International Seed Federation
Seed is Life

Detection of *Peronospora valerianellae* in corn salad (*Valerianella locusta*) seed

Validation report, April 2025

ISHI International Technical Group RBLV – G. Orgeur (GEVES, France)

ISHI VALIDATION REPORTS

This ISHI validation study has been conducted to determine the fitness of the described method for its intended purpose according to the ISHI Guidelines for the Validation of Seed Health Methods[1] and followed by an independent review of its outcome.

DISCLAIMER

ISF cannot guarantee that laboratories following the protocol described herewith will obtain similar results. Many factors, such as staff skills, laboratory equipment and conditions, reagents and sampling methods can influence the results. Consequently, in case of any litigation ISF will not accept any liability on the use of these tests.

Published by:

International Seed Federation (ISF)

Chemin du Reposoir 7, 1260 Nyon, Switzerland

Developed by ISHI

All rights reserved - @2025 ISF

[1] Available at: https://www.worldseed.org/our-work/seed-health/ishi-method-development-and-validation/

CONTENTS

Detection of *Peronospora valerianellae* in corn salad (*Valerianella locusta*) seed

SUMMARY

Peronospora valerianellae is a biotrophic, seed borne pathogen of corn salad (*Valerianella locusta*), which causes deformation and sporulation on leaves of seedlings. The primary way to prevent *P. valerianellae* infection on corn salad is to start with seeds that are free from *P. valerianellae*.

The goal of this project was to validate a method to detect *P. valerianellae* in corn salad seeds according to ISHI guidelines for the Validation of Seed Health Tests. This method is based on the detection of the fungus by grow-out identification, which combines detection and proof that the pest is alive and pathogenic.

Different criteria were evaluated to validate the method:

- The analytical specificity was evaluated on a collection of *P. valerianellae* infected seed lots. Typical symptoms (curling of cotyledons and/or white sporulation on cotyledons) were observed on every *P. valerianellae* positive seed lot tested. The results obtained met the analytical specificity requirements.
- The analytical sensitivity experiment indicated the limit of detection of the grow-out method and the capacity to detect one infected seed in a sample.
- The selectivity of the method was evaluated on different cultivars of corn salad seeds from different origins and was deemed fit for purpose.
- The repeatability was demonstrated by repeating the grow-out method with minimal variations in one laboratory three times on three different seed lots with a variable infection level.
- A comparative test (CT), including four participants, allowed for the evaluation of the reproducibility and diagnostic performance. Data from the CT indicated that the reproducibility and diagnostic performance of the method meet the requirements. Statistical analysis of quantitative data showed that the infection rates cannot be reproduced quantitatively in a sufficiently reliable manner, and that quantitative differences occur among laboratories. However, the presence of *P. valerianellae* can be identified with high reproducibility in each laboratory for all infection levels qualitatively.

Each validation criteria evaluated met the requirements and the method is deemed fit for purpose for detection of *P. valerianellae* in corn salad seed.

1. INTRODUCTION

Peronospora valerianellae (P. valerianellae) is a biotrophic, seed borne pathogen of corn salad (*Valerianella locusta*), which causes deformation and sporulation on leaves of seedlings (Koike, 2008; Figure 1). The primary way to prevent *P. valerianellae* infection on corn salad is to start with seeds free of *P. valerianellae*.

Figure 1. *Peronospora valerianellae* sporulation on corn salad seedling.

Several detection methods for *P. valerianellae* exist in the seed industry. Some are based on a grow-out (germination on substrate and observation of symptoms), while others are based on a seed wash with observation of oospores. The latter however does not reveal if the pest is alive. Therefore, the grow-out method, which combines detection and proof that the pest is alive and pathogenic, is preferred.

Following a comparison of different grow-out methods in use in 2018 (data not published), a standardized ISHI grow-out method for the detection of *P. valerianellae* in corn salad seed was developed. The method workflow is presented in Figure 2. The method is a grow-out of a sample of 400 seeds that demonstrates the viability and pathogenicity of the pathogen.

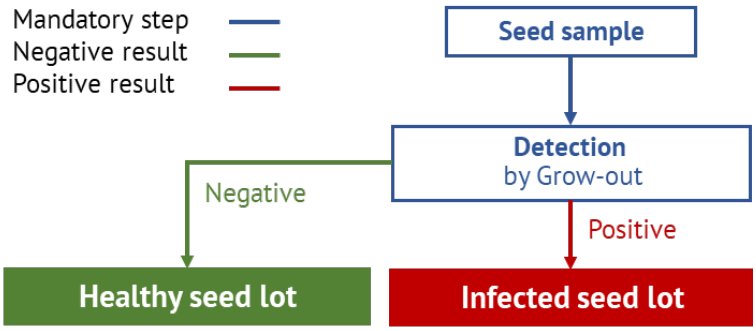

Figure 2. Workflow of the detection of *P. valerianellae* in corn salad seeds.

2. OBJECTIVES

The objectives of this study were to validate the standardized ISHI grow-out method to detect *P. valerianellae* in corn salad seed according to the ISHI guidelines for the Validation of Seed Health Tests (ISHI, 2020), and to show that the method is able to detect one infected seed in a sample of 400 seeds.

