International Seed Federation
Seed is Life

Detection of *Pseudomonas syringae* in *Cucurbita pepo* (squash) seed by grow-out

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CONTENT

1. INTRODUCTION

Pseudomonas syringae is a pathogen affecting a plethora of plant hosts and occurring in a multitude of environmental settings (Morris *et al.* 2019). Certain *P. syringae* pathovars are associated with cucurbits. Especially in *Cucurbita pepo*, hereafter referred to as squash, productions with seed-borne *P. syringae* infections have been reported (Marras & Corda 1973, Scortichini 1992). *Pseudomonas syringae* pv. *lachrymans,* which is typically encountered in cucumber was thought to be responsible for seed-borne infections of cucurbits (Bhat *et al.* 2010). However more recently, it has been determined that the population responsible for recent incidents was different from *pv. lachrymans*. Instead of *P. syringae* phylogroup 3, to which pv. *lachrymans* belongs, members of phylogroup 2 were identified as causative agents (Monteil *et al.* 2016, Newberry *et al.* 2017, Lybeert & Woudt. 2014).

Host range and worldwide strains distribution and diversity explain the origin of epidemics of the called "zucchini vein clearing disease" (Lacault *et al.* 2020).

The bacterium was isolated from very young, stunted plants of squash. Depending on the climatic conditions, the symptoms could be strong (from necrosis of cotyledons, to necrosis of leaves, vein clearing of leaves, and plants development hindering) or absent from the same source of seeds. Cool temperatures and humidity were the most favourable conditions for the expression of the disease. Artificial inoculation on plants and on different cucurbits showed that systemic symptoms were only observed on *Cucurbita pepo*.

There is currently no validated method to detect *P. syringae* strains, which cause the zucchini vein clearing disease in squash. Research projects helped to develop a specific selective media for this bacterium (Lybeert & Woudt 2014). Furthermore, grow-out-based testing was found appropriate to test squash seed lots for viable, infectious *P. syringae*. Figure 1 presents the method process flow.

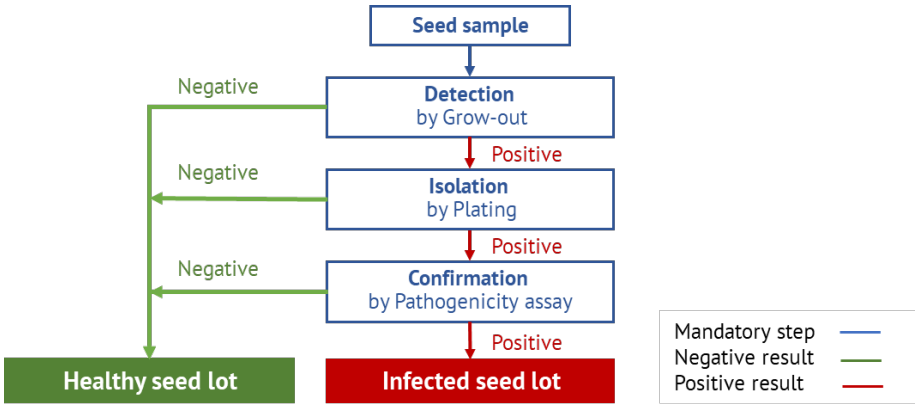

Figure 1. Method process flow.

2. OBJECTIVES

In this study, the validity of a protocol for the detection of *P. syringae* in squash seed by grow-out assay in greenhouses or climate chambers followed by isolation of the pathogen in pure culture and demonstration of its pathogenicity on plants was assessed according to the ISHI-Veg guidelines for the Validation of Seed Health Tests (ISHI-Veg 2020).

The protocol for the detection of *P. syringae* in squash seed is described in Annex A. Additional protocols used in this project are described in Annex B (16S sequencing, for identification of non-

P. syringae isolates used in specificity experiments) and in Annex C (seed spot inoculation assay, for testing the specificity and sensitivity of the grow-out assay). The inter-laboratory comparative test is described in the Annex D. Finally, raw data obtained from the comparative test can be found in Annex E.

3. METHOD VALIDATION

3.1. Analytical specificity

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

In the grow-out method, the detection of *P. syringae* in squash seed is done by growing out seeds under controlled environmental conditions (grow-out assay) followed by isolating the pathogen in pure culture from symptomatic seedlings (plating assay) and confirming its pathogenicity on plants (pathogenicity assay). The specificity of the grow-out method is evaluated based on the specificity of both the pathogenicity assay and the grow-out assay.

Analytical specificity requirements will be met when both the grow-out and pathogenicity assays yield positive results with pathogenic *P. syringae* isolates whilst excluding non-*P. syringae* isolates.

Experimental approach

1. Specificity of the pathogenicity assay

To determine specificity, 36 pathogenic *P. syringae* isolates derived from squash grow-outs and 14 non-*P. syringae* isolates derived from squash plant material (Table 1) were tested with the pathogenicity assay described in the grow-out protocol (Annex A). The pathogenicity assay was evaluated for symptoms (+) or no symptoms (-).

