Method Validation Reports on Rules Proposals for the International Rules for Seed Testing 2025 Edition

Introduction of a new pre-treatment for *Beta vulgaris* seeds

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Summary

The objective of this study was to demonstrate that an alternative pretreatment for *Beta vulgaris* seeds like soaking could be as effective as the use of running water for hours, to leach the inhibitory substances present on the surface of the seeds of this species, and propose a new methodology to be included in the ISTA Rules, considering the important need to conserve water. The experiment was carried out by six laboratories using four seed lots. Beta seeds were germinated between paper media (BP), and pleated paper media (PP) at 20°C. The first and final counts were made at four and fourteen days, respectively. All the methods tested gave comparable results in all the participating laboratories, and based on the statistical analysis and considering the results of repeatability and reproducibility, the new additional - dormancy breaking pretreatment to remove inhibitory substances proposed is: Presoak the seeds in water for two hours, using 250ml of water per 100 seeds at 20-25°C.

Introduction

Beta vulgaris is a widely cultivated species around the world due to its importance as an agricultural and forage crop for animal feed. Currently, ISTA Rules indicate- substrates TP; BP; S and 20<=>30°C; 15<=>25°C; and 20°C as the temperatures for testing this species.

In addition, Table 5A describes prewash (multigerm: 2 h; genetic monogerm: 4 h) and dry at maximum of 25 °C, as a recommendation for breaking dormancy. This procedure is carried out in running water as indicated in 5.6.3.3 of the ISTA Rules.

The seeds of this species maintain phenolic-type inhibitors that can remain for a long period of time both on the surface and inside the seed coat, therefore, this pretreatment is frequently used in laboratories that - test *Beta vulgaris* to leach these inhibitory substances and promote - germination.

Phenols can cause oxygen limitation and hypoxia, affecting the germination performance of seeds by depriving the embryo of access to the required amount of oxygen- (Devlin, H.R.; Harris, I.J, 1984).

On the other hand, we have a responsibility as a society to conserve natural resources, particularly water. In this regard, to develop a sustainable methodology to leach the inhibitors present on the surface of these seeds represents a challenge because it must be able to work effectively to promote the germination and also reduce the considerable water losses that are generated with every lot of *Beta vulgaris* tested under the current methodology, exposed to 2 or 4 hours of running water that is wasted during the process.

Materials and Methods

Four seed lots of *Beta vulgaris*, considering both monogerm and multigerm species were tested for this comparative study. These represented different levels of germination quality based on pre-test data, including forage and sweet varieties. The material was supplied by two companies: KWS (Germany) and SESVANDERHAVE (Belgium).

Each selected seed lot was mixed and divided into the required sample quantity according to Chapter 2 of the ISTA Rules. Each sample sent to the laboratories had approximately 4800 pure seeds (around 72 g), ensuring enough seed to perform all the germination tests on 400 seeds and enough seed for any retests required.

The preparation of all the samples was done by one analyst, to prevent any differences in the purity test.

For each test, a total of 400 pure seeds were tested and the participants used PSD 46 when preparing seeds for the germination tests. The first and final count were made on the fourth and fourteenth day, respectively. Seedlings were assessed according to 'Seedling Type E – Seedling Group A-2-1-1-1' of the ISTA Handbook on Seedling Evaluation, 4th Edition.
To avoid any risk of contamination, a fungicide treatment was applied before the sowing of the seeds. Each laboratory indicated on the reporting sheets the name of the chemical, the percentage of active ingredients and the method of treatment used.
The dormancy breaking methods tested are described in the following table:

Table 1. Germination Testing methods for *Beta vulgaris*

Test Number	Dormancy breaking pretreatment
1	Prewash (multigerm: 2 h; genetic monogerm: 4 h). Dry at maximum 25°C. As currently described at 5.6.3.3 and Table 5A of the ISTA Rules.
2	Presoak the seeds in water for two hours, using 250ml of water per 100 seeds. Then clean in running water and the surface blotted dry. The temperature of soaking and washing water should be at 20-25°C.
3	Sow directly with no pretreatment (only using fungicide).

If in this comparative study all combinations of temperatures and substrates could have been tested, considering the three proposed treatments described in Table 1, this would mean that each laboratory should have been performed a total of 27 tests for each lot, generating a total of 108 analyzes per laboratory, which is technically impractical.

On the other hand, even though TP and S are permitted substrates in the ISTA Rules for this species, in practice they are hardly used, mainly due to the difficulty they represent when testing multigerm Beta seeds units producing more than one seedling in a germination test.

In addition, very few laboratories use 15- <=>25 ° C for testing Beta seeds, so in practice it has been very difficult to find laboratories able to perform the test at this temperature regime.

For the reasons explained above and considering that 20°C is the temperature mainly used in most of the seed laboratories, it was decided to use PP, BP and 20°C as the test conditions for this validation study.

For the tests in BP, an envelope was made using two layers of paper (below and above the seeds) and placing the seeds into folded envelopes.

The following experimental design was proposed by Jean-Louis Laffont, Vice Chair of the ISTA Statistics Committee. This proposed analyzing 24 samples per laboratory, which was far fewer than the original 108 combinations and ensured that all laboratories performed the same tests.

Table 2. Breaking dormancy method for *Beta vulgaris*

Lot	Pre-treatment	Substrate	Temp °C
1	Prewash	PP	20
1	Presoak	PP	20
1	No pre treatment	PP	20
2	Prewash	PP	20
2	Presoak	PP	20
2	No pre treatment	PP	20
3	Prewash	PP	20
3	Presoak	PP	20
3	No pre treatment	PP	20
4	Prewash	PP	20
4	Presoak	PP	20
4	No pre treatment	PP	20
1	Prewash	BP	20
1	Presoak	BP	20
1	No pre treatment	BP	20
2	Prewash	BP	20
2	Presoak	BP	20
2	No pre treatment	BP	20
3	Prewash	BP	20
3	Presoak	BP	20
3	No pre treatment	BP	20
4	Prewash	BP	20
4	Presoak	BP	20
4	No pre treatment	BP	20

Participant laboratories

The following six 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:

GEVES-SNES Station Nationale d'Essais de Semences (France); SGS Mid-West Seed Services (USA); Naktuinbouw Seed Analysis (The Netherlands); LUFA Nord-West Institut für Düngemittel und Saatgut (Germany); OSTS, SASA, (Scotland); and ANASAC Seed Testing Laboratory (Chile).

Statistical analysis of the results

Data checking was performed according to ISTA Rules by calculating tolerances for germination test replicates. No results were out of out of tolerance.

A standard data report form was provided to each participant to obtain the same information in the same format. Germination results were checked to make sure that the sum of the percentages was equal to 100%. Tolerances were checked between replicates using Table 5B from the ISTA Rules.

Germination results obtained by the different laboratories

Figure 1 presents the germination percentage obtained for all the laboratories. The results provided by all the participants were comparable for all the lots and methods tested, therefore, they were considered in the analysis of the data.

Figure 2 shows the percentage of normal seedlings per laboratory, per lot.

All the laboratories obtained the best results with lot 2 (92,83%), followed by lot 1 very close to lot 3 with 91,37% and 91,35% respectively; and finally, lot 4 which showed the lowest germination results with an average of 88,64% of normal seedlings for all the tests performed by the participant laboratories.

Figure 3 shows the germination percentage per lot, and it can be observed that the median was very similar for lots 1, 2 and 3 and lower for lot 4 that also showed the lowest performance.

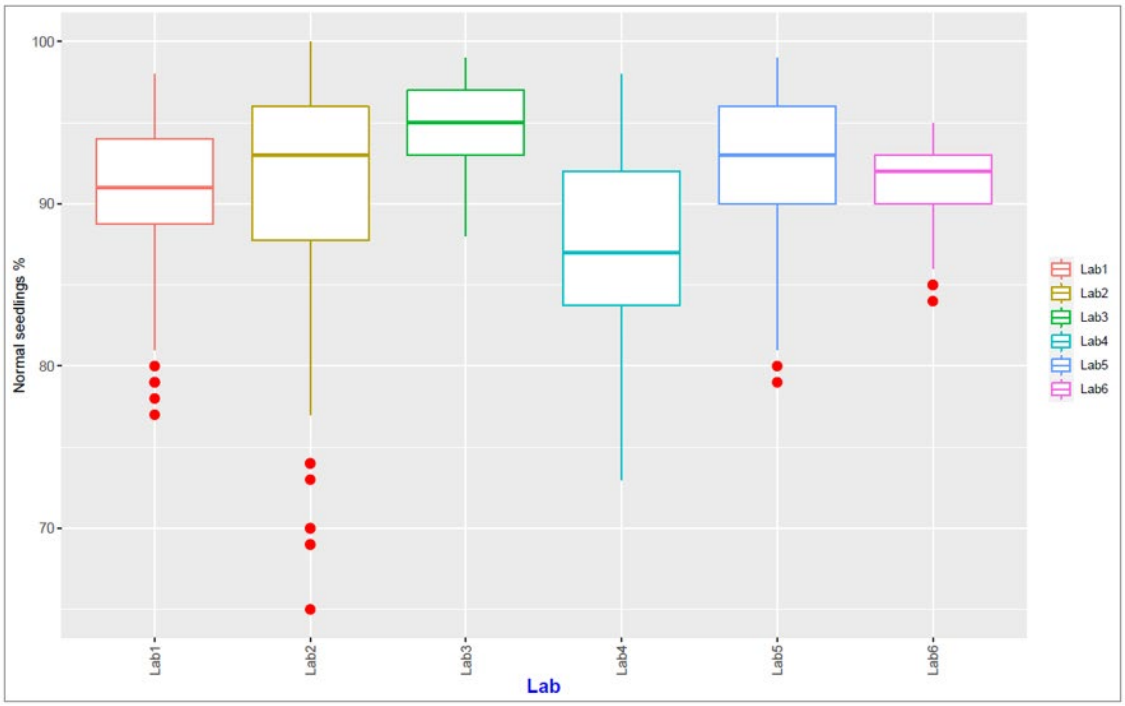

Figure 1. Percentage of normal seedlings for all the samples and all the methods, per laboratory.

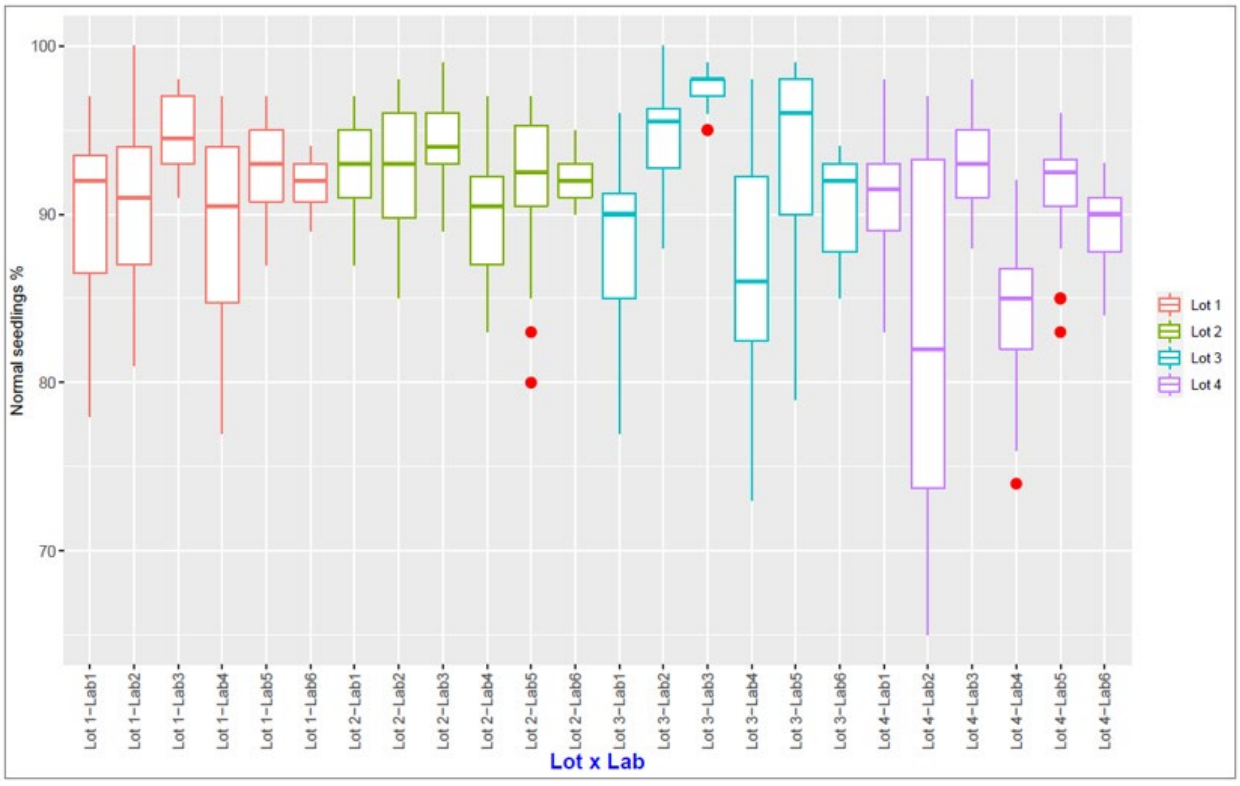

Figure 2. Percentage of normal seedlings per laboratory, per lot.