3. METHOD VALIDATION

The protocol used in this study is described in Annex A.

3.1. Analytical specificity

Definition ISHI guidelines: *The ability of an assay to detect the target(s) pathogens (inclusivity) while excluding non-targets (exclusivity).*

The analytical specificity requirements will be met when typical symptoms are observed on every *P. valerianellae* positive seed lot tested.

Typical symptoms caused by *P. valerianellae* are defined as one or more of the following characteristics: curling of cotyledons perpendicular to the axis of the stem (Figure 3A-F) without or with white sporulation on cotyledons (Figure 3A-C and Figure 3D-F, respectively) and an elongation of the stem of seedlings (Figure 3G-I).

Experimental approach

The proposed grow-out was carried out on 16 seed lots that were made available by ISHI members and were obtained from different regions to ensure that the conditions specified in the protocol are optimal against a potential wide range of isolates. The 16 seed lots were the only ones received from seed companies, without information about presence of target or non-target pathogen (unknow status). A total of 400 seeds from each seed lot was tested, as described in the protocol (Annex A). Observations on sporulation, rate of infection and other characteristic symptoms were assessed at the end of the incubation period.

A precise description of typical symptoms was noted, and the length of infected and healthy seedlings was measured qualitatively (similar, shorter or longer) to determine if this criterion is relevant.

As indicated in Table 1, five seeds lots were obtained as negative and eleven as positive for *P. valerianellae*. Infected seed lots were selected by the participants of the validation study exclusively based on grow-out, as it is known that the results of the seed-wash and grow-out are not always aligned, i.e., seed-wash positives have been observed to be grow-out negative, possibly due to the presence of dead oospores detected in the seed wash. The reverse has also been observed, possibly due to the presence of internal oospores or mycelium only inside the seeds.

Table 1. Characteristics of the corn salad seed lots included in the analytical specificity assessment.

Company	Lot #	Varieties	Infection rate	Qualitative results
1	Lot 1	Variety 1	6.00%	+
	Lot 2	Variety 2	3.00%	+
	Lot 3	Variety 3	5.00%	+
2	Lot 4	Variety 4	0.00%	-
	Lot 5		0.00%	-
	Lot 6		2.22%	+
	Lot 7		0.00%	-

Company	Lot #	Varieties	Infection rate	Qualitative results
	Lot 8	Variety 5	0.37%	+
	Lot 9		0.00%	-
	Lot 10	Variety 6	1.22%	+
	Lot 11		0.31%	+
	Lot 12	Variety 7	3.38%	+
	Lot 13	Variety 8	0.00%	-
3	Lot 14	Variety 9	Unknown	+
	Lot 15	Variety 10	Unknown	+
4	Lot 16	Variety 11	Unknown	+

Data analysis

The data from the experiment were analysed qualitatively (positive *vs*. negative) for the identification criteria, *viz*. curling cotyledons and/or white sporulation on cotyledons.

Results

Prior to this assay, it was determined by the ISHI project group that the symptoms caused by *P. valerianellae* on corn salad cotyledons, as detected by this grow-out analytical method, are typical and can hardly be confused with those of other pathogens (data not shown). These symptoms are characterized by curling of the cotyledons towards the stem (curling in the perpendicular axis of the stem; Figure 3A-C), which can be combined with the presence of white sporangiophores (Figure 3D-F). In the different tests described above, analytical specificity was determined by observations combined with pictures representing typical symptoms in order to define all their characteristics. Non-typical symptoms were also observed and compared against real symptoms, as for example non-typical curling of cotyledons in the vein axis direction (Figure 3J-L). In this case, no Pv was observed.

Moreover, a specific description of the sporulation was documented. The sporangiophore appearance is branched with thin ends and curved branchlets that do not have swollen tips. Conidia are ovoid, brownish and measured 15.9 to 22.2 µm-wide and 23.7 to 29.4 µm-long, as described by Koike (2008).

The seedling size can be an indication of infection, as indicated in Table 2. As observed in Figure 3G-I and 4, infected seedlings may be longer compared to healthy seedlings in some cases, whereas the healthy seedlings may be shorter.

Conclusion

Analytical specificity of the method allows for the detection of the target pathogen as described: slender seedling characterized by curled cotyledons towards the stem, with sporangiophore being branched with thin ends and ovoid, brownish conidia, which measured 15.9 to 22.2 µm-wide and 23.7 to 29.4 µm-long.