P. syringae isolates were characterized by sequencing of *gltA,* as described by Berge and co-workers (2014). Tested isolates included phylogroup 2 strains, which were reported to be involved in disease outbreaks in squash (Newberry *et al.* 2016). Tested non-*P. syringae* isolates represented typical saprophytes or commensals to be expected in squash grow-outs belonging to the *Pseudomonas* or *Xanthomonas* genera. They were identified by 16S sequencing (Annex B). These isolates displayed distinct patterns from *P. syringae* isolates involved in disease outbreaks in squash. Although only 14 non-target isolates are included in this study, which are less than the suggested amount in the ISHI-Veg Validation guidelines (i.e., 20-30), since they were isolated from squash grow-out assays, these are believed to be sufficiently representative of the typical saprophytes and commensals belonging to the *Pseudomonas* or *Xanthomonas* genera that are expected to occur in squash grow-outs.

2. Specificity of the grow-out assay

Specificity of the grow-out assay has been assessed using a seed spot inoculation method. This method enables testing of different isolates starting from inoculated seeds, simulating conditions during grow-out testing. The seed spot inoculation assay (Annex C) was performed on the same 50 isolates similarly to the pathogenicity assay. For the seed spot assay, 10^5-10^6 cells were applied to each of four 'Spineless Beauty' squash seeds per bacterial isolate tested. This concentration was based on the tested sensitivity in order to consistently yield symptoms. Fourteen days after sowing, seedlings raised in a greenhouse were assessed for symptoms.

Symptom rating was the average across the four seedlings tested per isolate (+++ severe lesions (more than 50% leaf/cotyledon surface); ++ intermediate lesions (25 to 50% leaf/cotyledon surface); + light lesions (up to 25% leaf/ cotyledon surface); - healthy seedling).

Results

All 36 *P. syringae* isolates yielded typical symptoms in both the pathogenicity assay and the seed spot assay (Table 1). The 14 other isolates tested (non-*P. syringae*) did not yield any symptoms under these conditions (Table 1).

Table 1. *Pseudomonas syringae* and non-*P. syringae* isolates tested for specificity in the pathogenicity assay and in the seed spot inoculation assay. *Syngenta (ZUM) collection, ** Non-*P. syringae* species as determined by 16S sequencing, *** Phylogroup determination applies only for *P. syringae*.

Isolate designation*	Country of origin	Year	Organism**	*P. syringae* phylogroup***	Seed spot assay	Pathogenicity assay
3716	Unknown	2005	*P. syringae*	2a	+++	+
4678	China	2012	*P. syringae*	2a	+++	+
4845	Unknown	2014	*P. syringae*	2a	+++	+
4229	China	2009	*P. syringae*	2a	+++	+
3584	Italy	2005	*P. syringae*	2b	+++	+
10	Honduras	2013	*P. syringae*	2b	+++	+
57	China	2012	*P. syringae*	2b	++	+
76	China	2012	*P. syringae*	2b	+++	+
110	China	2012	*P. syringae*	2b	+++	+
83	Peru	2013	*P. syringae*	2b	+++	+
4671	China	2012	*P. syringae*	2b	+++	+
4696	China	2011	*P. syringae*	2b	+++	+
8	China	2012	*P. syringae*	2b	++	+
49	China	2013	*P. syringae*	2b	+++	+
97	China	2011	*P. syringae*	2b	+++	+
178	China	2014	*P. syringae*	2b	+++	+
200	China	2013	*P. syringae*	2b	++	+
4555	China	2011	*P. syringae*	2b	++	+
4680	China	2012	*P. syringae*	2b	++	+
4503	China	2011	*P. syringae*	2b	+++	+
4134	China	2008	*P. syringae*	2b	++	+
4640	China	2011	*P. syringae*	2b	+++	+
3806	China	2003	*P. syringae*	2b	+	+
3807	China	2003	*P. syringae*	2b	+	+
3816	China	2003	*P. syringae*	2b	+	+
3817	China	2003	*P. syringae*	2b	+	+
160	China	2011	*P. syringae*	2b	+	+
168	China	2013	*P. syringae*	2b	+	+
220	Unknown	Unknown	*P. syringae*	2b	+	+

Isolate designation*	Country of origin	Year	Organism**	*P. syringae* phylogroup***	Seed spot assay	Pathogenicity assay
161	China	2011	*P. syringae*	2b	+	+
169	China	2013	*P. syringae*	2b	+	+
3973	France	2007	*P. syringae*	2d	+	+
3974	France	2007	*P. syringae*	2d	++	+
3975	France	2007	*P. syringae*	2d	++	+
4248	China	2009	*P. syringae*	2d	+	+
4847	Unknown	2014	*P. syringae*	2d	+	+
23-1	Unknown	2017	*P. fluorescens*	-	-	-
23-2	Unknown	2017	*P. moraviensis*	-	-	-
27-2	Unknown	2017	*P. alcaligenes*	-	-	-
3719	China	2005	*P. putida*	-	-	-
4073	China	2009	*P. viridiflava*	-	-	-
4074	China	2009	*S. maltophilia*	-	-	-
4284	China	2007	*S. maltophilia*	-	-	-
4285	China	2009	*S. maltophilia*	-	-	-
4286	China	2009	*S. maltophilia*	-	-	-
4386	China	2010	*P. viridiflava*	-	-	-
4387	China	2010	*P. viridiflava*	-	-	-
4388	China	2010	*P. thivervalensis*	-	-	-
4528	Italy	2011	*P. viridiflava*	-	-	-
4575	China	2011	*P. entomophila*	-	-	-

Conclusion

All 36 pathogenic *P. syringae* isolates yielded typical symptoms in the pathogenicity assay and in the seed spot assay. The 14 other non-*P. syringae* isolates tested did not yield any symptoms. Therefore, the pathogenicity and grow-out assay met the criteria for analytical specificity.