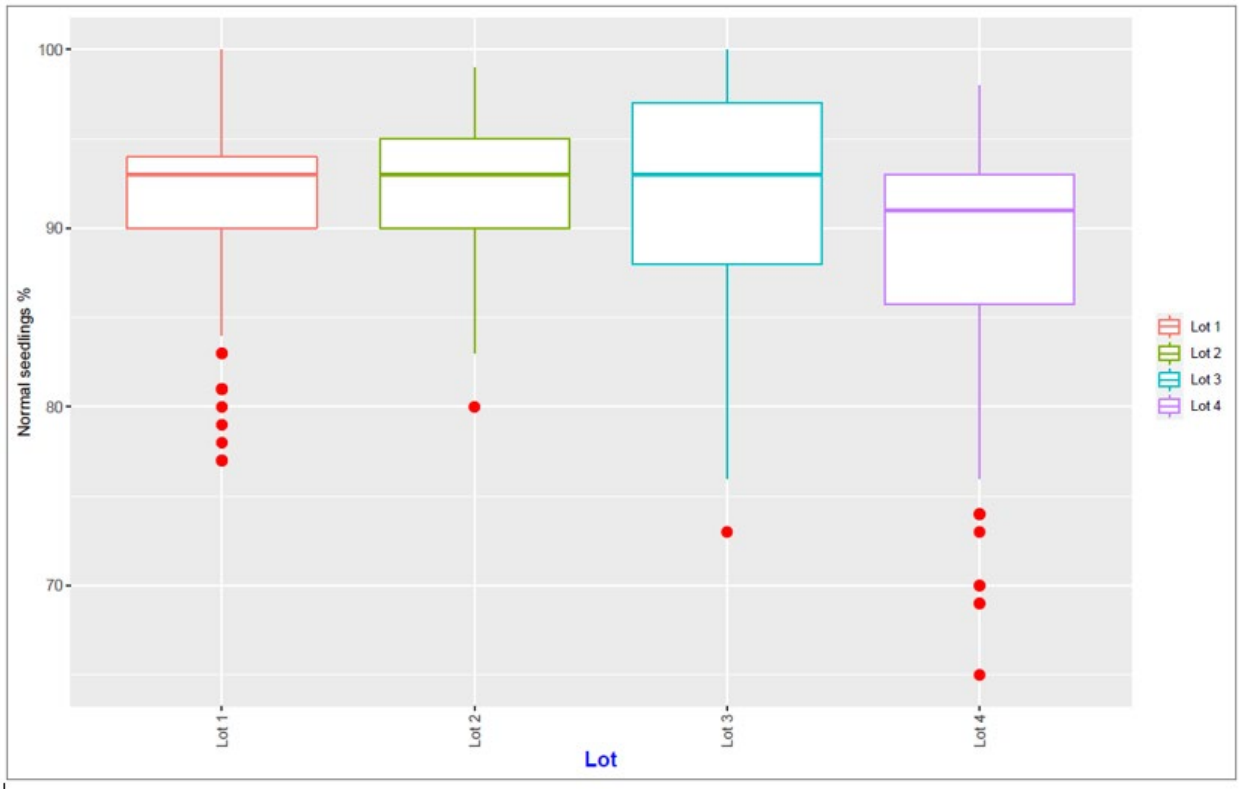

Figure 3. Data (% of normal seedlings) for all the laboratories and all the methods, per lot.

Germination results obtained with different testing methods

Effect of the testing method

Figure 4 presents the data of the percentage of normal seedlings obtained for all the samples and all the laboratories, depending on the seed testing method, and it was observed that regardless of the method used, very similar results were observed in prewashing and in pre-soaking, with both methods reaching an average of 91% normal seedlings for all treatments. The tests without pretreatment showed a lower percentage, equivalent to 88.8% compared to the methods that used water.

Figure 5 presents the percentage of normal seedlings depending on the seed test method, per lot and it was observed that for the 4 lots, the distribution was the same, showing practically identical results for the pre-soaking and pre-washing methods (regardless of the substrate used) and lower results for untreated methods. Consequently, lot 2 achieved the highest percentage germination for all the methods tested and lot 4 the lowest results regardless of the method tested. Figure 6 indicates the percentage of normal seedlings depending on the seed analysis method, by lot and laboratory.

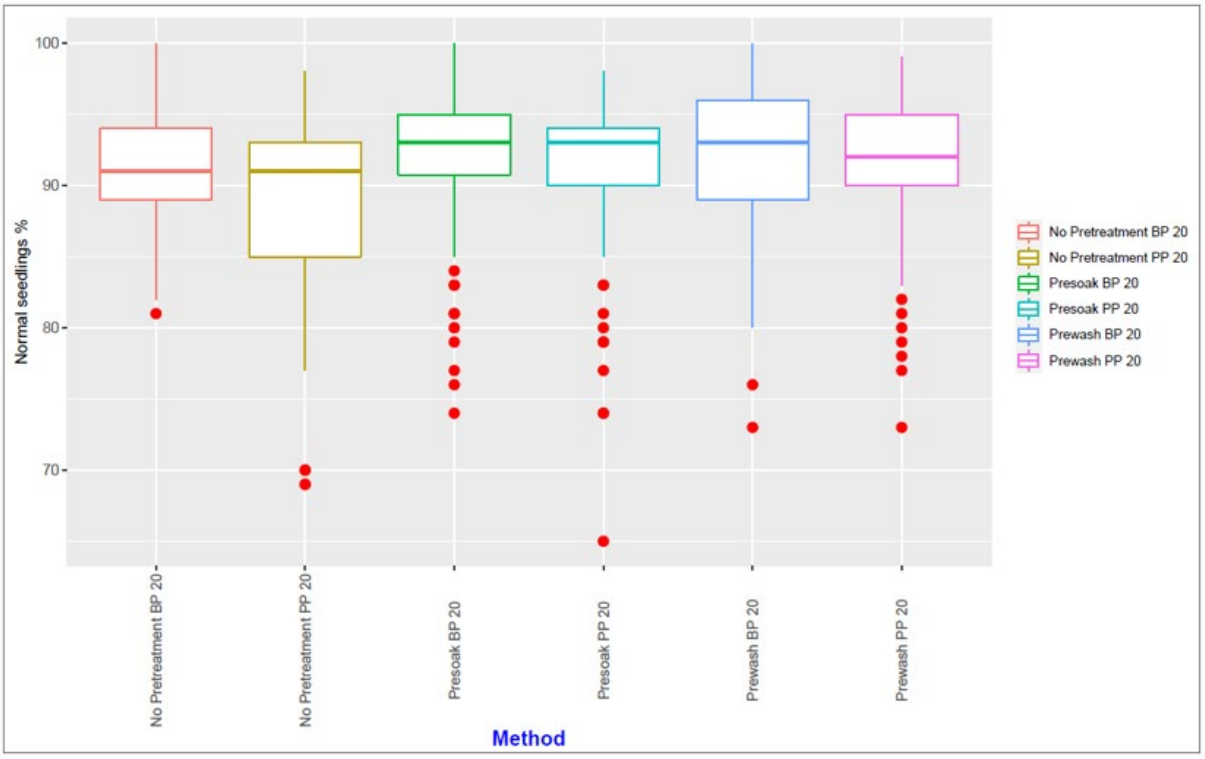

Figure 4. Effect of the testing method on germination (% of normal seedlings results)

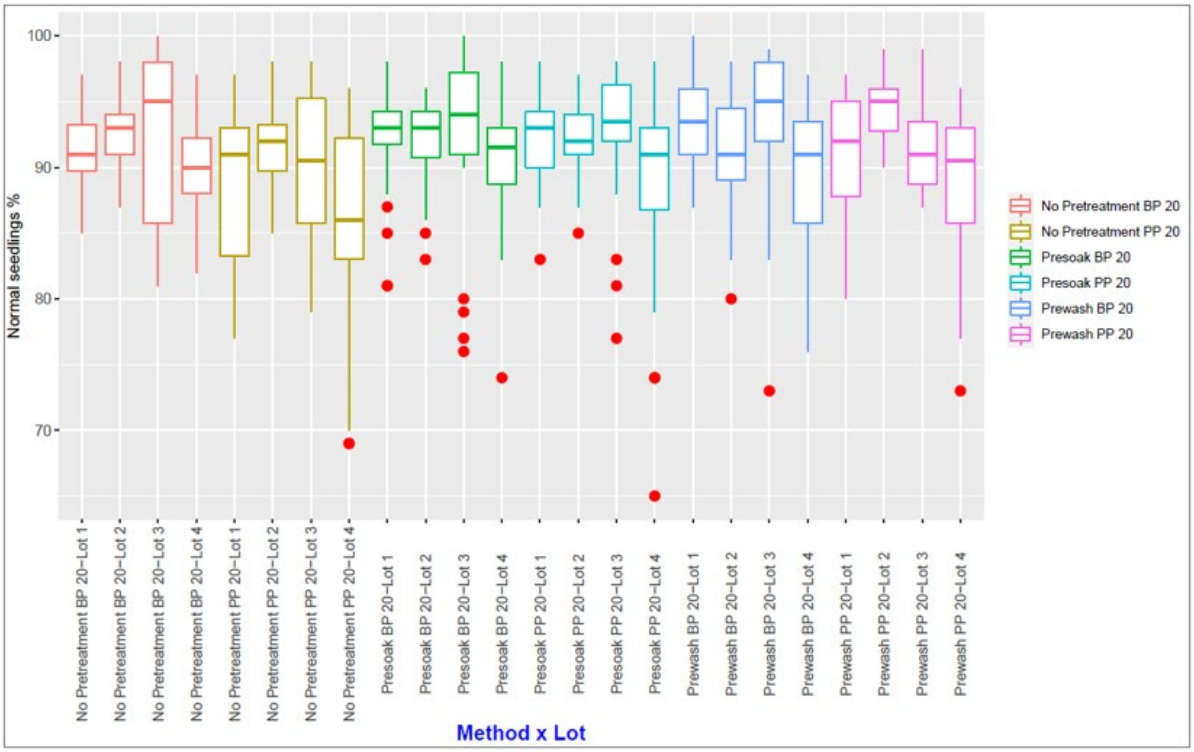

Figure 5. Percentage of normal seedlings depending on the seed testing method, per lot.

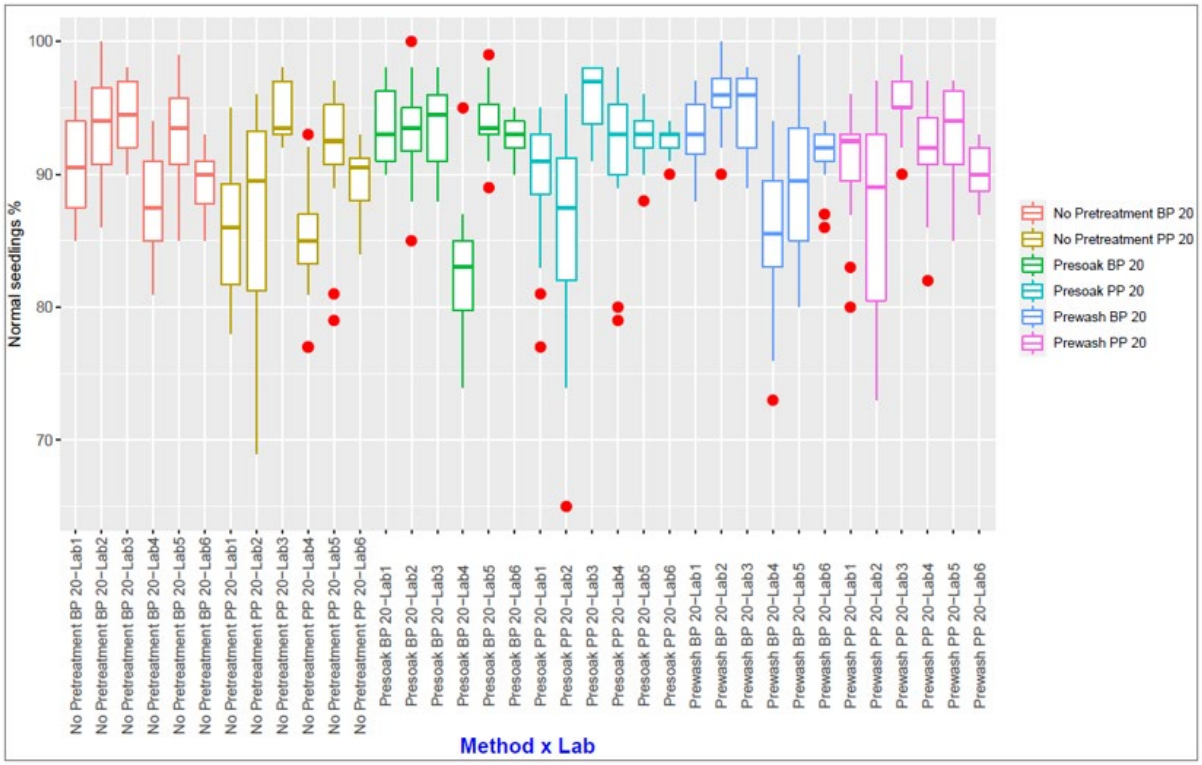

Figure 6. Percentage of normal seedlings depending on the seed testing method, per lot and laboratory.

Mixed model for comparing Method and Lot Means

Results from fitting the mixed model are displayed in the ANOVA for the fixed effects, indicating that only the lot effect was significant.

Table 3. Tests of fixed effects for "lot", "method" and "lot*method".

Source of variation	Sum of Squares	Mean Square	Num DF	Den DF	F value	Pr(>F)
Method	38.31	7.66	5	25	1.00	0.436434
Lot	64.69	21.56	3	15	2.82	0.074399
Method x Lot	80.92	5.39	15	75	0.71	0.770675

Repeatability/Reproducibility

Statistical analyses were performed using 'ISTAgermMV', the tool specifically developed by the ISTA Statistics Committee for germination validation studies. The outliers were detected using boxplots and removed accordingly as described in "Detection of outliers" specified in "*Appendix 1: Statistical Aspects of Method Validation"* of the *ISTA Method Validation for Seed Testing*. The figures with the boxplots (per lot, per method, per method x lot, and per laboratory x method), as well as the data checking, repeatability/reproducibility and the mixed model analyses were generated from this statistical tool, as well as an Analysis of Variance.

The methods were validated according to the repeatability and reproducibility results, where repeatability quantifies the average variability of results within a laboratory and reproducibility quantifies the average variability among laboratories.

For each method, 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 observed trait analyzed (%) 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 $S_R = \sqrt{\hat{\sigma}^2/K + \hat{\sigma}^2_{Lab} + \hat{\sigma}^2_{Lot\times Lab}/K}$ 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 analyzed and *n* is the number of seeds per Rep. If f_r > 1 one speaks of overdispersion because the data have larger variance than expected under the assumption of a binomial distribution.

The results of Repeatability and Reproducibility of the germination methods are summarized in the Table 4 and 5 respectively.

Table 4. Repeatability dispersion factor of the methods tested for breaking dormancy of *B. vulgaris*

Method	$\bar{p}_{...}$	$\hat{\sigma}_{Lab}$	$\hat{\sigma}_{Lot\times Lab}$	S_r	f_r	S_R
No Pretreatment BP 20	91	2.19	2.83	2.62	0.94	3.62
No Pretreatment PP 20	89	2.78	5.45	2.87	0.91	6.15
Presoak BP 20	92	4.29	2.34	2.65	0.96	4.92
Presoak PP 20	91	2.85	3.99	2.91	1.03	4.94
Prewash BP 20	92	3.22	3.13	2.85	1.04	4.53
Prewash PP 20	91	2.11	3.43	2.67	0.95	4.07

For all the methods tested, the fr values were very close or less than "1", which indicates that all the methods did not show repeatability problems.

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), and Miles's reference reproducibility dispersion factor is calculated as $f_{Miles} = 2.38 - 0.008321\bar{p}_{...}$ (Miles, 1963). These results are displayed below:

Table 5. Reproducibility dispersion factor of the methods tested for breaking dormancy of *B. vulgaris*

Method	$\bar{p}_{...}$	Excluded samples %	f_R	f_{Miles}
No Pretreatment BP 20	91	20.8	1.40	1.62
No Pretreatment PP 20	89	29.2	2.32	1.64
Presoak BP 20	93	12.5	1.77	1.60
Presoak PP 20	93	8.3	2.20	1.61
Prewash BP 20	93	20.8	2.26	1.61
Prewash PP 20	92	8.3	2.16	1.61

It can be seen that Presoak BP 20°C presented the highest % germination associated with a lower reproducibility dispersion factor value than the rest of the treatments (f_R=1.77), indicating that this method is more reproducible than Prewash.