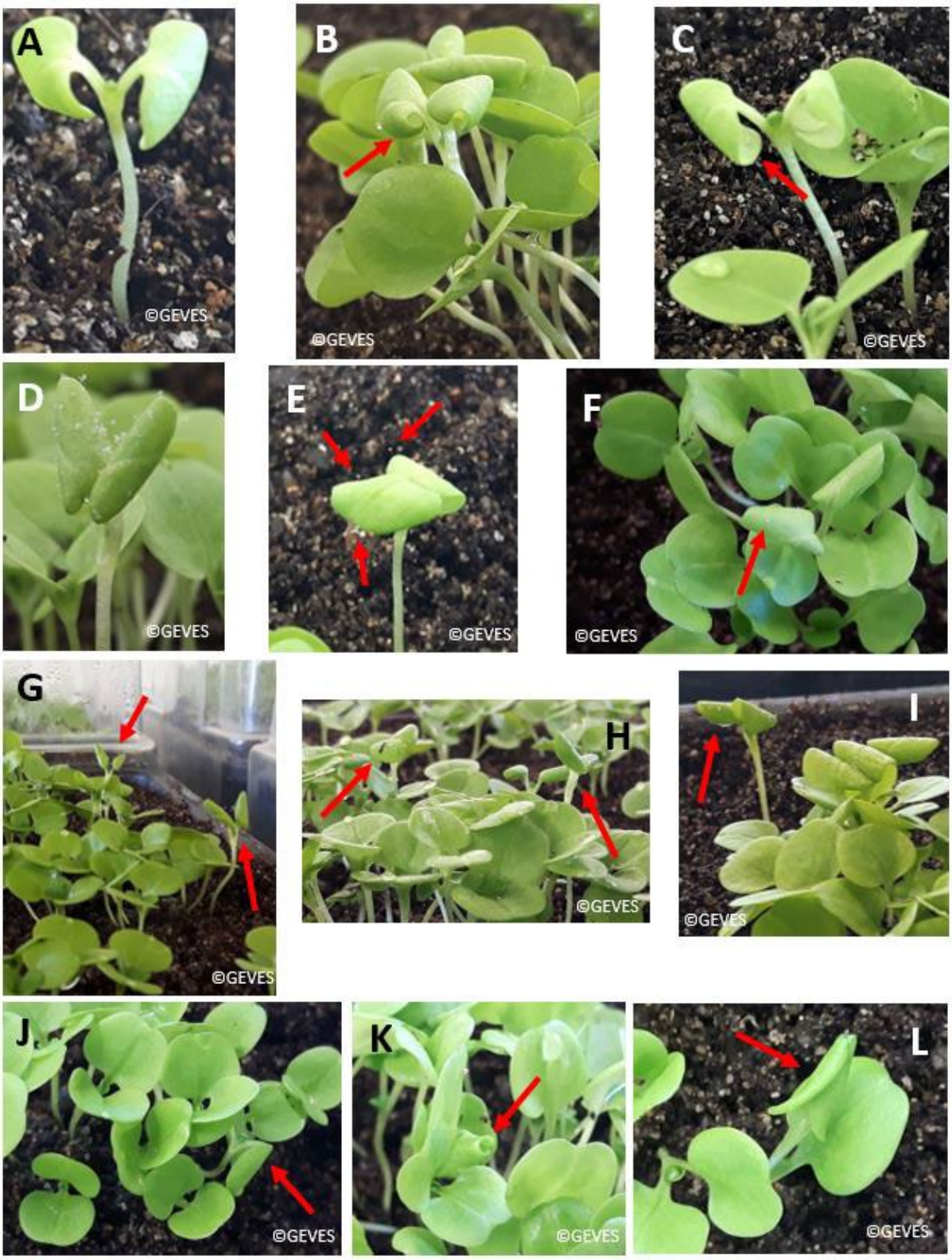

Figure 3. Seedling pictures of corn salad seedlings at 21 days after sowing. A, B and C: curling of cotyledons without sporangiophores typical of *P. valerianellae*; D, E and F: curling of cotyledons with sporangiophores typical of *P. valerianellae*; G, H and I: typical examples of seedlings infected with *P. valerianellae* showing elongation of the stem; J, K and L: non-typical curling of cotyledons, curling of the cotyledons in the vein axis direction.

Table 2. Seedling symptoms recorded on the 16 seed lots.

Seed lot status	# Seed lots	Symptoms	Seedling size
Positive	11	Typical curled cotyledons with and without sporulation	Longer or similar to other seedlings
Negative	5	No sporulation Some non-typical curled cotyledons	Shorter or similar to other seedlings

Figure 4. Variation in seedling size 14 days after sowing. Infected seedlings (represented by red arrow) are taller than healthy seedlings (represented by blue arrow) of the same seed lot.

3.2. Analytical sensitivity

Definition ISHI guidelines: *Smallest amount of the target pathogen that can be detected i.e., the limit of detection (LOD).*

The requirements for analytical sensitivity will be met when the LOD of the grow-out, determined using a dilution method, will be 0.25% of infection (correlating to at least one infected seed in a sample of 400 seeds).

Experimental approach

As *P. valerianellae* is an obligate pathogen, it is not possible to precisely create the required number of infected seeds by artificial inoculation. Therefore, naturally infected seed lots were used for this experiment. The percentage of infection of the positive seed lot was determined by testing eight replicates of 400 seeds by grow-out, as described in Annex A. The seed lot chosen for dilution had an infection rate of 3.25% and was diluted with seeds from known healthy seed lot to create the following three rates of infection: 0.25%, 1% and 2%. Process to obtain these rates is described in Table 3.

Table 3. Dilution of positive seed lot to obtain the different infection rates.

	Rate of infection			
	2%	**1%**	**0.25%**	**0%**
Dilution factor	0.62	0.31	0.08	0.00
Number of seeds from naturally infected seed lot (3.25% infection)	246	123	31	0
Number of seeds from healthy seed lot	154	277	369	400

Three replicates of 400 seeds from each level of infection, as well as from the healthy, and the undiluted infected sample were tested.