3.2. Analytical sensitivity

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

The analytical sensitivity requirements will be met when one *P. syringae* contaminated seed can be detected by grow-out assay.

The study assessed the number of bacterial cells needed to be present on a seed to cause an infection. The confirmation pathogenicity assay is not included in the analytical sensitivity testing, since for the detection of the artificially inoculated seed, no confirmation pathogenicity assay is needed.

Experimental approach

The *P. syringae* inoculum necessary to cause symptom development in individual seedlings was assessed by applying the seed spot inoculation, as this technique simulates the situation encountered during grow-out testing. See Annex C for the seed spot inoculation protocol.

Decreasing amounts of *P. syringae* isolates ZUM3584 and ZUM3806 were applied to squash seeds (see Table 2). A total of five replicate seeds per dilution were inoculated. Seedlings raised in a greenhouse were scored for symptoms 14 days after sowing. Seedlings displaying symptoms were scored as positive. As control, mock inoculations were performed with sterile NaCl solution. The number of bacteria in the dilutions were confirmed by plating in triplicate.

Results

Seedlings inoculated with ZUM3584 displayed symptoms down to one cell applied. ZUM3806 displayed symptoms down to 50 cells applied (Table 2). At these concentrations, the fraction of positive seedlings obtained was 20% for both. One hundred percent symptom development was observed for 10^3 cells for ZUM3584 and 10^5 cells for ZUM3806 (Table 2).

Table 2. Number of symptomatic seedlings obtained following inoculation of squash seeds (variety Spineless Beauty) with varying amounts of either *P. syringae* isolate ZUM3584 (top) or ZUM3806 (bottom).

Isolate	Cells per seed	Replicates found positive					Total number of positives	Ratio positives/total
		A	B	C	D	E		
ZUM3584	10^6	1	1	1	1	1	5	1
	10^5	1	1	1	1	1	5	1
	10^4	1	1	1	1	1	5	1
	10^3	1	1	1	1	1	5	1
	10^2	1	1	1	0	1	4	0.8
	50	1	0	1	1	1	4	0.8
	10	1	0	0	1	1	3	0.6
	5	1	1	0	0	0	2	0.4
	1	0	1	0	0	0	1	0.2
ZUM3806	10^6	1	1	1	1	1	5	1
	10^5	1	1	1	1	1	5	1
	10^4	1	1	1	1	0	4	0.8
	10^3	1	1	0	0	0	2	0.4
	10^2	1	0	0	0	0	1	0.2
	50	1	0	0	0	0	1	0.2
	10	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0
	1	0	0	0	0	0	0	0

Based on the fractions of symptomatic seedlings, the analytical sensitivity of the method was determined indicating a probability of p = 0.95 or p = 0.5 for observing symptomatic seedlings (Figure 2). Analytical sensitivity was determined as indicated in Table 3.

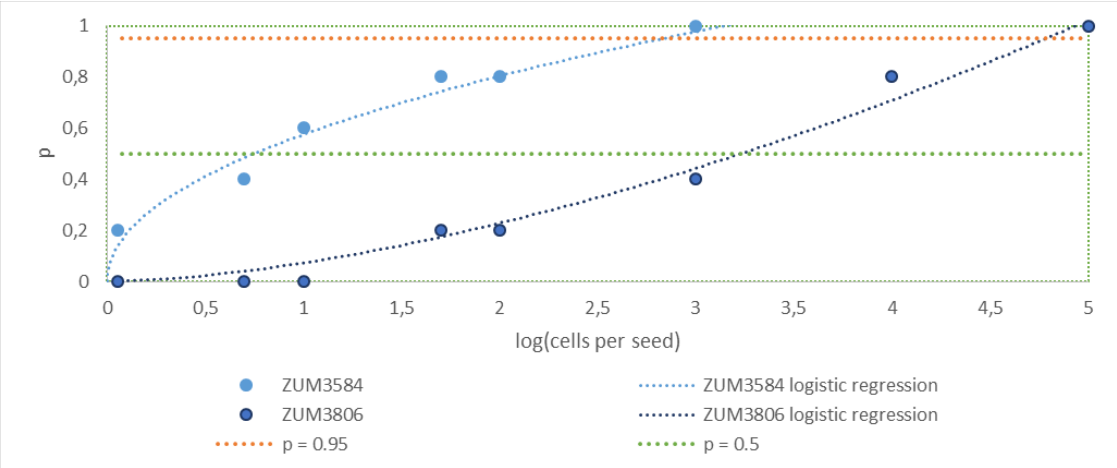

Figure 2. Fraction of seedlings displaying symptoms upon application of different amounts of either ZUM3584 (light blue) or ZUM3806 (dark blue) on seeds was used as an indication for the probability p for observing symptomatic seedlings. Round markers indicate values determined in Table 2. Logistic regression was used to interpolate. p = 0.95 and p = 0.5 are indicated as orange and green dotted lines.

Table 3. Analytical sensitivity for *P. syringae* isolates ZUM3584 and ZUM3806.