Conclusions

After analyzing the results, it was observed that regardless of the seed lot, using the Presoak method it was possible to obtain similar results to those obtained with the Prewash method. Both methods are also slightly better in terms of the percentage of normal seedlings in comparison with the results obtained when no method was used to eliminate the inhibitors present on the surface of the seeds.

Although not all temperatures and substrates were included in this study for practical reasons noted above, the temperatures and substrates used in this study did not reveal any interactions with Presoak. For this reason, the use of presoaking could be extrapolated with the rest of the test conditions prescribed also for *Beta vulgaris* in the ISTA Rules.

The use of this pretreatment for *Beta vulgaris* seeds is as effective as prewashing to leach the inhibitory substances present on the surface of the seeds and has the advantage of improving the efficiency in the use of water resources by avoiding the waste of hours of running water.

Considering the findings of this study, the additional method proposed for breaking dormancy in *Beta vulgaris* seeds to be included in Chapter 5 of the ISTA Rules is: **Presoak the seeds in water for two hours, using 250ml of water per 100 seeds. Then clean in running water and the surface blotted dry. Temperature of soaking and washing water should be at 20-25°C.**

References

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Miles S.R., 1963. In the "Handbook of tolerances and measures of precision for seed testing" Proceedings of the International Seed Testing Association, 28, 525-686.

ISTA validation report of the detection method of *Ascochyta rabiei (*(Pass.) Labrousse) on chickpea (*Cicer arietinum)* seeds.

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Summary

The aim of this validation was to provide a detection method and a pathogenicity test for *Ascochyta rabiei* (syn. *Phoma rabiei*), also known by its teleomorph name *Didymella rabiei* (syn. *Mycosphaerella rabiei*) responsible for *Ascochyta* blight on chickpea seeds. Tests in this validation study was carried out by six laboratories experienced in the detection of *Ascochyta rabiei* on media. The all participating laboratories obtained positive results (presence of typical symptoms) for the PPC and negative results (absence of symptoms) for the NPC. In addition, all the participants obtained positive results on the isolates isolated from the positive samples. Concordance of the results in this study among laboratories was 100%, so reproducibility is validated according to the qualitative results. It should be noted that pathogenicity testing was optional, therefore only 3 laboratories performed it. Results of the CT indicated that reproducibility met requirements. As a result, it was validated for both steps of the method.

Introduction

Classification for fungi is constantly changing, and they are increasingly called by the name of their sexual stage (teleomorph). Chickpea blight, caused by the fungus *Ascochyta rabiei* is one of the most serious diseases of the crop and severe epidemics have been reported worldwide (Nene, 1982; Nene and Reddy, 1987; Collard et al., 2001). This pathogen is very aggressive on chickpea crops and can spread quickly in the field once established and when weather conditions are suitable (Pearse, 2005). The crop reaction is based on the weather conditions, specific cropping practices and cultivar (Pearse, 2005).

The fungus is selectively attacking chickpea, then persists in the crop's residues, seeds, and weeds. There is a high rate of seed-to seedling transmission of *Ascochyta* in chickpea, even a small number of infected seed can result in significant seedling infection in the field, and seed-to – seedling transmission is high. Report indicated that a 0.1 per cent *Ascochyta*-infected seed lot (one infected seed in 1000 seeds), could potentially result into 175 infected seedlings per acre (Pearse, 2005). The use of *Ascochyta* blight-free seed and seed treatment with effective fungicides reduces the probability of transmitting seed-borne disease to the seedlings (Gan, et al. 2006). The infected seeds are often symptomless; therefore, a reliable seed health detection method is crucial to avoid plant infection.

Initial crop infection is due to the introduction of either infected seed or from movement of infected plant debris, with windborne spores (ascospores), machinery or animals. Spores of the fungus can survive for a short time on skin, clothing as well as machinery. Subsequent in-crop infection occurs when inoculum is moved higher in the canopy or to surrounding plants by wind or rain splash during wet weather (Cumming, et al. GRDC, 2009).

The ISTA method 7-005 for *A. pisi* can be adapted to detect *Ascochyta rabiei* using Malt Extract Agar Method, it has shown good results. Also, the Potato Dextrose Agar (PDA) media will be tested because it is often used to detect several fungi on seeds.

Objectives

The aim of this procedure is to validate a quantitative detection method of *Ascochyta rabiei* on chickpea seeds and a pathogenicity test of *Ascochyta rabiei* on chickpea, to support the development of this crop in organic and conventional production. This method will allow us to have a better understanding of the disease and allow the detection of this seed transmitted pest to circulate healthy seeds.

The method includes detection by plating 400 seeds on media (PDA or MA) to detect *Ascochyta rabiei*, followed by morphological identification under stereomicroscope and compound microscope.

This method can be followed by an optional step: pathogenicity test (as described in Fig 1).

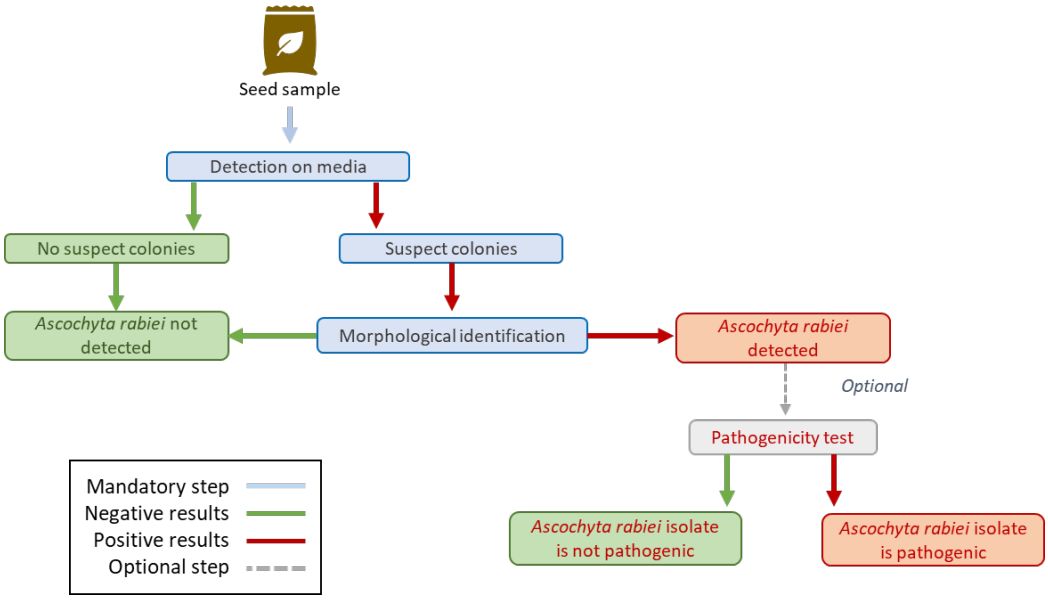


Fig 1 - Workflow of the method

Materials and methods

Chickpea seed samples

- Healthy samples
- Low infected samples
- Medium infected samples
- Highly infected samples
- Healthy susceptible chickpea seeds (Cultivar: Benito)

Manpower

- Validation of performance criteria: Mycology team of GEVES
- Reproducibility of the method: Participants of CT

Machines

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

Materials

- 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)
- Chickpea Seed Meal Dextrose Agar (CSMDA) media (Annex 3)
- Filter
- Beakers
- Microscopic slides
- Malassez Cell
- Sterile pipettes
- Plastic bags
- Trays, cups, and cover
- Steamed potting soil
- Reference strain of *Ascochyta rabiei* (PAS 2909)

Medium

- Incubator: 20 ±2 °C darkness (incubation of samples)
- Incubator: 20 ±2 °C UV (growing of reference material)
- Storage room: 10 ±2 °C for the storage of the samples
- Growth chamber to grow the plants (20 ± 2 °C, 8 hours light / 16 h darkness)
- Growth chamber for the pathogenicity test (20 ± 2 °C – 12h light / 12h darkness – 80% humidity)

Method

Experienced laboratories on detection of *Ascochyta rabiei* on media. Performance criteria will be studied in one lab. A minimum of six laboratories is required for validation of the detection and identification on media. A request for participation was sent to ISTA members and in the networks of the organizers.

Participating laboratories and contact persons				
Laboratories	**Contact persons**	**Isolate collection**	**Performance media/PP**	**CT**

GEVES	Isabelle Sérandat isabelle.serandat@geves.fr	X	X	X
Terres Inovia	Martine LEFLON m.leflon@terresinovia.fr	X		X
Top semence	Blaise Rolland b.rolland@topsemence.com			X
AGES	Helene Berthold helene.berthold@ages.at			X
SGS	Nicole Calliou nicole.calliou@sgs.com			X
SASA	Marian Mc Ewan Marian.McEwan@sasa.gov.scot			X

The main steps of the method are described below:

➔ *Detection method*

The method was tested on untreated chickpea seeds.
Pretreatment is necessary on chickpea 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 10 seeds of chickpea per Petri dish (90 mm Ø) containing the media (MA or PDA). Plate 40 Petri dishes per sample to analyze 400 seeds.

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 *Ascochyta rabiei.*

Sterility Control: Place a non -inoculated Petri-dish containing the media in a climate chamber T=20°C, to control the sterility of the medium.

Air control: Open a Petri dish containing the media for 2 min and incubate it with the samples to control the absence of contaminant in the air.

Incubation: 7 or 9 days at 20°C in darkness.

Examination: Carried out after 7 or 9 days.
Ascochyta rabiei will be identified under stereomicroscope using x20 – 25 magnification and under compound microscope using x100 – 400 magnifications to check the colony type and conidial shape, size and septation.

Description of the morphological criteria of *Ascochyta rabiei* in Annex 4.

➔ *Pathogenicity test*

Inoculum multiplication: Plate the isolates on CSMDA media, incubate at 20°C, NUV for at least 7 days.

Pretreatment: Immerse seeds of susceptible cultivar to *Ascochyta rabiei* of Chickpea in sodium hypochlorite (NaOCl) (1% available chlorine) for 10 mins, then drain and rinse well in sterile water and drain.

Germination: Place the disinfected seeds on a wet blotter paper. Roll the paper with the seeds and place it in a plastic bag. Incubate the closed bags at 20°C, darkness for 2-3 days to allow seed germination.

Inoculum preparation: Pour 2 mL of sterile water on the CSMDA plates containing the grown isolate and scrap the surface with a microscopic slide. Filter the inoculum and estimate the concentration using Malassez cell. Dilute, if necessary, in sterile water to obtain a concentration from 1.10^4 to 1.10^5 conidia/mL. Make sure to have a sufficient volume of inoculum to soak the seeds.

Inoculation: Cut the root tips (approx. 1cm) of 2-3-days-old, germinated chickpea seeds and soak 3 seeds in each inoculum for 10 min. Sow the seeds in potting soil.

Positive Process Control (PPC): Repeat with soaking of 3 seeds in inoculum prepared with a pathogenic isolate.

Negative Process Control (NPC): Repeat with soaking of 3 seeds in sterile water.

Incubation: 10 days at 20 ± 2˚C; RH = 100% (using a cover), 12h light, 12h dark.

Examination: After 10 days, take the plantlets out, check the presence of symptoms and compare to the positive and negative controls. Symptoms caused by *Ascochyta rabiei* are necrosis on the stem (all the time), seed blackened (most of the time), leaf wilting (often), plantlet rotting (sometimes).

Record the suspect colonies as pathogenic if at least one of the seedlings present black necroses on the stem.

Results

Results are recorded in the following annexes:

- Annex 5: Analytical specificity results for the validation of the detection method
- Annex 6: Analytical specificity results for the validation of the pathogenicity test
- Annex 7: Raw data of comparative test – detection
- Annex 9: Raw data of comparative test – pathogenicity test

<u>Detection method</u>

Seed lots selection: To validate the detection method, 15 naturally infected seed lots were available. A pretest on 400 seeds was done for each seed lot to evaluate their level of contamination.
Levels of contamination from 0% to 12.25% were obtained.

Three levels of infection have been determined: healthy, low and medium. The medium one was obtained by mixing 2 seed lots (4.25% and 4.75%).
The 3 seed lots have been homogenized and sampled then homogeneity was tested on 10 random samples.

Homogeneity test: Homogeneity has been tested for each level of contamination on 10 replicates of 400 seeds on MA, with a notation at 7 days. Results are recorded in Table 1.

Table 1 : Homogeneity results

Healthy 1

Repetition	Values
1	0.00
2	0.00
3	0.00
4	0.00
5	0.00
6	0.00
7	0.00
8	0.00
9	0.00
10	0.00

Low

Repetition	Values (Xi)	| Xi - M|	Status
1	0.75	0.250	OK
2	0.5	0.000	OK
3	0.25	0.250	OK
4	0.5	0.000	OK
5	0.75	0.250	OK
6	0.75	0.250	OK
7	0.75	0.250	OK
8	0	0.500	OK
9	0.25	0.250	OK
10	0.25	0.250	OK

Medium 1

Repetition	Values (Xi)	| Xi - M|	Status
1	3	0.000	OK
2	2.75	0.250	OK
3	4.25	1.250	OK
4	3.75	0.750	OK
5	4	1.000	OK
6	3	0.000	OK
7	3	0.000	OK
8	2.25	0.750	OK
9	4	1.000	OK
10	2	1.000	OK

Medium 2

Repetition	Values (Xi)	| Xi - M|	Status
1	5.25	0.250	OK
2	5.75	0.750	OK
3	5.75	0.750	OK
4	5.75	0.750	OK
5	5.50	0.500	OK
6	4.50	0.500	OK
7	4.00	1.000	OK
8	4.75	0.250	OK
9	4.25	0.750	OK
10	3.50	1.500	OK

The seed lots have been used for the validation of the performance criteria as followed:

- Healthy 1: Analytical sensitivity / Diagnostic sensitivity and specificity / Repeatability / Reproducibility
- Low: Diagnostic sensitivity and specificity / Repeatability / Reproducibility
- Medium 1: Robustness (First part) / Diagnostic sensitivity and specificity / Repeatability / Reproducibility
- Medium 2: Robustness (Second part)

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

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.

The performance of the analytical specificity was based on the morphological criteria (described in Annex 4) and performed on 2 media, MA and PDA and on a collection of 20 targets and 20 non-targets isolates (described in Annex 5), results are presented in Table 2 and Fig 2. Raw data are available in Annex 5.

Table 2 : Analytical specificity results

Target and non-target strains	Expected result + (Target)	Expected result - (Non-target)	Specificity
Obtained result +	20	0	100%
Obtained result -	0	20	

Analytical specificity of the detection method is validated and reaches 100% of performance.