Data analysis

The limit of detection was calculated by estimating the rate of infection, expressed in percentage (%), which is the number of infected seedlings against the total number of seeds sown and when all the replicates were detected positive.

Results

Infection of the corn salad seeds was evaluated after 21 and 28 days post inoculation (DPI) (incubation period). A summary of the results is presented in Table 4, showing that one out of 400 seeds on each three replicates for infected seed lot at 0.25% was detected as infected. The limit of detection of the grow-out assay is confirmed to be one infected seed out of 400.

For the seed lot infected at 0.25%, the sporulation of the infected seedling for replicate 2 was observed at 28 DPI. It is important to note that late sporulation may be observed after 21 DPI.

Note: If more than 25-30 % of the corn salad seedling show damping-off or do not germinate, this must be noted and indicated on the test result.

Table 4. Results obtained for the analytical sensitivity assessment. D+ : Days after sowing.

	Seed lot infection rate											
	2%			**1%**			**0.25%**			**0% (Healthy)**		
Replicates	1	2	3	1	2	3	1	2	3	1	2	3
Numbers germinated seedlings (/400)	375	374	376	363	354	359	349	341	342	400	398	399
Numbers curled cotyledons	10	6	12	8	5	4	1	1	1	0	0	0
Numbers sporulated at D+21	10	6	12	7	5	4	1	0	1	0	0	0
Numbers sporulated at D+28	10	6	12	7	5	4	1	1	1	0	0	0
% of positive seeds	2.50	1.50	3.00	2.00	1.25	1.00	0.25	0.25	0.25	0.00	0.00	0.00

Conclusion

As the infected seed lots were prepared with the naturally infected seed lot at 3.25% mixed with a negative seed lot, the percentage of infection obtained were not exactly as expected. For example, the subsamples of the infected seed lot were expected to display an infection of 2%, but obtained rates were 2.5%, 1.5% and 3.0%, with an average of 2.33%. The quantitative data

displayed variations due to a varying presence of true infected seeds in each subsample (respectively 10, 6 and 12 seeds in 400) and allowed for variations in the infection rate in the different subsamples.

To conclude, the grow-out assay is deemed fit to purpose as it allowed for the detection of a single infected seed of a sample of 400 seeds.

3.3. Selectivity

Definition ISHI guidelines: *The effect of different seed matrices on the ability of the method to detect target pathogen(s).*

The selectivity requirements will be met when *P. valerianellae* is detected on seed lots belonging to all tested varieties and production origins.

Experimental approach

Infected seed lots of different varieties of corn salad obtained from different production origins were tested. Depending on the availability of infected samples, at least three replicates of three varieties from three production origins were tested. The data from specificity were reused for part of the selectivity experiments. The expected results were determined by grow-out test.

Results

Results for the assessment of selectivity are presented in Table 5. Five seed lots (Lots 6, 8, 10, 11 and 12) corresponding to four different varieties were detected as contaminated by the grow-out assay. A 100% correlation is observed between expected results and obtained results. Due to confidentiality, the origins of production could not be obtained for each seed lot. Seed providers however confirmed that the seed lots 4 to 13 originated from three different countries (Germany, France, and New Zealand).

Table 5. Selectivity result of seed lots from four different varieties. +: seed lot positive for *P. valerianellae*.

Seed lot	Variety #	Expected results	Obtained infection rates (%)			Qualitative results
			Replicate 1	Replicate 2	Replicate 3	
Lot 6	1	+	2.2	1.1	2.2	+
Lot 8	3	+	0.4	0.7	0.4	+
Lot 10	4	+	1.2	0.6	0.9	+
Lot 11	4	+	0.3	0.3	0.6	+
Lot 12	5	+	3.4	2.4	4.1	+

Conclusion

Peronospora valerianellae was detected by the grow-out method in different cultivars of corn salad seeds and from different origins. Therefore, the selectivity of the method meets the requirements and is deemed fit for purpose.

3.4. Repeatability

Definition ISHI guidelines: *Degree of similarity in results of replicates of the same seed lots when the method is performed with minimal variations in a single laboratory.*

The requirements for repeatability will be met when the experiments performed in the same laboratory on three replicates of three seed lots with different infection levels on the same day by the same technician and using the same equipment produce the same results, giving rise to the same conclusion.

Experimental approach

The repeatability experiment was carried out using two naturally infected seed lots, one infected just above the LOD and the second at a higher level of infection, and a healthy seed lot. Three replicates of 400 seeds of each seed lot were tested.

Statistical analysis

The qualitative results were analysed with the method developed by Langton *et al.* (2002) for repeatability (accordance).

Results

The three replicates of the two naturally infected seed lots were consistently detected as positive for contamination with *P. valerianellae* (Table 6). The three replicates of the healthy seed lot were consistently detected as negative. Accordance for both the healthy and naturally infected seed lots was calculated to be 100%.

Table 6. Result of repeatability assay for the three seed lots. +: seed lot positive for *P. valerianellae*; –: seed lot negative for *P. valerianellae*.