Strain	Cells per seed, p = 0.95	Cells per seed, p = 0.5
ZUM3584	680	6
ZUM3806	60,931	1,715

Conclusion

Results show that one *P. syringae* infected seed can be detected by grow-out assay and therefore met the criteria for analytical sensitivity. However, for the typical strain ZUM3584, 700 bacterial cells were enough to obtain symptoms on one plantlet, for the other pathogenic strain, ZUM3806, 100 times more was necessary. Such a difference is probably due to differences in virulence between the two strains.

3.3. Selectivity

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

The present protocol has been developed with the aim of detecting seedborne *P. syringae* on squash species. Therefore, squash is the only matrix assessed here. The selectivity requirements will be met when *P. syringae* infection leads to symptom development in all squash varieties tested under the grow-out conditions.

Experimental approach

Fourteen different naturally infected squash seed lots representing at least ten different varieties, derived from six production countries and produced in five different production years were tested using the grow-out protocol mentioned in Annex A (Table 4). Qualitative results came from the routine testing of three different laboratories. The use of naturally infected seeds demonstrates the transmission of *Pseudomonas* from seeds to seedlings in different varieties under the grow-out conditions.

Table 4. Different squash varieties tested for *P. syringae* by grow-out method.

Squash variety	Production year	Country of origin	Test laboratory	Result
Spineless Beauty	2014	India	Lab A	Positive
Spineless Beauty	2014	China	Lab A	Positive
Spineless Beauty	2014	India	Lab A	Positive
Altea	2012	China	Lab A	Positive
Topazio	2014	Thailand	Lab A	Positive
Payroll	2014	China	Lab A	Positive
Golden Dawn	2006	Chile	Lab A	Positive
Noche	2014	China	Lab A	Positive
Spineless Perfection	2014	China	Lab A	Positive
Cora	2014	China	Lab B	Positive
Cora	2015	France	Lab B	Positive
Sinatra	2016	China	Lab B	Positive
Gloria	2016	Thailand	Lab B	Positive
X	2016	Peru	Lab C	Positive

Results

All 14 tested seed lots had positive results. Testing of additional varieties of seeds was not deemed necessary because of the difference in production locations, which gives seed already with a considerably different saprophytic seed background. Finally, the study of the matrix effect is less relevant for a biological assay compared to e.g. molecular assays since the seed background, since there is less risks of interferences with the test results.

Conclusion

These results suggest there are no significant variety-related effects within the squash species tested in the grow-out assay, and no effects of production conditions, as seed lots from different countries and years were tested. Therefore, the selectivity requirements are met.

3.4. Repeatability

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

The repeatability requirements will be met when the measure for this performance criterium, i.e. accordance within labs is >90%.

Experimental approach

Comparative test (CT) samples were tested to verify homogeneity and stability of the *Pseudomonas* infection before and after the CT by the CT organising laboratory. Obtained results were used to evaluate repeatability of the method. See annex A for the protocol of the method used.

The CT samples included ten repetitions of three seed lots of 100 seeds each (highly infected, medium infected and healthy seed lots). In the homogeneity test, nine, twenty and ten extra samples of 100 seeds representing the highly infected, medium infected and healthy seed lots,

respectively, were tested just before sending the samples to CT participants (see reproducibility 3.5).

In the stability test, ten extra samples of 100 seeds from each contamination level were tested after receiving the confirmation that all other CT participants started the test.

Accordance (repeatability) was evaluated using the method developed by Langton *et al.* (2002).

Results

Homogeneity and stability results together with CT results from the lab performing the homogeneity and stability tests are presented in Table 5.

Table 5. Repeatability data.

Lot	Homogeneity results	Comparative test result	Stability results
High	9 positive / 9 tested	10 positive / 10 tested	7 positive / 10 tested
Medium	11 positive / 20 tested	5 positive / 10 tested	4 positive / 10 tested
Healthy	0 positive / 10 tested	0 positive / 10 tested	0 positive / 10 tested

The Langton analysis gave a 100% accordance for the healthy seed samples. For the medium contaminated seed lot, the homogeneity test results showed that not all samples contained infected seeds. Furthermore, stability results of the highly infected seed lot showed that the level of contamination decreases with time. Therefore, no accordance can be calculated for both the highly and medium infected seed samples. Data of the CT for both the highly and medium infected seed lot will be compared with the data from the homogeneity and stability tests, as described below. Results from the CT should fall within the expected number of contaminated samples, as calculated based on the average percentage of infection obtained from the homogeneity and stability tests. The ratio of infection is calculated with the Seedcalc8 software (https://www.seedtest.org/en/services-header/tools/statistics-committee/statistical-tools-seed-testing.html, August 2021) using the computed % in sample at 95% confidence. The rate of infection of the highly infected seed lot, corresponding to 16 positive samples out of 19 totals, was 1.83% (Figure 3). The rate of infection of the medium infected seed lot, corresponding to 15 positive samples out of 30 totals, was 0.69% (Figure 4). This rate of infection is used for the calculation of the probability to obtain contaminated samples from the tested samples with "probability of *k* positive samples out of n" tool (https://www.seedtest.org/en/services-header/tools/seed-health-committee/seed-health-toolbox.html, August 2021).