Example of target:
Ascochyta rabiei – PAS 2950

Example of non-target:
Boeremia exigua – PAS 1847

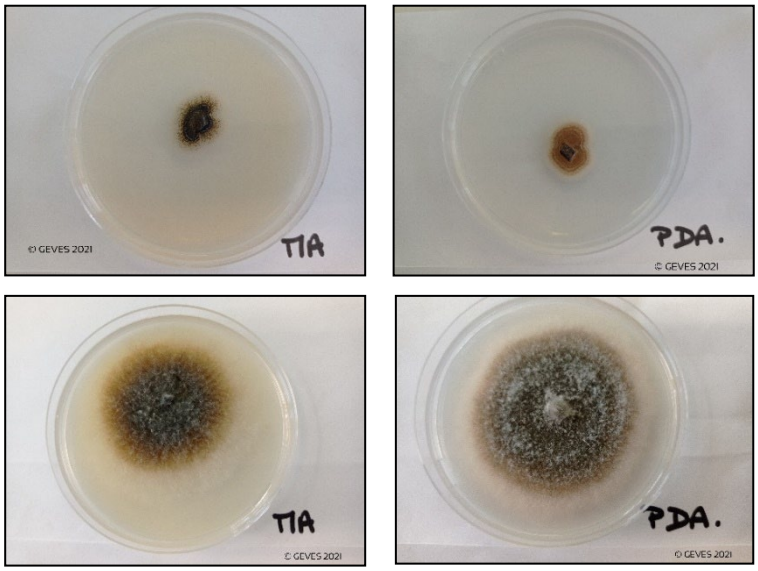


Fig 2 - Example of target and non-target strains
7 days, darkness

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).

To obtain contaminated seeds, artificial contamination using PDA mannitol was tested but the contamination didn't reach 100%.

The artificial contamination was done by soaking/shaking seeds free from *Ascochyta rabiei* in a suspension at 1.10^7 conidia/mL in 2% gum arabic solution for 10 min, then allowed the seeds to dry on filter paper.

To check the percentage of contamination, 50 seeds have been plated on MA and used as positive control, the obtained contamination rate was 100%.

Then 10 samples of 399 healthy have been spiked with 1 contaminated seed, results are recorded in Table 3.

Table 3 : Analytical sensitivity results

Seed sample	% of seeds contaminated by *Ascochyta rabiei*
1	0.25
2	0.25
3	0.25
4	0.25
5	0.25
6	0.25
7	0.25
8	0.25
9	0.25
10	0.25

The method allowed to detect 1 contaminated seed on all 10 samples of 400 seeds.
Analytical sensitivity of the detection method is validated and reaches 100% of performance.

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

In order to assess the robustness of the method we varied:

- Media: Incubation on MA and PDA have been compared on 3x400 seeds of a medium infected sample (Medium 1 ≈3.2%)
- Duration of incubation: Notations at 7 days and 9 days have been compared on 3x400 seeds of a medium infected sample. (Medium 1 ≈3.2%)
- During the analytical specificity, all the strains sporulated well in darkness so the light conditions (darkness or NUV light) haven't been tested.

Results are presented in : Table 4, Table 5, Fig 3 and Fig 4.

Table 4 : Robustness results (average)

Media / Notation	PDA	MA
7 days	4.1 %	3.9 %
9 days	4.7 %	4.3 %

Fig 3 - Robustness of detection method *Ascochyta rabiei* on Chickpea

Homogeneity test was done on MA, with a notation at 7 days (see results in 4.1.2)
The same samples were used for both notations (7 and 9 days).

Ascochyta rabiei is a slow growing fungus and might be located under the seeds, to allow a correct notation it is important to move the seed. This wasn't done at 7 days to avoid an overestimation by cross contamination.

Robustness has been tested a second time by using distinct samples of medium 2 seed lots (see homogeneity results in 4.1.2) for notation at 7 and 9 days, the results are presented in Table 5, Fig 4 and Fig 5.

Table 5 : Robustness results (average)

Media / Notation	PDA	MA
7 days	5.1 %	5.3%
9 days	5.6 %	6 %

Fig 4 - Robustness of detection method *Ascochyta rabiei* on Chickpea

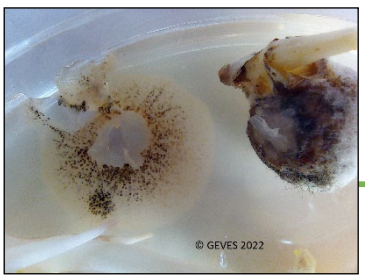




Fig 5 - Example of *Ascochyta rabiei* colonies
Left to right: MA – 7days; MA – 9 days; PDA – 7 days; PDA – 9 days

There are no significant differences between the results of the samples on PDA, MA and after 7 or 9 days of incubation.

Robustness of the detection is validated and highlight the importance of moving the seeds during notation, this information will be described in the final method.

Diagnostic sensitivity and specificity / Repeatability / Reproducibility: The diagnostic sensitivity is the verification that there are no false negatives and diagnostic specificity is the verification that there are no false positive.

It was tested on the following samples:

- 1 healthy sample
- 1 low contaminated sample (≈ 0.5% contamination)
- 1 medium contaminated sample (Medium 1 ≈ 3.2% contamination)

The low contaminated sample was taken out of the evaluation of this criteria because it shown 1 negative result out of 10 for homogeneity test, due to the very low level of contamination.

Samples were plated on MA and notation was done at 7 days.
Three replicates of each level of contamination were tested at the same time to evaluate the repeatability of the method and performed two times intra laboratory to evaluate the reproducibility. Results are recorded in Table 6.

Table 6 : Sensitivity and specificity diagnostic results

Samples	Date 1	Date 2
Healthy rep 1	0.00%	0.00%
Healthy rep 2	0.00%	0.00%
Healthy rep 3	0.00%	0.00%
Medium rep 1	2.50%	3.00%
Medium rep 2	2.00%	3.00%
Medium rep 3	2.25%	3.50%

To evaluate the diagnostic sensitivity and specificity, the analysis is a comparison between the expected results (known samples, validated by homogeneity test based on mean results) and the obtained results. Results are presented in Table 7 and Fig 6.

Table 7 : Qualitative analysis of the results:

	Expected result +	Expected result -	Diagnostic sensitivity	Diagnostic specificity

Obtained result +	6	0	100%	100%
Obtained result -	0	6		

Fig 6 - Quantitative results of accuracy presented with box plot

cur.

Performance criteria are validated with a diagnostic sensitivity that reaches 100% and a diagnostic specificity that reaches 100 %.

Pathogenicity test

Choice of pathogenicity test

A comparison was made between three pathogenicity tests:
1. Inoculation by soaking germinated seeds
2. Inoculation by deposit of conidial suspension on germinated seeds on agar medium
3. Inoculation by deposit of conidial suspension on germinated seeds in potting soil

All pathogenicity tests were performed on a pathogenic *Ascochyta rabiei* (PAS 2909) isolate.

	Advantages	Inconveniences
1. Soaking germinated seeds	Symptoms are more distinct.	One more step than the others (Cutting the germ).
2. Deposit of suspension agar	No need to use potting soil. Faster to develop.	Contamination of the media after inoculation by saprophytic flora.
3. Deposit of suspension soil	Realization is a bit faster.	Symptoms are weak.

According to these results the first pathogenicity test (soaking germinated seeds) was selected for validation. It has shown the best results and is quite simple to perform.

Analytical specificity: 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.

It was performed by testing target and non-target isolates from the collection in Annex 6.
Each isolate grown on CSMDA at 20°C 12h NUV / 12h darkness for 10 days was tested on one plant.

Results are presented in Table 8, Fig 8, Fig 9 and Fig 10.

The symptoms (Fig 7) caused by *Ascochyta rabiei* are:
- Leaf wilting (often)
- Presence of necrosis on the stem (all the time)
- Seed blackened (all the time)
- Plantlet rotting (sometimes)

Fig 7 - Symptoms

The most discriminating symptom is the presence of necrosis on the stems, which implies that the plantlets must be removed from the potting soil to make notation and conclude on pathogenicity of the isolate.

Fig 8 - Negative control

Fig 9 - Non target *Cladosporium* sp.

Fig 10 – Target *Ascochyta rabiei*

Table 8 : Analytical specificity results

Target and non-target strains	Expected result + (Target)	Expected result - (Non-target)	Specificity
Obtained result +	20	0	100%
Obtained result -	0	20	

The analytical specificity of the pathogenicity test is validated and reaches 100% of performance.

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

A fit for purpose concentration was chosen so that a maximum of seedlings shows symptoms. Concentration of the conidial suspension is about 1.10^5 conidia/mL so a dilution at $1/10^{th}$ was tested to evaluate the analytical sensitivity of the pathogenicity test. For each concentration 10 seedlings were tested and compared to the 10 seedlings of the negative control.

Results are presented in Table 9, Fig 11, Fig 12 and Fig 13.

Table 9 : Analytical sensisitivity results

Dilution	0	$1/10^{th}$	**Negative control**	**Comments**
Concentration	10^5	10^4	NC	
Ascochyta rabiei - PAS 2909	10+/10	10+/10	0+/10	Symptoms are more severe and typical on the highest concentration (1.10^5)

According to the results each concentration fits for the evaluation of the pathogenicity, but we noticed that the symptoms are less visible with the diluted concentration (1.10^4 condia/mL).

Fig 11 - *Ascochyta rabiei* 1.10^4 conidia/mL

Fig 12 - *Ascochyta rabiei* $1/10^5$ conidia/mL

Fig 13 - Negative control

The selected concentration is 1.10^5 conidia/mL, the symptoms are more intense and severe at this concentration than at 1.10^4 conidia/mL.

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

To assess the robustness of the method, we varied temperatures and light conditions according to these details:

- 20°C - 12h light (Neon lights) / 12h darkness
- 20°C - 8h light (LED) /16h darkness
- 25°C - 12h light (LED) / 12h darkness
- 25°C - 8h light (LED) /16h darkness

Each condition was tested on 5 seeds, results are presented in Table 10 and Fig 14.

Table 10: Results of the robustness

Temperature / Light	20°C	25°C
8h light / 16h darkness	5+ / 5	5+ / 5
12h light / 12h darkness	5+ / 5	5+ / 5

Fig 14 – Results of the robustness
Left to right :
20°C 12h/12h Néon; 20°C 8h/16h LED;
25°C 12h/12h LED; 25°C 8h/16h LED

The negative controls were all negatives, they are validated. Symptoms were weaker on the plantlets tested at 25°C. No difference was observed between LED and Neon lights.
The robustness of the pathogenicity test is validated and reaches 100% of performance.

Diagnostic Sensitivity and specificity: Diagnostic Sensitivity and specificity have been tested and validated at the same time as the analytical specificity on the 20 targets and 20 non targets of the isolate collection.

Repeatability/Reproducibility: To evaluate the repeatability and reproducibility of the method one target (*Ascochyta rabiei* PAS 2948) and one non-target (*Botrytis cinerea* PAS 2910) were selected.

Three replicates of each selected strain were tested at the same time to evaluate the repeatability of the method and performed two times intra laboratory to evaluate the reproducibility.

Reproducibility was performed in one lab by changing some parameters:

- Two different dates of analysis

- Inoculation by two different operators
- Notation by two different operators

Results are recorded in Table 11 and presented in Table 12 and Fig 15.

Table 11 : Raw results of the repeatability and intra laboratory reproducibility

Samples	Replicate 1	Replicate 2
Target	3+/3	3+/3
Non-target	0+/3	0+/3
Negative control	0+/3	0+/3

All plantlets inoculated by the target (*Ascochyta rabiei* PAS 2948) shown symptoms. While all plantlets inoculated by the non-target (*Botrytis cinerea* PAS 2910) didn't show the typical symptoms caused by *Ascochyta rabiei*.

Fig 15 – Results of repeatability/reproducibility
Replicate 1, left to right: Negative controls – *Ascochyta rabiei* PAS 2948 – *Botrytis cinerea* PAS 2910

Table 12 : Results of the repeatability and intra laboratory reproducibility

	Expected result +	Expected result -	Repetability	Reproducibility
Obtained result +	18	0	100%	100%
Obtained result -	0	18		

Repeatability and reproducibility of the pathogenicity are validated and reaches 100% of performance.

Comparative Test (CT)

CT Organization:

The aim of this CT was to validate the performance criteria (repeatability and reproducibility) of the method for detection and pathogenicity test of *Ascochyta rabiei* on chickpea seeds.
The principle was to detect *Ascochyta rabiei* on chickpea seeds on medium and to assess pathogenicity test on *Ascochyta rabiei* isolated from samples.

Table 13 - Timeline of the pre-comparative test

Step	Schedule
Sending of samples	Week 22 of 2023
Deadline to start analysis	Within 3 weeks after receipt
Deadline to send results	August 4th 2023
Sending of report	October 2023

Six laboratories participated to those tests and were randomly allocated a number, so that results remained anonymous.

Notation of results: For detection method, the laboratories indicated: quantitative and qualitative (positive, negative) results for each sample and information about the method used.
For pathogenicity test, the laboratories indicated: qualitative (positive, negative) results for each isolate and information about the method used.

Composition of the sample panel: 9 samples of 400 chickpea seeds have been sent to each of the six participating laboratories as in Table 14.

Table 14 - Samples characteristics

Code	Level of contamination	Number of samples	Expected values
A	Medium	3	Positive
B	High	3	Positive
C	Healthy	3	Negative

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

Pretests: To have enough quantity of chickpea seeds to obtain each level of contamination, 44 seed lots have been tested. A pretest on 400 seeds was done for each seed lot to evaluate their level of contamination.

Levels of contamination from 0% to 14.75% were obtained. 11 samples with contamination levels from 4.25% to 6% were chosen to compose Lot A, 10 samples from 10.5% to 14.75% were chosen to compose Lot B and 1 healthy sample was chosen as Lot C, see composition of samples in Table 15.

Table 15 - Composition of samples

Code	Sample number	% *Ascochyta rabiei*
Lot A	1	6
	2	4.25
	3	5
	4	5
	5	6
	6	4.5
	7	5.75
	8	4.25
	9	4.75
	10	4.75
	11	5
Lot B	12	13
	13	10.5
	14	14.75
	15	12.25
	16	13.25
	17	11
	18	11.5
	19	11.75
	20	11.5
	21	12.25
Lot C	22	0

Homogeneity tests: Homogeneity tests were done after sampling and packaging and before shipping of the seed samples to the participating laboratories. The method was provided to the participants. Homogeneity was tested on 10 samples of 400 seeds for each level of infection between January and March 2023. Qualitative results, minimum, maximum and average values are given in Table 16. Quantitative results were analyzed by Hampel's method (Table 17), by repartition against the mean (Fig 16).