Seed lot	Expected results	Obtained infection rate (%)			Qualitative result
		Replicate 1	Replicate 2	Replicate 3	
11 (LOD)	+	0.3	0.3	0.6	+
12 (High)	+	3.4	2.4	4.1	+
13	-	0.0	0.0	0.0	-

Conclusion

The repeatability for the grow-out method meets the requirement showing that seed lots tested by the same laboratory by the same technician and using the same equipment yield the same outcome.

3.5. Reproducibility

Definition ISHI guidelines: *Degree of similarity in results when the method is performed across laboratories with replicates of the same subsamples.*

The reproducibility requirements will be met when the measure for this performance criteria, i.e., concordance among participant laboratories is ⩾90%.

Experimental approach

A comparative test (CT) was organized with four participating laboratories. Each participant received samples of the healthy, medium, and high infected seed lots. Three samples per level of infection were included. The participants processed the samples according to the protocol (Annex A).

Before sending the samples to participants, the test organizer tested them for homogeneity.

After the last participant had started the test, within a two month-period after reception of samples, a set of the samples was tested by the organizer for determining the stability of infection.

Data analysis

Homogeneity and stability test results were used to evaluate the results of participants at the qualitative level (detected/non detected) using the method developed by Langton *et al.* (2002) for reproducibility (concordance). Quantitative data, i.e. percentage of infection of the seed lot, were analysed using box plots.

Results

Homogeneity test

The three seed lots were tested in 10 replicates of 400 seeds each.

At the qualitative level, expected results were obtained for all three seed lots. All subsamples of the negative seed lot were detected as negative (0+/10 tested). All subsamples of the two positive seed lots (medium and high infection) were detected as positive (10+/10 tested) (Table 7).

Table 7. Qualitative results and infection rate obtained for each lot for the homogeneity assessment. +: seed lot positive for *P. valerianellae*; –: seed lot negative for *P. valerianellae*.

Replicates	Healthy lot		Medium infection lot		High infection lot	
	Qualitative result	Infection rate (%)	Qualitative result	Infection rate (%)	Qualitative result	Infection rate (%)
1	-	0.00	+	4.25	+	4.75
2	-	0.00	+	2.25	+	6.25
3	-	0.00	+	4.00	+	9.25
4	-	0.00	+	4.75	+	5.25
5	-	0.00	+	3.50	+	7.50
6	-	0.00	+	4.75	+	6.25
7	-	0.00	+	4.00	+	4.50
8	-	0.00	+	3.25	+	4.50
9	-	0.00	+	3.25	+	4.25
10	-	0.00	+	3.25	+	7.25
Mean		0.00		3.73		6.00
Qualitative results	0+/10		10+/10		10+/10	

Stability test

The three seed lots were tested by the organizing laboratory on three replicates of 400 seeds.

All the qualitative results of the two infected lots (medium and high infection with 3+/3) and the negative seed lots (0+/3) were as expected (Table 8).

The qualitative results confirmed homogeneity and stability of the three different seed lots.

Table 8. Qualitative results and infection rate obtained for each lot for the stability assessment. +: seed lot positive for *P. valerianellae*; –: seed lot negative for *P. valerianellae*.

Replicate	Healthy lot		Medium infection lot		High infection lot	
	Qualitative result	Infection rate (%)	Qualitative result	Infection rate (%)	Qualitative result	Infection rate (%)
1	-	0.00	+	2.00	+	5.75
2	-	0.00	+	4.00	+	6.50
3	-	0.00	+	2.50	+	4.25
Mean		0.00		2.83		5.50
Qualitative result	0+/3		3+/3		3+/3	

Comparative test

Each participant received three replicates from the healthy, medium, and high infected seed lots and tested them by grow-out assay, as described in Annex A. Results were analysed at qualitative level. The results obtained by each participant are presented in Table 9.

All negative samples were detected as negative (0+/3), as expected, for all participants. For the infected seed lot, one participant (participant 4) detected only two replicates of the three as positives for both infected seed lots (medium and high). After discussion with participant 4, two deviations from the protocol were identified: 1) only 200 seeds were tested instead of 400 seeds, and 2) the incubation period prior to seedlings observation was not followed (D+14 instead of D+21/D+28). As such, it was decided to exclude participant 4 from the analysis when calculating the test's concordance.

Result from qualitative data analysed by Langton *et al.* (2002), with the exclusion of the data from participant 4, confirmed a concordance at 100%. Additionally, the quantitative data of the homogeneity, CT participants (excluding laboratory 4) and stability experiment, expressed in rate of infection of the seed lot, was analysed using box plots (Figure 5). For the medium infected seed lot, a significant deviation in the infection rates was observed (Figure 5A). For the high infected lot, results from participant 2 deviated from the other results (Figure 5B).

Conclusion

The homogeneity test showed that at the qualitative level the three seed lots were homogeneous and the stability test revealed that the infection of the three seed lots were stable. The qualitative analysis, by Langton *et al.* (2002), of the results obtained from the CT participants, excluding one participant that did not respect the protocol, allowed to confirm a concordance at 100%, for which the reproducibility of the method meets the requirements.