Considering each value with a probability higher than 5%, the CT should give between 7 to 10 positives out of the 10 samples tested in the CT for the highly contamination seed lot (Figure 5) and between 3 to 7 positives on the 10 samples tested for the medium contamination seed lot (Figure 6). Results are summarised in Table 6.

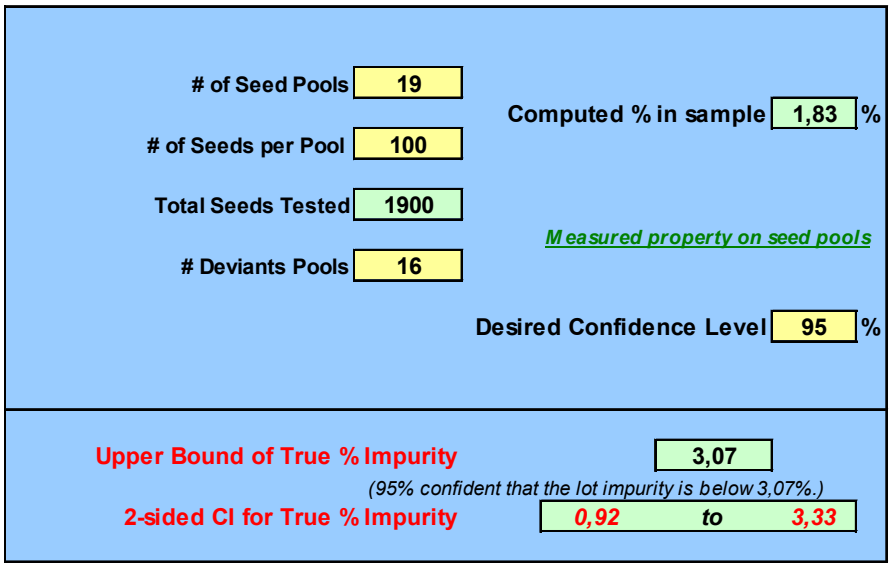

Figure 3. Results of the highly infected seed lot with the Seedcalc 8 software.

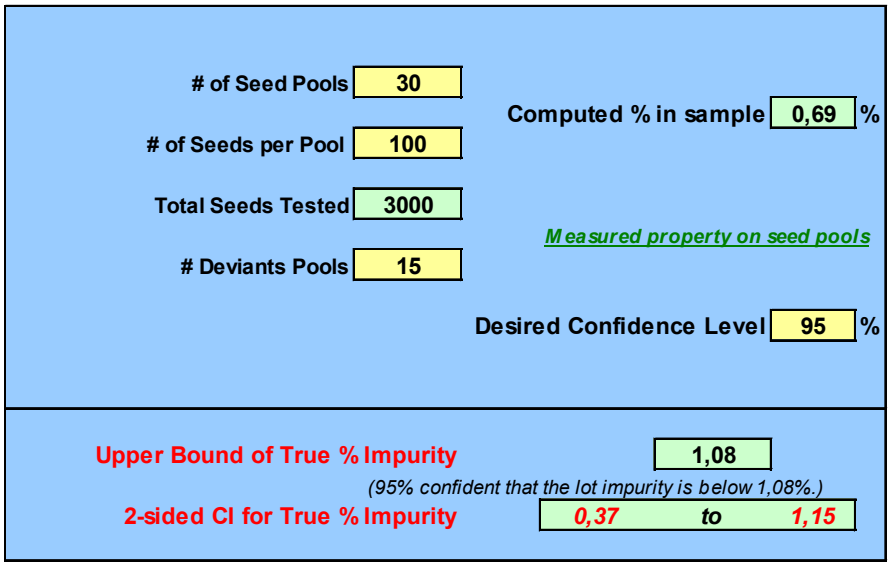

Figure 4. Results for the medium infected seed lot using the Seedcalc 8 software.

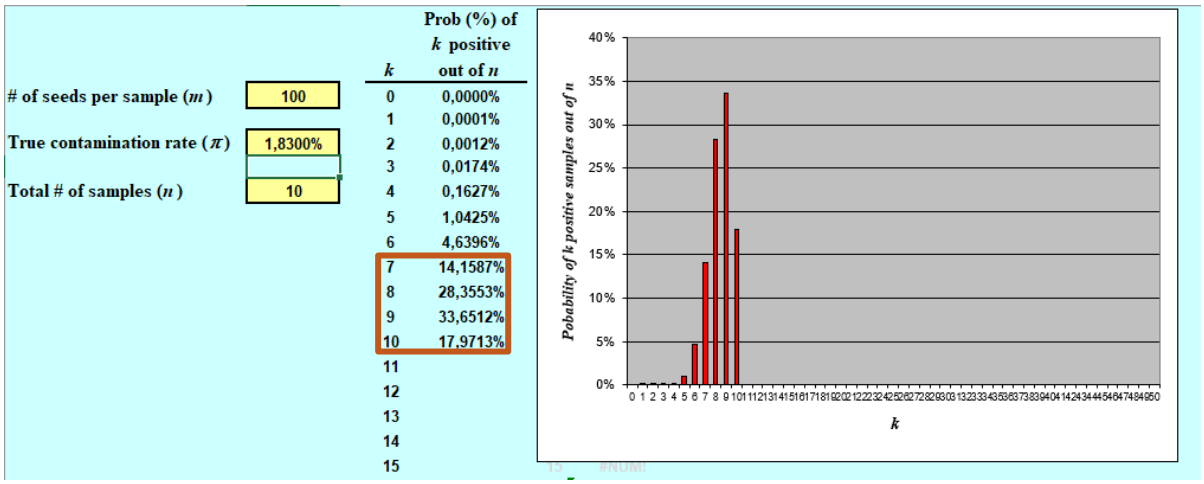

Figure 5. Expected number of contaminated samples for the CT highly infected seed lot according to infection rate with the "probability of *k* positive samples out of *n*" tool.