Table 16 - Homogeneity test results

Seed lot	Level of infection	Expected result	Quantitative results		Standard deviation	Coeffcient of Variation	Qualitative result	Conformity
			Min.-Max. (%)	Mean (%)				
A	Medium	Detected	3.5-5.75	4.90	0.82	16.7%	10+/10	Conform
B	High	Detected	5.5-11.25	8.28	1.77	21.4%	10+/10	Conform
C	Healthy	Not detected	0	0			0+/10	Conform

Table 17 - Homogeneity tests results Lot A and B using the Hampel's method for outlier detection.

Lot A

Repetition	Values (Xi)	| Xi - M|	Status
1	5.25	0.25	OK

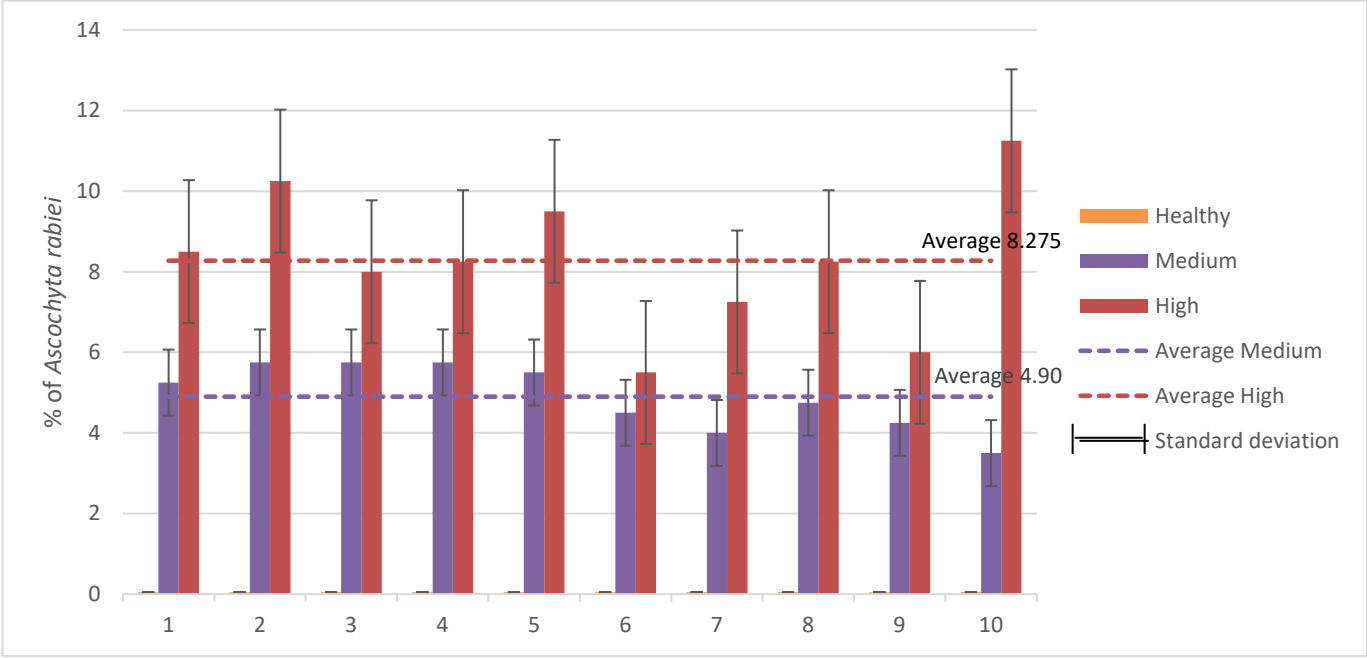
Lot B

Repetition	Values (Xi)	| Xi - M|	Status
1	8.50	0.250	OK

2	5.75	0.75	OK
3	5.75	0.75	OK
4	5.75	0.75	OK
5	5.5	0.5	OK
6	4.5	0.5	OK
7	4	1	OK
8	4.75	0.25	OK
9	4.25	0.75	OK
10	3.5	1.5	OK

2	10.25	2.000	OK
3	8.00	0.250	OK
4	8.25	0.000	OK
5	9.50	1.250	OK
6	5.50	2.750	OK
7	7.25	1.000	OK
8	8.25	0.000	OK
9	6.00	2.250	OK
10	11.25	3.000	OK

Fig 16 - Homogeneity test results, repartition against the mean, for medium and high level



Conclusions of homogeneity test results:

Medium and 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.
Given that no outliers have been detected and assuming a Coefficient of Variation threshold of 25% to ensure homogeneity, we conclude that samples are homogeneous.

Stability tests: Stability test started on June 26th, 2023, after all the lab confirmed starting of analysis. It was tested on 4 samples of 400 seeds for each level of infection. Qualitative results minimum, maximum and average values are given in Table 17. Quantitative results were analyzed by Hampel's method, analysis of variance (Table 19) and repartition against the mean (Fig 17).

Table 18 – Stability test results

Level of contamination	Code	Expected result	Value mini - maxi (%)	Average (%)	Qualitative result
Medium	Lot A	Detected	4.5-8.5	6.19	$4^+/4$
High	Lot B	Detected	9-13	11.5	$4^+/4$
Healthy	Lot C	Not detected	0	0	$0^+/4$

Table 19 - Stability tests results Lot A and B using the Hampel's method and F-test

Lot A - Medium

Repetition	Values (Xi)	| Xi - M|	Status
1	8.50	3.250	Outlier
2	5.50	0.250	OK
3	4.50	0.750	OK
4	5.00	0.250	OK

Median (M):	**5.250**
MAD:	**0.500**
5.2 X MAD	**2.600**

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Test	1	2.716	2.716	2.074	0.175
Residuals	12	15.713	1.309		

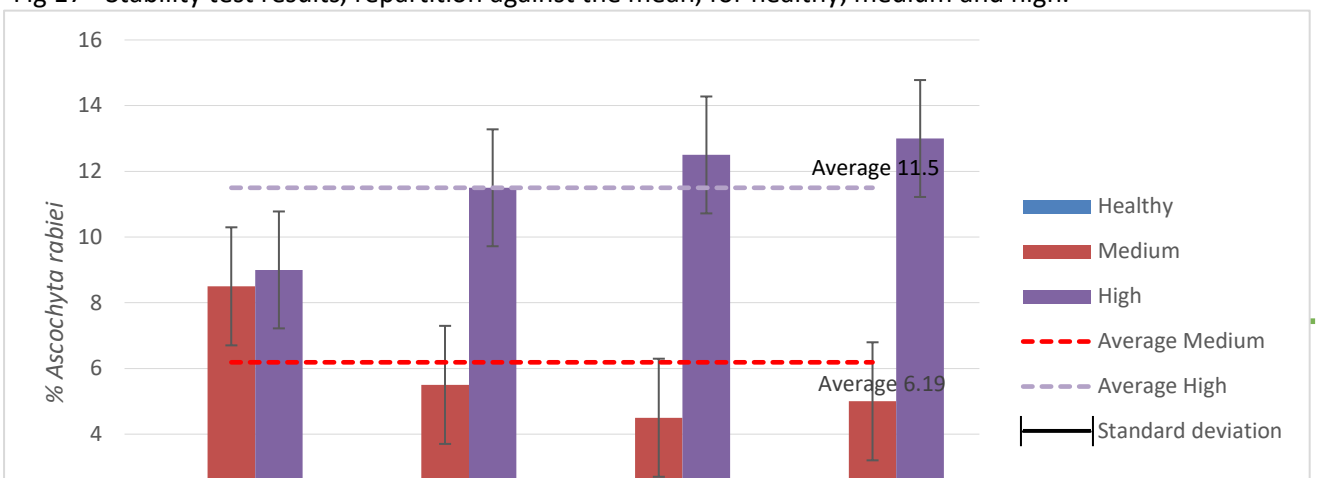
Lot B - High

Repetition	Values (Xi)	| Xi - M|	Status
1	9.00	3.000	OK
2	11.50	0.500	OK
3	12.50	0.500	OK
4	13.00	1.000	OK

Median (M):	**12.000**
MAD:	**0.750**
5.2 X MAD	**3.900**

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Test	1	29.72	29.716	9.432	0.0097
Residuals	12	37.81	3.151		

Fig 17 - Stability test results, repartition against the mean, for healthy, medium and high.

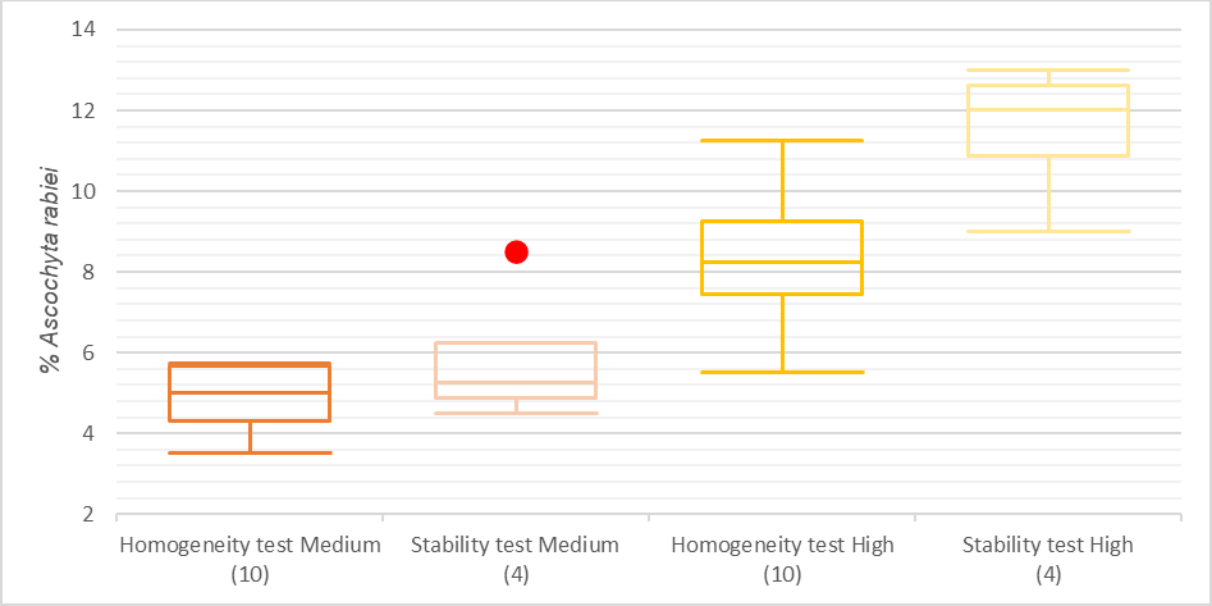


Comparison between homogeneity and stability results for medium and high level is given in Table 20 and Fig 18.

Table 20 - Comparison between homogeneity and stability results

Level of contamination	% of *Ascochyta* rabiei		
	Homogeneity results	Stability results	Deviation
Medium	4.90	6.19	+ 1.29
High	8.275	11.5	+ 3.225
Healthy	0	0	0

Fig 18 - Comparison between homogeneity and stability tests for medium and high level



Conclusion of stability test:

Medium level: Percentage of contamination was stable in time (no significant difference between homogeneity and stability tests). The outlier result (8.5) was due to the few numbers of samples used to test the stability and the use of naturally contaminated samples that may lead to variations. Regarding the results of the participants along with the stability test, this data was not considered outlier.
High level: Percentage of contamination does not decrease over time.
Healthy level: All samples were negative, there were no cross-contamination over time.
Stability is validated.

CT results
Detection method

Qualitative results

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 reproducibility (concordance). Results are given in Table 21, Table 22 and Table 23. Raw data are available in Annex 7.

Table 21 – Summary results of detection method

Lab code	Obtained results		
	Medium	High	Healthy
01	3+/3	3+/3	0+/3
02	3+/3	3+/3	0+/3
03	3+/3	3+/3	0+/3
04	3+/3	3+/3	0+/3
05	3+/3	3+/3	0+/3
07	3+/3	3+/3	0+/3
Reproducibility: Concordance	**100%**	**100%**	**100%**

For each of the six participating laboratories, all negative samples were detected as negative (0+/3) as expected, as well as all positive samples were obtained positive (6+/6) as expected. Therefore, concordance of results for the negative and positive seed lots was calculated by Langton et al. and result is 100%.

Table 22 – Concordance calculated for the negative seed lot qualitative results

Laboratories	Repetition			Positive results	Negative results	# of according positive pairs	# of according negative pairs	Concordance (%)
	1	2	3					
01	-	-	-	0/3	3/3	0	153	100
02	-	-	-	0/3	3/3			
03	-	-	-	0/3	3/3			
04	-	-	-	0/3	3/3			
05	-	-	-	0/3	3/3			
07	-	-	-	0/3	3/3			

Table 23 – Concordance calculated for the positive seed lot qualitative results

Labs	Repetition						Positive results	Negative results	# of according positive pairs	# of according negative pairs	Concordance (%)
	1	2	3	4	5	6					

01	+	+	+	+	+	+	6/6	0/6	630	0	100
02	+	+	+	+	+	+	6/6	0/6			
03	+	+	+	+	+	+	6/6	0/6			
04	+	+	+	+	+	+	6/6	0/6			
05	+	+	+	+	+	+	6/6	0/6			
07	+	+	+	+	+	+	6/6	0/6			

Reproducibility is then validated according to the qualitative results.

Quantitative results: Analysis of results of each level has also been carried out for each laboratory at the quantitative level (rate of seed infection) with the box plot tool (ISTA Seed Health Toolbox). Qualitative analysis of results by Hampel's method and Box Plot is given in Table 24, Table 25, Fig 19 (Medium level) and Fig 20 (High level).

The six participating laboratories obtained statistically identical results in accordance with Hampel's method and Boxplot for medium and high levels.
A global analysis confirmed that there was no significant difference between the homogeneity test and the results of participants (See annex 8).

For each lot, the following linear model is fitted:

$$y_{ij} = \mu + L_i + e_{ij}$$

in which:
. y_{ij} is the observed % *Ascochyta rabiei* in Sample *j* of Lab *i*.
. μ is the intercept.
. L_i is the random effect of Lab *i*. L_i ~ i.i.d. $N(0, \sigma^2_{Lab})$.
. e_{ij} are the residuals. e_{ij} ~ i.i.d. $N(0, \sigma^2)$.
Repeatability relative standard-deviation is then given by $CV_r = \sqrt{\hat{\sigma}^2}/\hat{\mu}$ and reproducibility standard-deviation by $CV_R = \sqrt{\hat{\sigma}^2_{Lab} + \hat{\sigma}^2}/\hat{\mu}$ where K is the number of samples.