The analysis of the quantitative data showed that the infection rates cannot be reproduced in a sufficiently reliable manner, and that quantitative differences occurred among laboratories.

However, the presence of *P. valerianellae* can be identified with high reproducibility in each laboratory for all infection levels qualitatively.

Table 9. Qualitative results and infection rate obtained per participant for each lot in the CT. +: seed lot positive for *P. valerianellae*; –: seed lot negative for *P. valerianellae*.

Participant #	Replicates #	Healthy		Medium		High	
		Qualitative result	Infection rate (%)	Qualitative result	Infection rate (%)	Qualitative result	Infection rate (%)
1	1	-	0.00	+	1.89	+	8.73
	2	-	0.00	+	2.24	+	5.60
	3	-	0.00	+	2.54	+	3.20
Mean			0.00		2.22		5.84
Qualitative result		0+/3		3+/3		3+/3	
2	1	-	0.00	+	0.75	+	3.00
	2	-	0.00	+	1.25	+	2.75
	3	-	0.00	+	1.00	+	3.00
Mean			0.00		1.00		2.92
Qualitative result		0+/3		3+/3		3+/3	
3	1	-	0.00	+	2.50	+	2.75
	2	-	0.00	+	1.25	+	5.50
	3	-	0.00	+	1.75	+	6.50
Mean			0.00		1.83		4.92
Qualitative result		0+/3		3+/3		3+/3	
4	1	-	0.00	-	0.00	+	2.00
	2	-	0.00	+	0.50	-	0.00
	3	-	0.00	+	1.00	+	2.00
Mean			0.00		0.50		1.33
Qualitative result		0+/3		2+/3		2+/3	

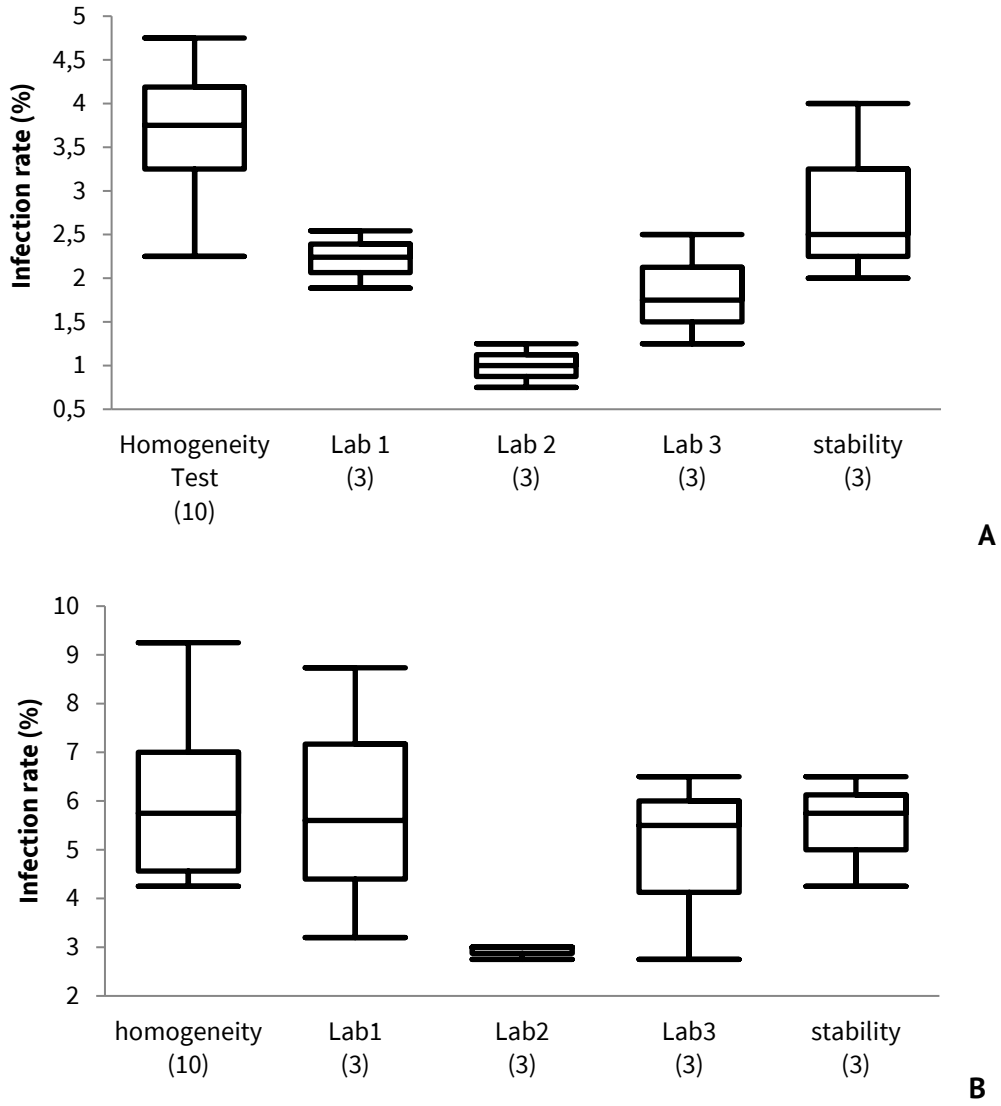

Figure 5. Comparative analysis by box plot of the homogeneity, CT participants (without laboratory 4) and stability test data on (A) medium infected and (B) high infected seed lot.