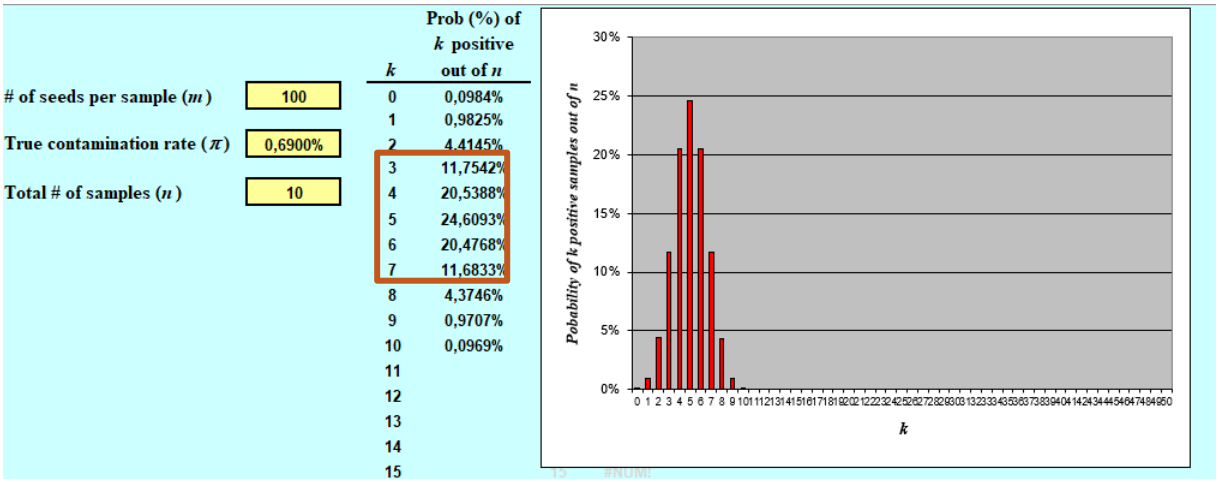

Figure 6. Expected number of contaminated samples for the CT medium infected seed lot according to infection rate with the "probability of *k* positive samples out of *n*" tool.

Table 6. Expected number of positive subsamples based on the homogeneity and stability results.

Lot	Homogeneity results	Stability results	Estimated contamination level[1]	Number of positive subsamples expected[2]	Obtained results with CT samples
High	9 / 9	7 / 10	1.83 %	7 to 10	10
Medium	11 / 20	4 / 10	0.69 %	3 to 7	5

[1] Calculated with the Seedcalc8 software.

[2] Calculated with the "probability of k positive samples out of n" tool.

Conclusion

Accordance for the healthy seed lot is 100%. The observed 10 and 5 positive samples in the CT for the high and medium infected lot, respectively (Table 5), are within the expected range (Table 6). Therefore, repeatability of the method met requirements.

3.5. Reproducibility

Definition ISHI-Veg guidelines: *Degree of similarity in results when the method is performed across labs with replicate seed subsamples.*

The requirements will be met when concordance of the method is >90%.

Experimental approach

Six laboratories participated in the CT (Table 7). Participants had a different level of experience with this method and conducted the grow-out either in the greenhouse (two laboratories) or in a climate chamber (four laboratories). They were randomly allocated a number, so that results remained anonymous.

Each participating laboratory received three coded samples of 1,000 seeds each. The samples were composed of a healthy, a medium infected and a highly infected seed lot. An artificially infected seed sample was also used as a positive control.

Typical or doubtful *Pseudomonas* symptoms on plants were confirmed isolating the pathogen in pure culture, as described in the protocol, and demonstrating its pathogenicity on plants. See

Annex A for the protocol, and Annex D for the inter-laboratory CT plan. Reproducibility of the method was calculated using the method developed by Langton *et al*. (2002).

Table 7. Laboratories participating in the comparative test.

Laboratories
BAYER, NL (now BASF)
Enza Zaden, NL
HM-Clause, FR
Monsanto, USA (now BAYER)
Rijk Zwaan, NL
Syngenta, NL

Results

Raw CT data of all laboratories are presented in the Annex E. All participants prepared the Positive Process Control (PPC) by artificially inoculating 50 healthy seeds with a provided *P. syringae* strain, as described in the appendix of the CT plan (see Annex B). However, three of the participating labs reported issues with the PPC and with the *P. syringae* strain, which could be due to a decrease in viability and virulence of the positive control strain during storage and transport. Furthermore, differences were observed in the contamination level of the PPC between the different laboratories (Table 8).

Data from the three laboratories reporting contamination issues and invalid positive control (1, 4 and 5) were excluded from the statistical analysis. A summary of the CT results of the remaining three participants in provided in Table 9. The Langton analysis gave a 100% concordance for the healthy seed samples, as all three labs detected all healthy seed samples to be negative.