- Lot A: $\hat{\mu} = 6.44$, $CV_r = 18.4\%$, $CV_R = 27.1\%$

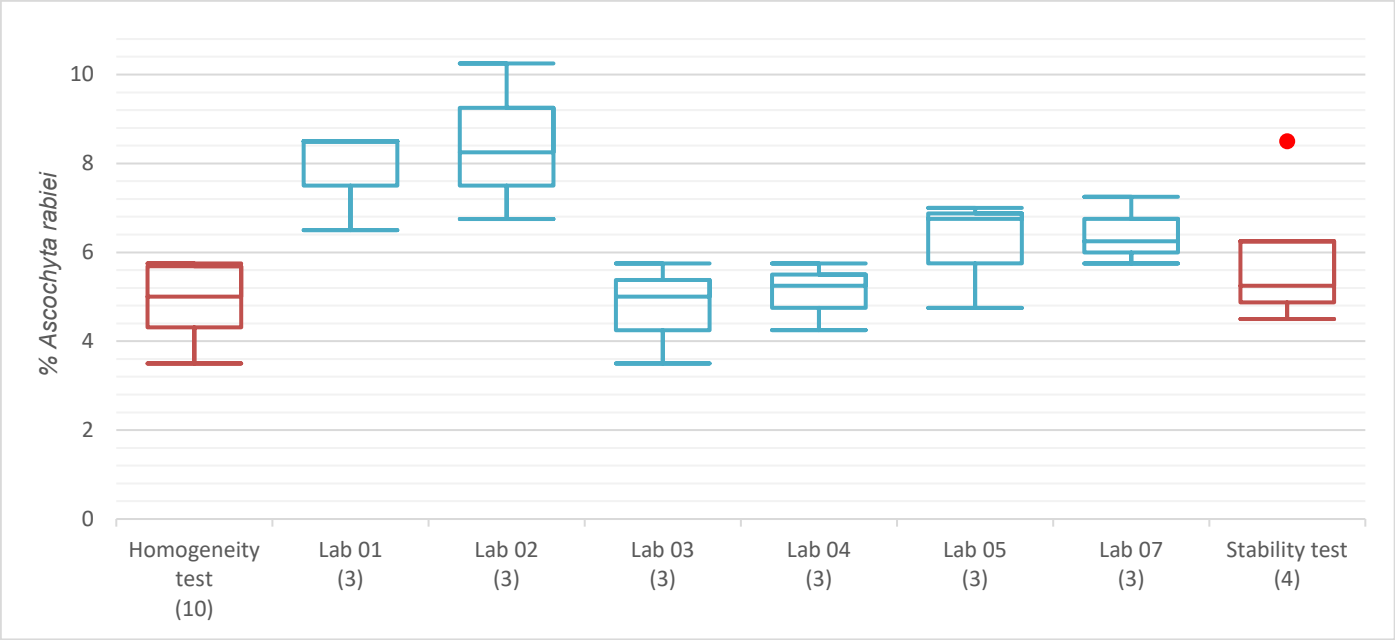
Table 24 – Results of Hampel's method on lot A (medium level)

Lot A

Laboratories	Values (Xi)	| Xi - M|	Status		
01	8.5	2.125	OK	**Median (M):**	**6.375**
	8.5	2.125	OK	**MAD:**	**1.000**
	6.5	0.125	OK	**5.2 X MAD**	**5.200**
02	6.75	0.375	OK		

	8.25	1.875	OK
	10.25	3.875	OK
03	5	1.375	OK
	5.75	0.625	OK
	3.5	2.875	OK
04	5.25	1.125	OK
	4.25	2.125	OK
	5.75	0.625	OK
05	4.75	1.625	OK
	6.75	0.375	OK
	7	0.625	OK
07	6.25	0.125	OK
	5.75	0.625	OK
	7.25	0.875	OK

Fig 19 – Box plot comparison of homogeneity, participating laboratories, and stability test on medium level.



- Lot B: $\hat{\mu} = 13.21$, $CV_r = 13.9\%$, $CV_R = 13.9\%$.

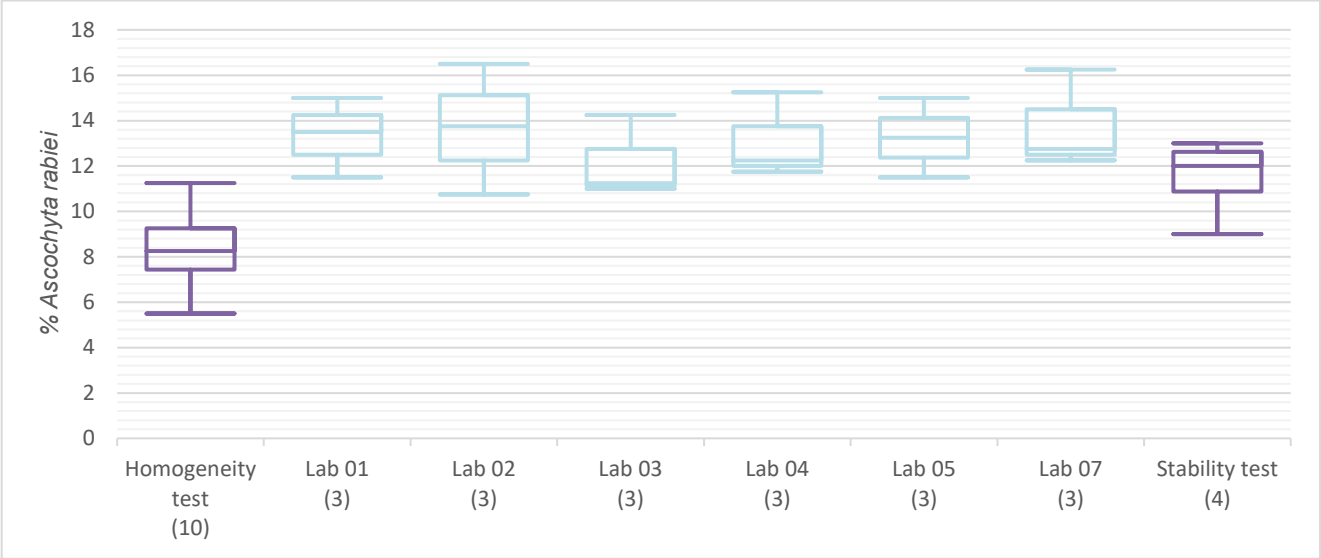
Table 25 – Results of Hampel's method on lot B (high level)

Lot B

Repetition	Values (Xi)	| Xi - M|	Status		
01	15	2.000	OK	**Median (M):**	13.000

	13.5	0.500	OK	**MAD:**	1.375
	11.5	1.500	OK	**5.2 X MAD**	7.150
02	13.75	0.750	OK		
	16.5	3.500	OK		
	12.75	0.250	OK		
03	11.25	1.750	OK		
	14.25	1.250	OK		
	11	2.000	OK		
04	12.25	0.750	OK		
	15.25	2.250	OK		
	11.75	1.250	OK		
05	15	2.000	OK		
	13.25	0.250	OK		
	11.5	1.500	OK		
07	12.25	0.750	OK		
	16.25	3.250	OK		
	12.75	0.250	OK		

Fig 20 - Box plot comparison of homogeneity, participating laboratories, and stability test on high level.



Conclusion of detection method:

Based on homogeneity test, each seed lot was characterized as homogeneous qualitatively and quantitatively.
Stability test has shown that each seed lot were stable in time, both qualitatively and quantitatively.

Qualitative and quantitative results of both medium and high level of the participants are as expected. Concordance analysis, Hampel's method and box plot analysis showed satisfying results of each participant and in accordance with the homogeneity test results and the expected results.

The repeatability relative standard-deviations are below 20% and the reproducibility relative standard-deviations are below 30%: we conclude that the method meets minimum performance criteria[1].

Pathogenicity test:
Quantitative results—Participation to pathogenicity test was optional, therefore, only Labs 03, 05 and 07 performed it.

Results are presented in Table 26.

Table 26 - Results of the pathogenicity test

Lab number	Obtained results		
	Isolate	NPC	PPC
Lab 03	All isolates are pathogenic	Negative	Positive
Lab 05	All isolates are pathogenic	Negative	Positive
Lab 07	All isolates are pathogenic	Negative	Positive
Reproducibility: Concordance	100%	Conform	Conform

Table 27 – Concordance calculated for the positive isolates from positive samples, qualitative results.

Laboratories	Repetition						Positive results	Negative results	# of according positive pairs	# of according negative pairs	Concordance (%)
	1	2	3	4	5	6					
03	+	+	+	+	+	+	6/6	0/6	153	0	100
05	+	+	+	+	+	+	6/6	0/6			
07	+	+	+	+	+	+	6/6	0/6			

Conclusion of detection method:

According to the results, each participant obtained positive results (presence of typical symptoms) for the PPC and negative results (absence of symptoms) for the NPC. Furthermore, all the participants obtained positive results on the isolates isolated from the positive samples. Concordance of these results reaches 100%, so reproducibility is validated according to the qualitative results.

Conclusion

This comparative test (CT) organized with 6 participants allowed to evaluate reproducibility of the method (detection and pathogenicity test). Pathogenicity test was optional, therefore only 3 laboratories performed it. Results of the CT indicated that reproducibility met requirement and is validated for both step of the method.

Appendix (Supporting information for seed health validation report):

List of annexes provided with report:

- Annex 1: Malt-agar media (MA)
- Annex 2: Potato Dextrose Agar media (PDA)
- Annex 3: Chickpea Seed Meal Dextrose Agar (CSMDA)
- Annex 4: Description of the morphological criteria for the detection method
- Annex 5: Analytical specificity results for the validation of the detection method

[1] ENGL considers an acceptable threshold of 30% for CV_r and 33% for CV_R in GMO testing. Specific thresholds should be defined in the future for SH CTs, but for now, we can consider the ENGL thresholds.

- Annex 6: Analytical specificity results for the validation of the pathogenicity test
- Annex 7: Raw data of comparative test – detection
- Annex 8: Hampel's analysis of participants results along with homogeneity test
- Annex 9: Raw data of comparative test – pathogenicity test

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Proposal for the addition of *Glycine max* [(L.) Merrill)] as a species to which the radicle emergence test for seed vigour can be applied.

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Summary

The objective of the current proposal was to conduct a comparative test to determine whether the radicle emergence (RE) test applied to soybean [*Glycine max* [(L.) Merrill)] seed lots is sufficiently repeatable within laboratories and reproducible between laboratories. The comparative test was carried out by five laboratories using six seed lots. Counts of radicle emergence (2.0 mm or more radicle visibly protruding through the seed coat) were recorded after both 24 hours at 25±1°C and 48 hours at 20±1°C. The RE test carried out in both test conditions differentiated those lots with higher RE values (lots 1, 2 and 3) from those with lower RE values (lots 4, 5 and 6) in all participating laboratories. Both test conditions performed correctly regarding repeatability and reproducibility. However assessment and counting of RE was easier for the longer radicles after 48 hours at 20±1°C for 48 hours which is therefore considered as more appropriate to be included as a vigour test for soybean seed lots.

Introduction

The radicle emergence (RE) is an ISTA vigour test validated for some crop species and included in the International Rules for Seed Testing, namely *Zea mays* (Matthews *et al*., 2011), *Brassica napus* (Powell *et al*., 2014), *Raphanus sativus* (Powell and Mavi, 2015) and *Triticum aestivum* subsp. *aestivum* (Khajeh-Hosseini *et al*., 2019). This test is based on differences in the mean germination time (MGT) of seed lots. The MGT is calculated by the regular counts of germinated seeds (Ellis and Roberts, 1980) and describes the average time for a seed to germinate, or the lag period from the start of imbibition to radicle emergence due to the need for more time for metabolic repair during the imbibition period (Matthews and Khajeh Hosseini, 2007; Matthews and Powell, 2011).

There are three vigour tests validated by ISTA for soybean seed: electrical conductivity (EC), accelerated ageing (AA) and tetrazolium (TZ) (ISTA, 2022). Although these tests have already proven their accuracy and efficiency for vigour status determination, the RE test has advantages since it is faster, simpler, and cheaper than other procedures. It also it does not require the use of chemicals, additional equipment, or analysts' experience in addition to knowledge about the standard germination test. While all ISTA-validated vigour tests for soybean species are accurate and reliable, the incorporation of RE would provide a further rapid and accurate tool for vigour assessment in soybean seed lots.

The RE test has previously been shown to identify the same differences in vigour as does the validated TZ vigour test, with high correlations between RE and TZ of 0.96 after 48 hours at 20°C, and 0.92 for RE after 24 hours at 25°C (Gallo *et al.*, 2022). More recently the RE test carried out for nine seed lots in the same test conditions as above has been shown to relate to both the TZ test results and seedling emergence in the field (FE) (Gallo *et al*, 2023). Thus the coefficients of determination of 0.74 between the RE count after 48 hours at 20°C and FE and of 0.68 after 24 hours at 20°C were very highly and highly significant respectively, indicative of good prediction of seed vigour by the RE test. The relationships

between RE, TZ and field emergence data in both studies therefore identified the RE test as a promising alternative vigour test for soybean seeds.

The objective of the current work was to conduct a comparative test to determine whether the RE test applied to soybean is both sufficiently repeatable within laboratories and reproducible between laboratories and the possibility of the test being validated by ISTA for this species.

Materials and methods

Seed material

Samples of six seed lots of *Glycine max* (non-GMO), from two cultivars, were obtained from Oliveros Experimental Station of National Institute of Agricultural Research, Argentina. All seed lots had germination above 80% (L1, 97%; L2, 95%; L3, 95%; L4, 86%; L5, 89% and L6, 93%). The six selected seed lots, identified by the numbers, were sent out to all participating laboratories with the corresponding protocol.

Participant laboratories

Coded samples of the seed lots were sent from Oliveros, Argentina to the participating laboratories, namely National Seed Institute, Canelones, Uruguay; National Institute of Agricultural Research, La Estanzuela, Uruguay; Urma Pampa Seed Lab, Argentina; Ing. Broda Seed Lab, Argentina and Los Brotes Seed Lab, Argentina. All laboratories had previous experience with testing *Glycine max.*

Testing procedure

Recipients were asked to store the seed in a moisture-proof container (polythene bags) at a low temperature (10°C) prior to use.

A run of the test was completed within approximately two weeks after receiving the seeds.

Radicle emergence test: the germination was carried out using four replicates of 50 seeds. The seeds were placed on two moistened paper towels. The seeds were placed apart from each other to facilitate the observation of the radicles. The seeds were then covered with another moistened paper towel, rolled up and placed into a polythene bag; the bags were placed upright in containers. The test was set up at both 20±1°C and 25±1°C. Counts of radicle emergence (2 mm or more radicle visibly protruding through the seed coat) were recorded 24 hours after sowing at 25±1°C and 48 hours after sowing at 20±1°C. The radicle emergence results were expressed as the percentage of seeds with an emerged radicle of at least 2 mm length.

The data were analysed with R Core Team (2022), ISTAgermMV package (2022) developed by ISTA Statistics Committee.

Results

Radicle emergence results obtained by the different laboratories.

Radicle emergence results obtained by the different laboratories using both test conditions identified the separation of lots into two groups: lots 1, 2 and 3 were the most vigorous lots, while lots 4, 5 and 6 were significantly less vigorous (table 1).

