**Yes**		
F. culmorum	945	Yes	Yes		Yes		**Yes**		
F. culmorum	2673	Yes		Yes	Yes		**Yes**		
F. culmorum	2716	Yes	Yes		Yes		**Yes**		
F. graminearum	2674	Yes		Yes	Yes		**Yes**		
F. graminearum	2693	Yes	Yes		Yes		**Yes**		
F. graminearum	2694	Yes	No		No		**Doubt**	*F. graminearum*	PDA Difco : The mycelium is aerial, red and sterile.

TARGET		Match with target's criteria							
Strain	**N° PAS**	**MA**	**PDA Difco**	**PDA Oxoid**	**SNA**	**CLA**	**General conclusion on the isolate**	**PCR Validation by CREA/SASA**	**Comments**
F. graminearum	2695	Yes	No		No		**Doubt**	*F. graminearum*	PDA Difco and MA : The mycelium is aerial, pale red and sterile.
F. graminearum	868	Yes		Doubt	Yes	Doubt	**Yes**	*F. graminearum*	MA : The mycelium is aerial, abundant, pink. Sporodochia, macroconidia and microconidia are absent. Chlamydospores are present.
F. graminearum	2715	Yes		Yes	Yes		**Yes**		
F. langsethiae	27	Yes		Yes	Yes		**Yes**		
F. langsethiae	30	Yes	Yes		Yes		**Yes**		
F. langsethiae	32	Yes	Yes		Yes		**Yes**		
F. langsethiae	2686	Yes		Yes	Yes		**Yes**		
F. langsethiae	2720	Yes	Yes		Yes		**Yes**		
F. poae	43	Yes	Yes		Yes		**Yes**		
F. poae	2678	Yes		Yes	Yes		**Yes**		
F. poae	2696	Yes	Yes		Yes		**Yes**		
F. poae	2697	Yes	Yes		Yes		**Yes**		
F. poae	2719	Yes	Yes		Yes		**Yes**		
F. pseudograminearum	2698	Yes		Yes	Yes	Yes	**Yes**		
F. pseudograminearum	2699	Yes	Doubt		Yes		**Yes**		

TARGET		Match with target's criteria							
Strain	**N° PAS**	**MA**	**PDA Difco**	**PDA Oxoid**	**SNA**	**CLA**	**General conclusion on the isolate**	**PCR Validation by CREA/SASA**	**Comments**
F. pseudograminearum	2700	Yes	Yes		Yes		**Yes**		
F. sporotrichioides	47	Yes	Yes		Yes		**Yes**		
F. sporotrichioides	2532	Yes		Yes	Yes	Yes	**Yes**		
F. sporotrichioides	2701	Yes	Yes		Yes		**Yes**		
F. sporotrichioides	2707	Yes		Yes	Yes	Yes	**Yes**		
F. sporotrichioides	2718	Yes	Yes		Yes		**Yes**		
F. tricinctum	48	Yes		Yes	Yes	Yes	**Yes**		
F. tricinctum	50	Yes	No		No		**Yes**	*F.tricinctum*	PDA : The mycelium is slow growing, flat and yellow to red. No sporodochia and macroconidia are falcate with basal cell no foot shaped. Microconia napiform and conidiogenous cells are monophialides.
F. tricinctum	2522	Yes	Yes		Yes		**Yes**		
F. tricinctum	2717	No	No		Doubt		**Doubt**	*F.tricinctum*	MA : Mycelium short and white. Presence of long microconidia absence of typical microconidia lemon to pear-shape. PDA Difco and SNA : Dense white cottonlike mycelium, weakly pink at the edges.Microconidia pear-shaped and some short two-celled "comma-shaped"
F. tricinctum	143	No	No		No		**No**	*F.tricinctum*	Isolate removed from the data analysis : Sterile and presence of bacteria

TARGET		Match with target's criteria							
Strain	N° PAS	MA	PDA Difco	PDA Oxoid	SNA	CLA	General conclusion on the isolate	PCR Validation by CREA/SASA	Comments
F. tricinctum	2929	Yes		Yes	Yes	Yes	**Yes**		