Table 8. Details of the CT per participant.

Lab	Sowing date	Incubator	Incubation duration	Issues with controls	PPC contamination	High	Medium	Healthy
1	21-11-18	Greenhouse	21 days	Possible contamination, and PPC negative outcome.	14%*	10/10	10/10	7/10
2	01-11-18	Growth chamber	14 days	-	64%	10/10	3/10	0/10
3	19-10-18	Greenhouse	21 days	-	48%	10/10	10/10	0/10
4	01-10-18	Growth chamber	21 days	PPC did not show usual symptoms. Furthermore, PC caused less severe symptoms than sample isolates.	16%	5/10	1/10	0/10
5	03-12-18	Growth chamber	14 days	No PC included in path. Assay, culture was dead by the time of the test.	2%	5/10	1/10	0/10
6	08-10-18	Growth chamber	14 days	-	16%	10/10	5/10	0/10

*After the grow-out assay, 14% of plants showed symptoms, but the final PPC outcome, after performing the pathogenicity assay, was negative.

Table 9. CT results of the numbers of positive subsamples per seed lot per participating laboratory.

Seed Lot	Expected positive subsamples (See Table 6)	CT results participant laboratories		
		Lab 2	Lab 3	Lab 6
High	7 to 10	10	10	10
Medium	3 to 7	3	10	5
Healthy	0	0	0	0

For the high and medium infected samples, concordance could not be calculated due to the homogeneity and stability test results. Here, expected number of positive samples according to the infection rate is calculated per participant with the "probability of *k* positive samples out of *n*" tool (https://www.seedtest.org/en/services-header/tools/seed-health-committee/seed-health-toolbox.html, August 2021). The infection rate comes from the homogeneity and stability test results, see section 3.4 and Table 6. The number of detected samples is compared to the calculated expected number of positive samples.

For the highly infected seed samples, all three labs performed as expected as their results fell within the expected range as shown in Table 9 (i.e., 7 to 10). For the medium infected seed samples, average results from the three labs (18 of 30 i.e., 6 of 10 subsamples) also fell within the expected range, as shown in Table 9 (i.e., 3 to 7). However, labs 2 and 6 performed as expected, but lab 3 reported more positive samples than expected (Table 9). This may be due to the fact that lab 3 performed the grow-out in a greenhouse and scored symptoms after 21 days of growth, while lab 2 and 6 performed the grow-out in a growth chamber and scored symptoms after 14 days of growth (Table 8), as this was also the case for the homogeneity and stability tests. Although both evaluation at 14 and 21 days is valid, the later test plants are evaluated, the higher the chances to detect contact infections, instead of infections coming from infected seeds. Therefore, early evaluation is closer to the true level of infection of the seed lot.

Conclusion

Due to problems with the Positive Process Control and/or Positive Control by participants, only data from three participants could be included in the analysis. Although the statistical power is not as strong as initially foreseen, similar trends were observed for both labs 4 and 5. Taken together, it is believed that data is sufficient to provide a conclusion on the fitness of the method.

With a concordance of 100% for the healthy seed lot and the detection of the number of positive samples in agreement with expectation for the highly infected seed lot, the reproducibility requirements are met for the healthy and high contamination seed lot.

With regards to the medium contaminated samples, although one out of three laboratories detected more positives samples than expected, results were considered appropriate when taking into account the differences in the protocol execution (greenhouse *vs.* growth chamber and duration of the incubation). Therefore, reproducibility requirements are met.

3.6. Diagnostic performance

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

Diagnostic performance requirements will be met when diagnostic sensitivity reach 100% and specificity is ≥ 90%.

Experimental approach

The diagnostic performance was calculated based on the inter-laboratory CT data, as compared to the expected results (see Annex D for the CT plan).

Diagnostic sensitivity and diagnostic specificity of the assay were calculated according to the following mathematical formulas:

		Validated method result / independent assessment	
		Positive	**Negative**
Test outcome	**Positive**	True positive (TP)	False positive (FP)
	Negative	False negative (FN)	True negative (TN)
		DIAGNOSTIC SENSITIVITY = (TP / (TP + FN)) x 100%	**DIAGNOSTIC SPECIFICITY = (TN / (FP + TN)) x 100%**

Results

Analysis of the CT results from the three labs combined are presented in Table 10. Note that the expected number of positive results, calculated based on the homogeneity and stability results (Table 6) were taken into account when defining true and false positives and negatives for the high and medium contaminated seed samples. Raw CT data can be found in Annex E.

Table 10. Analysis of qualitative results for the healthy, medium and high contaminated seed lots.

	Expected + result	**Expected - result**	**Diagnostic Sensitivity**	**Diagnostic Specificity**
Obtained + result	57 (TP)	3 (FP)	100 %	91 %
Obtained – result	0 (FN)	30 (TN)		
Total	57	33		

Conclusion

The diagnostic sensitivity was found to be 100%, and the diagnostic specificity ⩾ 90 % (Table 10). The diagnostic performance is therefore considered to be met.

4. CONCLUSION

The performance criteria measured during method validation confirm that the grow-out method for the detection of *P. syringae* in squash seeds is suitable to detect contaminated seed lots with viable and infectious *P. syringae* bacteria in squash.