3.6. Diagnostic performance

Definition ISHI guidelines: *An evaluation of the ability of the test to discriminate between positive and negative seed lots.*

The diagnostic performance requirements will be met when the accuracy, the proportion of true results, is ⩾90%.

Experimental approach

In the absence of a reference method, the qualitative data (+/-) generated in the CT, *viz*. the expected and obtained results for all participants (except laboratory 4), was used for estimating diagnostic performance (Table 10). Comparison of true positives and negatives *vs*. false positives and negatives were recorded. The expected results were determined by the organizer of the CT after completion of the evaluation of the performance characteristic (Sections 3.1 to 3.5) and during the homogeneity testing.

Table 10. True positives and negatives or false positives and negatives.

	Expected result + (i.e., target)	Expected result - (i.e., non-target)
Obtained result +	True positive (TP) +/+	False positive (FP) +/-
Obtained result -	False negative (FN) -/+	True negative (TN) -/-

Diagnostic sensitivity (Dsen), diagnostic specificity (Dspec) and accuracy are calculated according to the following mathematical formulas:

Diagnostic Sensitivity = ΣTP/(ΣTP+ΣFN)×100

Diagnostic Specificity = ΣTN/(ΣTN+ΣFP)×100

Accuracy = (ΣTN+ΣTP)/(ΣTP+ΣTN+ΣFP+ΣFN)×100

Ideally accuracy should be 100%, indicating that the method results leads to no false positives, and no false negatives. However, as there are many sources of variations, such as the origin of the seed lots, infection levels and testing laboratories among others, an accuracy of at least 90% is deemed fit for purpose.

Results

The analysis of the comparative test results to evaluate diagnostic performance of the method is presented in Table 11. Results from participant 4 were not taken into account, due to the deviations from the protocol (see Section 3.5). All 18 contaminated seed samples were detected as positive by all participants (i.e., including the two levels of infection), resulting in a diagnostic sensitivity of 100%. All nine healthy seed samples were detected as negative by all participants, resulting in a calculated diagnostic specificity of 100%. Accuracy was 100%.

Table 11. Diagnostic performance analysis. Dsen: diagnostic sensitivity, Dspec: diagnostic specificity.

	Expected result +	Expected result -
Obtained result +	True positive (TP) = 18	False positive (FP) = 0
Obtained result -	False negative (FN) = 0	True negative (TN) = 9
	Dsen = 18 / (18+0) × 100= 100%	Dspec = 9 / (0+9) × 100 = 100%
	Accuracy = (9+18) / (18+9+0+0) × 100 = 100%	

Conclusion

The diagnostic performance of the method meets the requirements with diagnostic specificity, diagnostic sensitivity and accuracy at 100%.

4. CONCLUSIONS

The analytical specificity was evaluated on a collection of corn salad seed lots with and without infection of *P. valerianellae* by the grow-out method. The method allows for the detection of the target pathogen as described: slender seedling characterized by curled cotyledons towards the stem, with sporangiophore being branched with thin ends and ovoid, brownish conidia, which measured 15.9 to 22.2 µm-wide and 23.7 to 29.4 µm-long.

The analytical sensitivity experiment was evaluated for the grow-out assay showing that the method is fit for purpose, as it could detect a single infected seed in a 400-seed sample.

The selectivity of the method was evaluated on different cultivars of corn salad seeds from different origins and met the set requirements. The method can detect *P. valerianellae* in different cultivars of corn salad seeds and from different origins.

The repeatability met the requirement and was deemed fit for purpose for the method with 100% of accordance, as calculated by the Langton *et al.* (2002) analysis.

Initially, the comparative test (CT) included four participants. However, following the exclusion of Laboratory 4 (see Section 3.5), the evaluation of reproducibility and diagnostic performance based on qualitative analysis was conducted with three laboratories only. Data from the CT indicated that the reproducibility and the diagnostic performance of the method met the requirements and are fit for purpose with a concordance of 100%, according to the Langton *et al.* (2002) analysis, and a diagnostic specificity, diagnostic sensitivity, and accuracy at 100%. It should be noted that the analysis of the quantitative data of the CT showed that the infection rates could not be reproduced in a sufficiently reliable manner, and that quantitative differences occur among laboratories. However, the presence of *P. valerianellae* can be identified with high reproducibility in each laboratory for all infection levels qualitatively.

All the validation criteria evaluated meet the requirements and the method to detect *P. valerianellae* in corn salad by grow-out is deemed fit for purpose for testing corn salad seed for the presence of *P. valerianellae,* according to the ISHI guidelines for the Validation of Seed Health Tests (ISHI, 2020).

5. REFERENCES

ISHI (2020). ISHI-Veg Guidelines for the Validation of Seed Health Tests. Version 3. International Seed Federation, Nyon, Switzerland. Available at: https://www.worldseed.org/wp-content/uploads/2020/12/MVGuidelines_v3_November-2020.pdf

Koike, S. T. (2008). Downy Mildew Caused by *Peronospora valerianellae* on Corn-Salad (*Valerianella locusta*) in California. *Plant Disease,* **92**, 1470.