NON TARGET

Strain	N° PAS	Different from targets's criteria		Different from targets's criteria
		Malt-agar	PDA	
Helminthosporium gramineum	52	Yes	Yes	Yes
Helminthosporium teres	771	Yes	Yes	Yes
Bipolaris sorokiniana (Helminthosporium sativum)	2728	Yes	Yes	Yes
Helminthosporium avenae	2234	Yes	Yes	Yes
Parastagonospora nodorum	2196	Yes	Yes	Yes
Parastagonospora avenae	2755	Yes	Yes	Yes
F.equiseti	2680	Yes	Yes	Yes
F.acuminatum	2687	Yes	Yes	Yes

F.fujikuroi (F.verticillioides, F.subglutinans)	2685	Yes	Yes	Yes
F.proliferatum	1616	Yes	Yes	Yes
F.oxysporum	2723	Yes	Yes	Yes
F.solani	244	Yes	Yes	Yes
F. lateritium	2676	Yes	Yes	Yes
Epicoccum sp.	828	Yes	Yes	Yes
F. venenatum	2681	Yes	Yes	Yes
F. caeruleum	2683	Yes	Yes	Yes
Alternaria tenuissima	829	Yes	Yes	Yes
Cladosporium sp.	1237	Yes	Yes	Yes
Penicilium sp.	1005	Yes	Yes	Yes

Annex 7: Analytical sensitivity raw data

NC = Negative control PC = Positive control

A1,B1,C1 = Samples *F.culmorum*
Y = Contaminated with *F. culmorum*

A2,B2,C2 = Samples *F.tricinctum*
Z = Contaminated with *F.tricinctum*

FIESC: *Fusarium incarnatum - equiseti* species complex
FTSC: *Fusarium tincinctum* complex

Code n°	Crop	Media	Done by	% *F. culmorum*	% *F. tricinctum*	% *F. sp.*	% *F. avenaceum*	% *F. graminearum*	% *F. crookwellense (F. cerealis)*	% *F. poae*	% *F. sporotrichioides*	% *F. acuminatum*
A1	Wheat	MA	GEVES	0.25		0.25				0.25		
B1	Wheat	MA	GEVES	0.25								
C1	Wheat	MA	GEVES	0.25								
PCY	Wheat	MA	GEVES	100								
NCA 1	Wheat	MA	GEVES									
NCB 1	Wheat	MA	GEVES									
NCC 1	Wheat	MA	GEVES									
A1	Barley	MA	GEVES	0.27								
B1	Barley	MA	GEVES	0.29				0.85				
C1	Barley	MA	GEVES	0.27						1.35		
PCY	Barley	MA	GEVES	100								
NCA 1	Barley	MA	GEVES			0.75						
NCB 1	Barley	MA	GEVES				0.75	0.5				
NCC 1	Barley	MA	GEVES				1			1.75		
A1	Oat	MA	GEVES	0.25								
B1	Oat	MA	GEVES	0.25								
C1	Oat	MA	GEVES	0.25			0.25			0.25		
PCY	Oat	MA	GEVES	100								
NCA 1	Oat	MA	GEVES									
NCB 1	Oat	MA	GEVES							0.25		
NCC 1	Oat	MA	GEVES									
A2	Wheat	MA	GEVES		0.25	0.25						
B2	Wheat	MA	GEVES		0.25							

NC = Negative control PC = Positive control

A1,B1,C1 = Samples *F.culmorum*
Y = Contaminated with *F. culmorum*

A2,B2,C2 = Samples *F.tricinctum*
Z = Contaminated with *F.tricinctum*

FIESC: *Fusarium incarnatum - equiseti* species complex
FTSC: *Fusarium tincinctum* complex