Table 1. Radicle emergence (RE) data assessed at 20±1°C for 48 hours (a) and 25±1°C for 24 hours (b) of six soybean seed lots in five laboratories.

20°C 48 hours	Lot					
Lab	1	2	3	4	5	6
A	81	78	84	61	57	68
B	73	70	79	28	36	36
C	69	70	79	55	55	64
D	85	86	94	67	56	74
E	82	81	84	62	55	66
Mean	78 A	77 A	84 A	54 B	52 B	61 B
SD	7	7	6	16	9	15

(a)

Each point is a mean of four replications and the significant difference was analysed by the Tukey test (Degrees of freedom test conditions: Lenth 2023). Means with a letter in common are not significantly different ($P \geq 0.05$)

25°C 24 hours	Lot					
Lab	1	2	3	4	5	6
A	25	25	34	10	11	10
B	24	19	26	5	8	10
C	4	2	12	3	5	5
D	29	23	30	6	5	3
E	4	8	4	2	2	2
Mean	17 AB	15 ABC	21 A	5 C	6 BC	6 BC
SD	12	10	13	3	4	4

(b)

Each point is a mean of four replications and the significant difference was analysed by the Tukey test (Degrees of freedom test conditions: Lenth 2023). Means with a letter in common are not significantly different ($P \geq> 0.05$)

Calculation of the z-scores (table 2) revealed that all data for the RE (%) did not exceed the value 2, therefore all results are considered satisfactory for the two test conditions.

Table 2. Comparison of z-scores of radicle emergence values for six seed lots of soybean tested by five laboratories using two test conditions for the RE test 20±1°C at 48 hours (a) and 25±1°C at 24 hours (b).

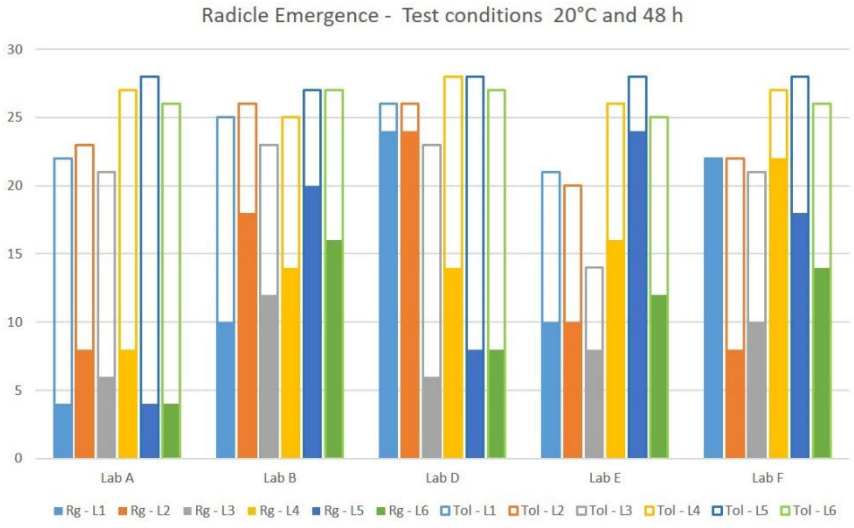
20°C 48 hours	Lot					
Lab	1	2	3	4	5	6
A	0,4	0,1	0,0	0,4	0,6	0,4
B	-0,7	-1,0	-0,8	-1,7	-1,8	-1,7
C	-1,4	-1,0	-0,8	0,0	0,4	0,1
D	1,0	1,3	1,7	0,8	0,5	0,8
E	0,6	0,6	0,0	0,5	0,3	0,3

(a)

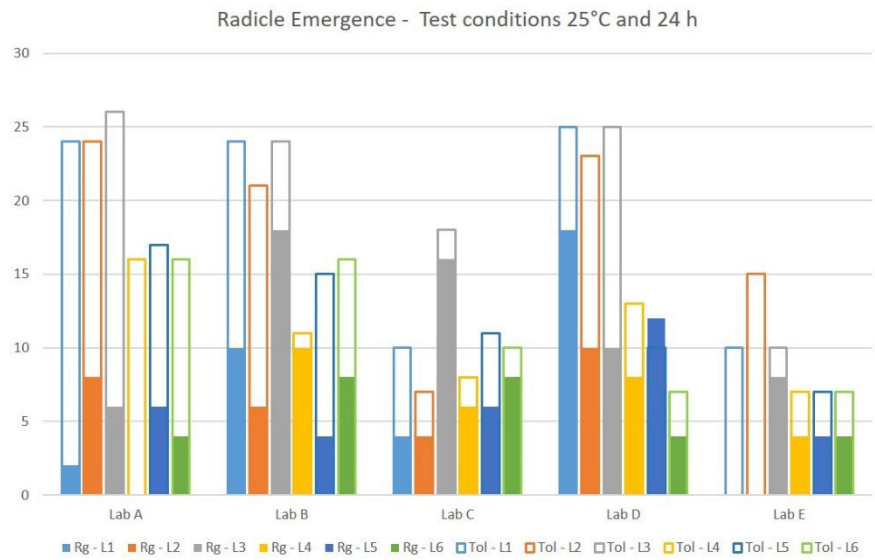
25°C 24 hours	Lot					
Lab	1	2	3	4	5	6
A	0,67	1,00	1,01	1,54	1,38	1,06
B	0,54	0,34	0,38	-0,06	0,55	1,06
C	-1,10	-1,34	-0,75	-0,71	-0,28	-0,33
D	0,96	0,74	0,70	0,26	-0,41	-0,84
E	-1,06	-0,73	-1,34	-1,03	-1,24	-0,96

(b)

The radicle emergence data obtained in both test conditions (20±1°C/48 hours and 25±1°C/24 hours), in all seed lots and in all laboratories, were within tolerance as shown in figures 1a and 1b. The only out-of-tolerance radicle emergence data corresponded to lot 5 evaluated by laboratory D in the conditions of 25±1°C/24 hours (figure 1b).



(a)



(b)

Figure 1. Data range (Rg) and tolerances (Tol) of radicle emergence for all soybean seed lots and test conditions, per laboratory. a) test conditions 20±1°C and 48 hours and b) test conditions 25±1°C and 24 hours.

Radicle emergence results obtained with different testing conditions.

Figure 2 presents the distributions of the percentage of radicle emergence obtained for all the samples and all the laboratories, depending on the seed testing conditions. The conditions of 20±1°C / 48 hours showed higher RE values compared to the conditions of 25±1°C/24 hours. In both test conditions, seed lots 1, 2 and 3 showed higher RE values than the seed lots 4, 5 and 6.

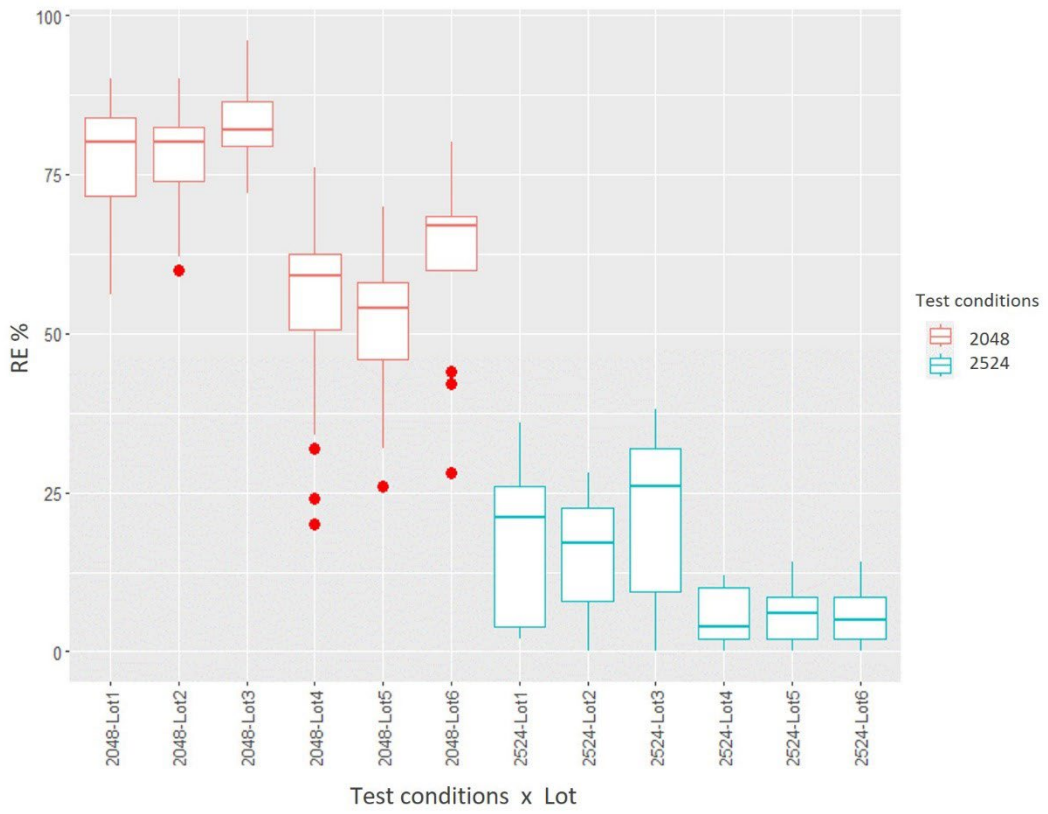


Figure 2. Box plots of the percentage of radicle emergence results per test conditions and per lot.

Figure 3 shows the same data of radicle emergence depending on the seed testing conditions and per laboratory. In the conditions of 20±1°C/48 hours, laboratories B and C had the lowest median RE values, while the lowest values at 25±1°C/24 hours, were seen for laboratories C and E.

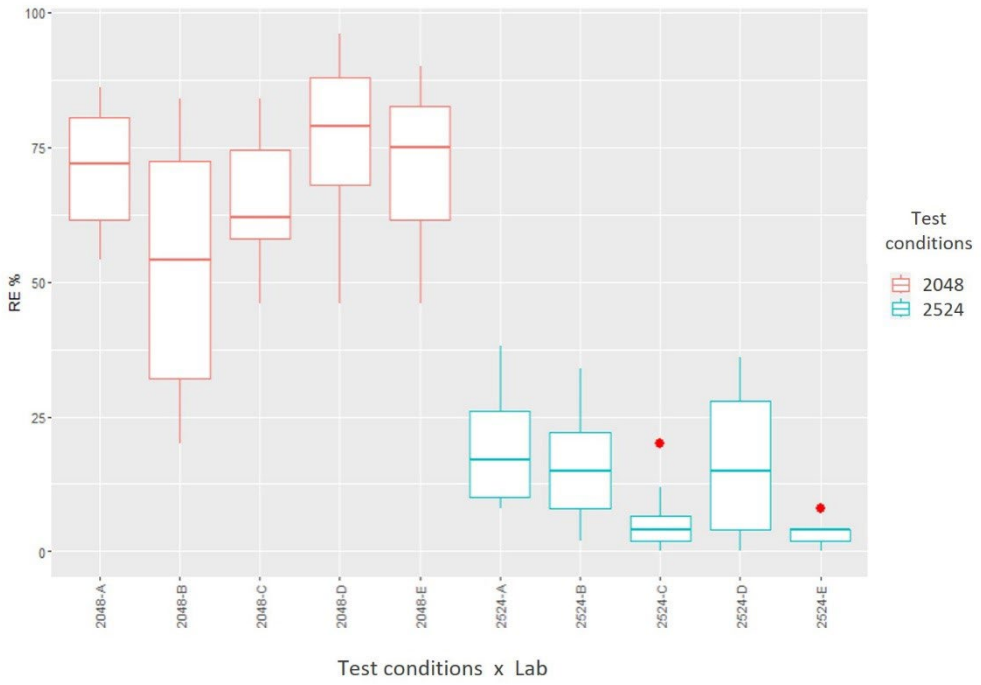


Figure 3. Box plots of the percentage of radicle emergence per test conditions and per laboratory.

Mixed model for comparing test conditions and mean seed lot values.
Results from fitting the mixed model are displayed in table 3 indicating that the interaction between test conditions and seed lots had a significant effect on radicle emergence results.
Table 3. Tests of fixed effects for seed lot in two test conditions: 20±1°C at 48 hours (a) and 25±1°C at 24 hours (b).

20°C 48 hours	Sum of Squares	Mean Square	Num DF	Den DF	F value	Pr(>F)
Lot	4907,5	981,51	5	20	23,462	<0.05

(a)

25°C 24 hours	Sum of Squares	Mean Square	Num DF	Den DF	F value	Pr(>F)
Lot	519,85	103,97	5	20	7,759	<0.05

(b)

Repeatability/Reproducibility

Repeatability and Reproducibility were estimated using a Linear Mixed model, using the R package ISTAgermMV. The results are shown in table 4 below.

Table 4. Estimate of repeatability and reproducibility of two evaluated test conditions for for determining RE in soybean seed.

Method	Mean	s_repeatability	dispersion	s_Reproducibility	s_Lab	s_LotxLab
20°C/48 h	68	6,5	0,98	12,02	8,6	5,4
25°C/24 h	12	3,7	0,81	9,18	6,6	5,2

The overall average percentage (mean) of the radicle emergence was different for the two test conditions due to the greater radicle development after 48 hours germination at 20±1°C. The development of the radicle allowed more rapid evaluation of the radicle emergence. The results indicated that the mean radicle count at 48 hours showed a higher number of seeds (68%) with radicles longer than 2.0 mm. After 24 hours at 25±1°C, there was a low mean percentage of the seeds (12%) with a radicle longer than 2.0mm.

The dispersion factor is less than 1 for both test conditions which indicates that both test conditions did not show problems regarding repeatability. The reproducibility standard deviation is function of the

$$\sigma^2$$

,

$$\sigma^2_{Lab}$$

, and

$$\sigma^2_{LabxLot}$$

. Even though both test conditions share the same measure unit, they are not comparable because the magnitude of the measurements for both test conditions differed. In the conditions of 20±1°C/48hours conditions, the Lot by Lab interaction standard deviation is relatively low, compared to the mean value, indicating consistent results across laboratories. In contrast, the Lot by Lab interaction standard deviation is high for 25±1°C/24hours when it is compared to the mean value for these test conditions.

Conclusion

Both RE test conditions identified the same seed lots as having high (lots 1,2,3) or low (lots 4,5,6) vigour. However, it was easier to quickly assess RE and to differentiate between the lots with the higher RE values when the test was carried out for 48 hours at 20±1°C compared to 24 hours at 25±1°C. The radicle emergence conditions of 20±1°C for 48 hours is therefore more suitable to be included as a vigour test for soybean seed than the conditions of 24 hours at 25±1°.