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

6. ANNEXES

Annex A: Protocol for detection of *Peronospora valerianellae* in corn salad (*Valerianella locusta*) seed.

GROW-OUT

The test is performed on a sample of 400 seeds, performed on a single subsample.

Materials

- Containers: potting trays/boxes with a lid
- Potting soil substrate (containing up to 1/3 sand)
- Ethanol 70% (v/v) or equivalent disinfectant
- Controls (See Table A.1)
- Growth chamber (12 ± 3 °C) supplemented with light
- Water

Table A.1. Types of controls used.

Control type	Description
Positive process control (PPC)	Known *P. valerianellae* infected seed sample
Negative process control (NPC)	Known healthy seed sample

1. Sowing

1.1. Disinfect the containers with 70% (v/v) ethanol or equivalent.

1.2. Fill containers with potting soil substrate (containing up to 1/3 sand). Different sizes of containers with lids can be used.

1.3. Sow 400 seeds evenly at the rate of 1 seed / 1.5 to 2 cm per cm^2. The number of containers required for 400 seeds will depend on their dimensions and the prescribed sowing density.

1.4. Water the containers taking into account their dimension (e.g., 100 mL for a 10×15 cm box).

Note: Optimising the quantity of water required during sowing and during plant growth is essential. The relative humidity in the containers can greatly influence the outcome of the test and must be controlled for and monitored for the duration of each test.

1.5 Close the container with the lid.

2. Positive process control (PPC)

2.1. Prepare another container for the infected seed in the PPC using the same conditions as for the test samples.

3. Negative Process Control (NPC)

3.1. Prepare a container for the healthy seed sample, the NPC using the same conditions as for the test samples. Preferably prepare the NPC after the samples to be tested and PPC.

4. Incubation

4.1. Incubate the sample and controls at 12 ±3 °C and 12 h light for 21 days. Record the temperature in the closed container with a temperature probe. Check relative humidity regularly (optimal 100%), condensation should be present on the complete lid of the container during the entire duration of the test.

5. Examination of the seedlings

5.1. Examine PPC containers. Seedlings from the PPC seeds should show cotyledon curling and/or white sporulation on cotyledons (Figure A.1). The number of seedlings of the PPC that are infected is dependent on the rate of infection of the seeds. Examine seedlings for their length and note if they are similar/shorter/longer than the NPC and PPC seedlings.

5.2. Examine the seedlings from the test samples for typical sporulation by comparing them with the PPC seedlings. If only curling cotyledons are observed, prolong the incubation for one extra week.

5.3. Count the number of infected seedlings (curling and/or sporulation) and the total number of seedlings that germinated.

5.4. At the end, examine the NPC containers. No disease symptoms should be observed on the NPC seedlings. In case of positive plantlet, it proved cross contamination. The test cannot be validated.

5.5. The results are indicated as the percentage of seeds infected by *Peronospora valerianellae* calculated as the number of infected seedlings against the total number of seeds sown.

Note: If more than 25-30% of the corn salad seedling show damping-off or do not germinate, this must be noted and indicated on the test result.

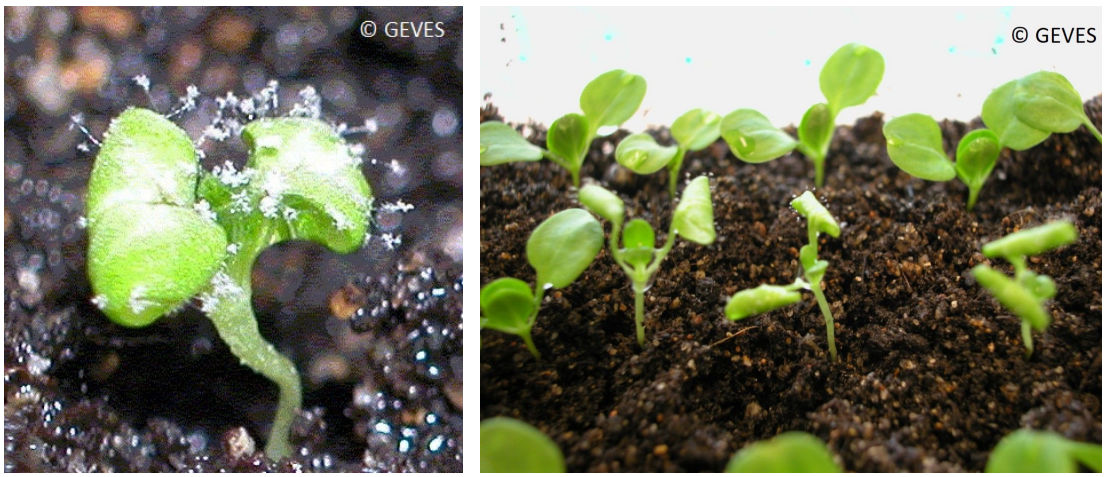

Figure A.1. Typical sporulation of *P. valerianellae* (left and right) and cotyledon curling (right) on infected corn salad seedlings.