Code n°	Crop	Media	Done by	% *F. culmorum*	% *F. tricinctum*	% *F. sp.*	% *F. avenaceum*	% *F. graminearum*	% *F. crookwellense (F. cerealis)*	% *F. poae*	% *F. sporotrichioides*	% *F. acuminatum*
C2	Wheat	MA	GEVES		0.25						0.25	
PCZ	Wheat	MA	GEVES		100							
NCA 2	Wheat	MA	GEVES							0.5		
NCB 2	Wheat	MA	GEVES									
NCC 2	Wheat	MA	GEVES									
A2	Barley	MA	GEVES		0.25	0.5		0.25				
B2	Barley	MA	GEVES		0.25	1		0.5				
C2	Barley	MA	GEVES		0.25			0.25		1	0.5	
PCZ	Barley	MA	GEVES		100							
NCA 2	Barley	MA	GEVES									
NCB 2	Barley	MA	GEVES			1.5		0.5				
NCC 2	Barley	MA	GEVES			0.75	1			0.75		
A2	Oat	MA	GEVES		0.25							
B2	Oat	MA	GEVES		0.25							
C2	Oat	MA	GEVES		0.25							
PCZ	Oat	MA	GEVES		100							
NCA 2	Oat	MA	GEVES									
NCB 2	Oat	MA	GEVES									
NCC 2	Oat	MA	GEVES									
A1	Wheat	PDA	NIBIO/KIMEN						0.25	0.25		
B1	Wheat	PDA	NIBIO/KIMEN						0.25			
C1	Wheat	PDA	NIBIO/KIMEN						0.25			
PCY	Wheat	PDA	NIBIO/KIMEN						100			
NCA 1	Wheat	PDA	NIBIO/KIMEN									
NCB 1	Wheat	PDA	NIBIO/KIMEN									
NCC 1	Wheat	PDA	NIBIO/KIMEN									

NC = Negative control PC = Positive control

A1,B1,C1 = Samples *F.culmorum*
Y = Contaminated with *F. culmorum*

A2,B2,C2 = Samples *F.tricinctum*
Z = Contaminated with *F.tricinctum*

FIESC: *Fusarium incarnatum - equiseti* species complex
FTSC: *Fusarium tincinctum* complex

Code n°	**Crop**	**Media**	**Done by**	**% *F. culmorum***	**% *F. tricinctum***	**% *F. sp.***	**% *F. avenaceum***	**% *F. graminearum***	**% *F. crookwellense (F. cerealis)***	**% *F. poae***	**% *F. sporotrichioides***	**% *F. acuminatum***
A2	Wheat	PDA	NIBIO/KIMEN			0.25					0.25	
B2	Wheat	PDA	NIBIO/KIMEN			0.25						
C2	Wheat	PDA	NIBIO/KIMEN			0.25						
PCZ	Wheat	PDA	NIBIO/KIMEN			100						
NCA 2	Wheat	PDA	NIBIO/KIMEN								0.25	
NCB 2	Wheat	PDA	NIBIO/KIMEN									
NCC 2	Wheat	PDA	NIBIO/KIMEN									
A1	Oat	PDA	SASA	0.25								
B1	Oat	PDA	SASA	0.25								0.25
C1	Oat	PDA	SASA	0.25						0.25		
PCY	Oat	PDA	SASA	100								
NCA 1	Oat	PDA	SASA									
NCB 1	Oat	PDA	SASA									
NCC 1	Oat	PDA	SASA	0.25						0.5		
A2	Oat	PDA	SASA									0.25
B2	Oat	PDA	SASA									0.25
C2	Oat	PDA	SASA									0.25
PCZ	Oat	PDA	SASA			100						
NCA 2	Oat	PDA	SASA									
NCB 2	Oat	PDA	SASA									
NCC 2	Oat	PDA	SASA									
A1	Barley	PDA	CREA	0.25								
B1	Barley	PDA	CREA	0.25	0,25 FTSC	1.50		0.75		2.00		
C1	Barley	PDA	CREA	0.25	0,50 FTSC	0.25		0.25				
PCY	Barley	PDA	CREA	100.00								
NCA 1	Barley	PDA	CREA								0.50	

NC = Negative control PC = Positive control

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Y = Contaminated with *F. culmorum*

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Z = Contaminated with *F.tricinctum*

FIESC: *Fusarium incarnatum - equiseti* species complex
FTSC: *Fusarium tincinctum* complex

Code n°	Crop	Media	Done by	% *F. culmorum*	% *F. tricinctum*	% *F.* sp.	% *F. avenaceum*	% *F. graminearum*	% *F. crookwellense (F. cerealis)*	% *F. poae*	% *F. sporotrichioides*	% *F. acuminatum*
NCB 1	Barley	PDA	CREA			0.25						
NCC 1	Barley	PDA	CREA							3.00		
A2	Barley	PDA	CREA							0.25		
B2	Barley	PDA	CREA			1.00						
C2	Barley	PDA	CREA	0.25		0.25				1.75		
PCZ	Barley	PDA	CREA			100.00						
NCA 2	Barley	PDA	CREA									
NCB 2	Barley	PDA	CREA			1.50				0.50		
NCC 2	Barley	PDA	CREA		0,25 FIESC or FTSC	0.50				0.75		