Acknowledgements

We would like to thank Ing. Agr. M.Sc. Juan Martín Enrico of the Department of Soybean Crop, Oliveros Experimental Station of National Institute of Agricultural Research, Argentina to provide the seed lots.

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Appendix

Appendix 1: Malt-agar media (MA)

Malt agar: According to manufacturer's instructions
Distilled/deionised water: 1000 mL
Streptomycin: may be used between 50 mg and 130 mg, depending on the level of saprophytic bacterial contamination commonly encountered.

Preparation

1. Weigh out ingredients into a suitable autoclavable container.
2. Add 1000 mL of water.
3. Dissolve completely the ingredients in water by stirring.
4. Autoclave at 15 p.s.i. and 121 °C for 15 min.
5. Allow agar to cool to approximately 50 °C, add Streptomycin sulphate dissolved in sterile water.
6. Pour 18-20 mL of malt agar into 90 mm Ø Petri dish and allow to solidify before use.

Appendix 2: Potato Dextrose Agar media (PDA)

Potato Dextrose Agar: According to manufacturer's instructions
Distilled/deionised water: 1000 mL
Streptomycin: may be used between 50 mg and 130 mg, depending on the level of saprophytic bacterial contamination commonly encountered.

Preparation

1. Weigh out ingredients 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 p.s.i. and 121 °C for 15 min.
5. Allow agar to cool to approximately 50 °C, add Streptomycin sulphate dissolved in sterile water.
6. Pour 18-20 mL of PDA into 90 mm Ø Petri dish and allow to solidify before use.

Appendix 3: Chickpea Seed Meal Dextrose Agar (CSMDA)

Chickpea seed meal :40 g
D-glucose: 20 g
Agar: 14 g
Distilled/deionised water: 1000 mL

Preparation

1. Weigh out ingredients 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 p.s.i. and 121 °C for 15 min.
5. Allow agar to cool to approximately 50 °C.
6. Pour 18-20 mL of CSMDA into 90 mm Ø Petri dish and allow to solidify before use.

Appendix 4: Description of the morphological criteria for the detection method

- ***Ascochyta rabiei*** (Pass.) Labr. syn. *Didymella rabiei* Kovatsch. ex Arx, syn. *Mycosphaerella rabiei* Kovatsch. ex Gruyter.

On malt-agar and PDA, **mycelium** develops slowly, white to green colored, development of pycnidia occurs at the surface of the substrate, they are pale brown to dark brown on both media. At 7 days, colony diameter varies between 10-35 mm.

Pycnidia immersed becoming erumpent, globose, dark brown, 140-200 µm [3], 65-245 µm [5] diam.; wall composed of 1-2 layers of elongated pseudo parenchymatic cells, ostiole 30 - 50µm [3][5] wide. Pycnidia usually release orange mucilaginous mass of pycnidiospores (cirrus).

Pycnidiospores hyaline, straight or slightly curved, (0–) 1 septate, some unicellular, slightly or not constricted at the septum, rounded at each end, 10–16 × 3–5 µm [3], 8.2-10 x 4.2-4.5 µm [5] formed from hyaline ampulliform phialides.

Fig 21 - *Ascochyta rabiei* on MA, 18 days darkness

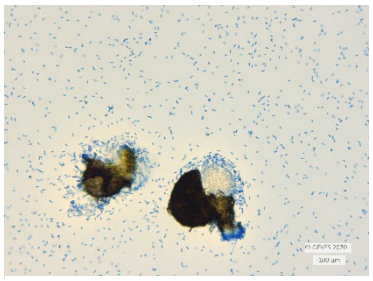

Fig 22 – Pycnidia and pycnidiospores (x10)

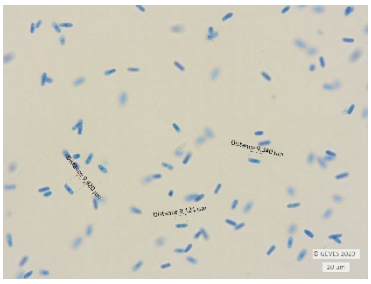

Fig 23 – Pycnidiospores (x40)

Appendix 5: Analytical specificity results for the validation of the detection method

: Meets criteria as described in Annex 4

Different from criteria as described in Annex 4

Strain	N° PAS	MA	PDA
Mycosphaerella rabiei	2908	✓	✓
Mycosphaerella rabiei	2909	✓	✓
Mycosphaerella rabiei	2919	✓	✓
Mycosphaerella rabiei	2920	✓	✓
Mycosphaerella rabiei	2921	✓	✓
Mycosphaerella rabiei	2922	✓	✓
Mycosphaerella rabiei	2923	✓	✓
Mycosphaerella rabiei	2924	✓	✓
Mycosphaerella rabiei	2925	✓	✓
Mycosphaerella rabiei	2926	✓	✓
Mycosphaerella rabiei	2930	✓	✓
Mycosphaerella rabiei	2931	✓	✓
Mycosphaerella rabiei	2947	✓	✓
Mycosphaerella rabiei	2950	✓	✓
Mycosphaerella rabiei	2958	✓	✓
Mycosphaerella rabiei	2963	✓	✓
Mycosphaerella rabiei	2964	✓	✓
Mycosphaerella rabiei	2982	✓	✓
Mycosphaerella rabiei	2983	✓	✓
Mycosphaerella rabiei	2984	✓	✓
Fusarium oxysporum	178	✓	✓
Didymella fabae	179	✗	✗
Phoma valerianellae	218	✗	✗
Didymella pinodes	250	✗	✗
Didymella pinodella	251	✗	✗
Stemphylium botryosum	263	✗	✗
Curvularia lunata	265	✗	✗
Alternaria tenuis	826	✗	✗
Epicoccum sp.	828	✗	✗
Ostracoderma sp.	1002	✗	✗
Penicillium sp.	1005	✗	✗
Trichoderma sp.	1006	✗	✗
Stysanus sp.	1009	✗	✗
Cladosporium sp.	1237	✗	✗
Strain	**N° PAS**	**MA**	**PDA**

Didymella pisi	1557	✗	✗
Aspergillus sp.	1849	✗	✗
Stagonosporopsis cucurbitacearum	2517	✗	✗
Botrytis cinerea	2910	✗	✗
Sordaria sp.	3064	✗	✗

Appendix 6: Analytical specificity results for the validation of the pathogenicity test

✓: Symptoms meets criteria as described in 4.2.2 Analytical specificity

✗ Symptoms are different from criteria as described in 4.2.2 Analytical specificity

Strain	N° PAS	Pathogenicity test
Mycosphaerella rabiei	2908	✓
Mycosphaerella rabiei	2909	✓
Mycosphaerella rabiei	2919	✓
Mycosphaerella rabiei	2920	✓
Mycosphaerella rabiei	2921	✓
Mycosphaerella rabiei	2922	✓
Mycosphaerella rabiei	2923	✓
Mycosphaerella rabiei	2924	✓
Mycosphaerella rabiei	2925	✓
Mycosphaerella rabiei	2926	✓
Mycosphaerella rabiei	2930	✓
Mycosphaerella rabiei	2931	✓
Mycosphaerella rabiei	2947	✓
Mycosphaerella rabiei	2950	✓
Mycosphaerella rabiei	2958	✓
Mycosphaerella rabiei	2963	✓
Mycosphaerella rabiei	2964	✓
Mycosphaerella rabiei	2982	✓
Mycosphaerella rabiei	2983	✓
Mycosphaerella rabiei	2984	✓
Fusarium oxysporum	178	✗
Didymella fabae	179	✗
Phoma valerianellae	218	✗
Didymella pinodes	250	✗
Didymella pinodella	251	✗
Stemphylium botryosum	263	✗
Curvularia lunata	265	✗
Alternaria tenuis	826	✗
Epicoccum sp.	828	✗
Strain	**N° PAS**	**Pathogenicity test**

Ostracoderma sp.	1002	
Penicillium sp.	1005	
Trichoderma sp.	1006	
Stysanus sp.	1009	
Cladosporium sp.	1237	
Didymella pisi	1557	
Phoma exigua	1847	
Aspergillus sp.	1849	
Stagonosporopsis cucurbitacearum	2517	
Botrytis cinerea	2910	
Sordaria sp.	3064	

Appendix 7: Raw data of comparative test - detection

Lab number	Level of contamination expected	Coded samples	Quantitative results	Mean	Qualitative results
Lab 01	Medium	47	8.5	7.8	+
	Medium	67	8.5		+
	Medium	72	6.5		+
	High	19	15	13.3	+
	High	35	13.5		+
	High	41	11.5		+
	Healthy	36	0	0.0	-
	Healthy	39	0		-
	Healthy	42	0		-
Lab 02	Medium	6	6.75	8.4	+
	Medium	46	8.25		+
	Medium	68	10.25		+
	High	12	13.75	13.7	+
	High	24	16.5		+
	High	63	10.75		+
	Healthy	11	0	0.0	-
	Healthy	50	0		-
	Healthy	65	0		-
Lab 03	Medium	4	5	4.8	+
	Medium	14	5.75		+
	Medium	31	3.5		+
	High	9	11.25	12.2	+
	High	28	14.25		+
	High	62	11		+
	Healthy	29	0	0.0	-
	Healthy	44	0		-
	Healthy	64	0		-

Lab number	Level of contamination expected	Coded samples	Quantitative results	Mean	Qualitative results
Lab 04	Medium	2	5.25	5.1	+
	Medium	18	4.25		+
	Medium	37	5.75		+
	High	1	12.25	13.1	+
	High	16	15.25		+
	High	49	11.75		+
	Healthy	8	0	0.0	-
	Healthy	10	0		-
	Healthy	43	0		-
Lab 05	Medium	27	4.75	6.2	+
	Medium	56	6.75		+
	Medium	57	7		+
	High	21	15	13.3	+
	High	48	13.25		+
	High	69	11.5		+
	Healthy	13	0	0.0	-
	Healthy	33	0		-
	Healthy	59	0		-
Lab 07	Medium	3	6.25	6.4	+
	Medium	70	5.75		+
	Medium	71	7.25		+
	High	20	12.25	13.8	+
	High	22	16.25		+
	High	58	12.75		+
	Healthy	25	0	0.0	-
	Healthy	30	0		-
	Healthy	53	0		-

Appendix 8 : Hampel's analysis of participants results along with homogeneity test

Medium

Results	Lab	Lab Values (Xi)	| Xi - M|	Status
Samples	1	8.5	2.750	OK
	2	8.5	2.750	OK
	3	6.5	0.750	OK
	4	6.75	1.000	OK
	5	8.25	2.500	OK
	6	10.25	4.500	OK
	7	5	0.750	OK
	8	5.75	0.000	OK
	9	3.5	2.250	OK
	10	5.25	0.500	OK
	11	4.25	1.500	OK
	12	5.75	0.000	OK
	13	4.75	1.000	OK
	14	6.75	1.000	OK
	15	7	1.250	OK
	16	6.25	0.500	OK
	17	5.75	0.000	OK
	18	7.25	1.500	OK
Homogeneity test	19	5.25	0.500	OK
	20	5.75	0.000	OK
	21	5.75	0.000	OK
	22	5.75	0.000	OK
	23	5.5	0.250	OK
	24	4.5	1.250	OK
	25	4	1.750	OK
	26	4.75	1.000	OK
	27	4.25	1.500	OK
	28	3.5	2.250	OK

Median (M):	**5.750**
MAD:	**1.000**
5.2 X MAD	**5.200**

High

Results	Lab	Lab Values (Xi)	| Xi - M|	Status
Samples	1	15	3.375	OK
	2	13.5	1.875	OK
	3	11.5	0.125	OK
	4	13.75	2.125	OK
	5	16.5	4.875	OK
	6	12.75	1.125	OK
	7	11.25	0.375	OK
	8	14.25	2.625	OK
	9	11	0.625	OK
	10	12.25	0.625	OK
	11	15.25	3.625	OK
	12	11.75	0.125	OK
	13	15	3.375	OK
	14	13.25	1.625	OK
	15	11.5	0.125	OK
	16	12.25	0.625	OK
	17	16.25	4.625	OK
	18	12.75	1.125	OK
Homogeneity test	19	8.5	3.125	OK
	20	10.25	1.375	OK
	21	8	3.625	OK
	22	8.25	3.375	OK
	23	9.5	2.125	OK
	24	5.5	6.125	OK
	25	7.25	4.375	OK
	26	8.25	3.375	OK
	27	6	5.625	OK
	28	11.25	0.375	OK

Median (M):	**11.625**
MAD:	**2.125**
5.2 X MAD	**11.050**

Appendix 9: Raw data of comparative test – pathogenicity test

Lab number	Isolate or control	Results	Quantitative results	Pathogenicity
Lab 03	PPC	2+/2	+	Pathogen
	NPC	0+/2	-	Not pathogen
	4-3-1	3+/3	+	Pathogen
	4-22-1	3+/3	+	Pathogen
	4-33-1	3+/3	+	Pathogen
	9-9-1	3+/3	+	Pathogen
	9-16-1	3+/3	+	Pathogen
	9-21-1	3+/3	+	Pathogen
	14-9-1	3+/3	+	Pathogen
	14-20-1	3+/3	+	Pathogen
	14-33-1	3+/3	+	Pathogen
	28-1-1	3+/3	+	Pathogen
	28-8-1	3+/3	+	Pathogen
	28-13-1	3+/3	+	Pathogen
	31-21-1	3+/3	+	Pathogen
	31-22-2	3+/3	+	Pathogen
	31-34-1	3+/3	+	Pathogen
	62-8-1	3+/3	+	Pathogen
	62-34-1	3+/3	+	Pathogen
	62-35-1	3+/3	+	Pathogen
Lab 05	PPC	2+/3	+	Pathogen
	NPC	0+/3	-	Not pathogen
	21	3+/3	+	Pathogen
	27	3+/3	+	Pathogen
	48	3+/3	+	Pathogen
	56	3+/3	+	Pathogen
	57	3+/3	+	Pathogen
	69	3+/3	+	Pathogen
Lab 07	PPC	2+/2	+	Pathogen
	NPC	0+/2	-	Not pathogen
	3-12-1	3+/3	+	Pathogen
	3-23-1	3+/3	+	Pathogen
	3-71-1	3+/3	+	Pathogen
	20-31-1	3+/3	+	Pathogen
	20-42-1	3+/3	+	Pathogen
	20-61-1	3+/3	+	Pathogen
	22-1-1	3+/3	+	Pathogen
	22-51-1	3+/3	+	Pathogen
	22-63-1	3+/3	+	Pathogen
	58-11-1	3+/3	+	Pathogen
	58-25-1	3+/3	+	Pathogen
	58-78-1	3+/3	+	Pathogen
	70-2-1	3+/3	+	Pathogen
	70-34-1	3+/3	+	Pathogen

	70-41-1	3+/3	+	Pathogen
	71-23-1	3+/3	+	Pathogen
	71-51-1	3+/3	+	Pathogen
	71-73-1	3+/3	+	Pathogen