All participants found the healthy lot to be healthy and the two contaminated lots (highly and medium infection) to be positive for *P. syringae*. The ten repetitions of 100 seeds resulted in 1,000 seeds tested, the recommended minimum sample size to detect *P. syringae* on squash seeds.

In order to avoid potential cross-contamination, it is recommended to follow ISHI-Veg Best Practices when performing the grow-out assay.

5. ACKNOWLEDGEMENTS

This report contains experimental data contributed by laboratories of HM-Clause, Syngenta, Monsanto (Bayer), BASF, Rijk Zwaan and Enza Zaden.

6. REFERENCES

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6. ANNEXES

Annex A: Protocol for the detection of *Pseudomonas syringae* on *Cucurbita pepo* (squash) seed using a grow out assay

Detection of *Pseudomonas syringae* in *Cucurbita pepo* (squash) Seed

The recommended minimum sample size is 1,000 seeds.

I. DETECTION BY GROW-OUT ASSAY, ISOLATION BY PLATING AND CONFIRMATION BY PATHOGENICITY ASSAY

For grow-out and pathogenicity assays, in-house method optimization is often necessary by changing certain parameters as described in Best Practices for Sweat Box and Grow-Out assays and Best Practices for Biological Assays in Seed Health Tests.

Materials

- NaCl solution (recipe see Table A.1)
- 70% (v/v) ethanol
- Controls (Table A.2)
- Growth chamber / Greenhouse compartment (25-30°C with relative humidity ⩾ 70%)
- Laminar airflow cabinet
- Commercial sterilized or virgin potting mix
- Trays
- Healthy squash seeds
- Small grinding plastic bags and a press grinder (or equivalent)
- Forceps
- Scalpel
- Inoculation loop
- Cotton swab
- Petri dishes
- Microliter pipettes
- Plates of LBC + AL medium (recipe see Table A.3)
- Lab disposables

Table A.1. NaCl solution 0.85%.

Compound	Amount / L
Sodium chloride (NaCl)	8.5 g
De-ionized water up to a volume of	1 L

Note: Autoclaved at 121 °C, 15 psi for 15 min.

Table A.2. Types of controls used.

Control type	Description	Assay
Positive Process Control (PPC)	A known *P. syringae*-positive seed sample	Grow-out
Negative Process Control (NPC)	A known *P. syringae*-negative seed sample	
Positive Control (PC)	A reference *P. syringae* strain	Pathogenicity assay
Negative Control (NC)	NaCl solution	

Table A.3. Composition of the LBC+AL medium[a].

Compound	Amount / L
Yeast extract	2 g
Bacto-peptone	5 g
Sucrose	50 g
Boric acid	1.5 g
Bacto agar	15 g
NaOH 1N	2 mL
Nystatin[b] (20 mg/mL in 50% DMSO/50% (v/v) ethanol)	20 mg (1.0 mL)
Cephalexin[b] (10 mg/mL in distilled or de-ionized water)	80 mg (8 mL)
Lincomycin[b] (50 mg/mL in distilled water)	50 mg (1 mL)
De-ionized water up to a volume of	1 L

[a] Adjusting pH is not required

[b] Added after autoclaving (Temp < 50 °C)

Antibiotics stock solutions and other supplements prepared in distilled/de-ionized water must be sterilized using a 0.2 µm bacterial filter. Alternatively, add the antibiotic powder to the autoclaved distilled/de-ionized water. Solutions prepared in 70% (v/v) ethanol need no sterilization.

Storage conditions and duration may affect antibiotic activity, which can influence the test performance.

1. Grow-out

1.1. Prepare growth chamber or greenhouse compartment. Clean and disinfect all surfaces and equipment before starting the assay. Disinfect hands between seed samples sown. Separate trays from one sample to another to avoid cross-contamination.

1.2. Sow seeds, together with PPC and NPC (Table A.2), in a commercial sterilized or virgin potting mix and incubate for 48 hours at 25 °C for seedling emergence.

Seedling density should be such to allow unrestricted seedling development for a period of three weeks, corresponding to a maximum of 900 seeds per square meter

For a routine test, plants coming from a same seed lot can be close.

1.3. After seedling emergence:

Temperature and relative humidity should be uniform across the area where plants are raised. Maintain relative humidity at 70% or higher, from the time seedlings emerge to final reading. Relative humidity should not be lower than 50% for more than 12 hours.

Maintain temperatures at 25-30 °C during the day and 15-18 °C during the night until final inspection. Temperatures should not be out of this range for more than 12 hours. Record temperature and relative humidity, preferably above plant canopy for the duration of the test.

Supplement light for 12 hours per day, if necessary, in greenhouse. In a growth chamber, light should be moderate with respect to heat radiation.

Overhead watering, just sufficient to create uniform leaf wetness, shortly before onset of the daily cooler (night) period will speed up symptom development.

1.4. Inspect seedlings 11, 14 and 21 days after sowing. To avoid cross contamination, disinfect hands between seed samples during each observation. Final inspection is done when seedlings have two developed true leaves. In a growth chamber final inspection may be done earlier (14 days). Do not remove plants showing symptoms during intermediate observation.

Symptoms typical of *Pseudomonas syringae* occur on cotyledons and true leaves: marginal necrosis and water-soaked to dark necrotic lesions (Figure A.1).

1.5. Record number of plants showing symptoms and number of plants observed per inspection days.

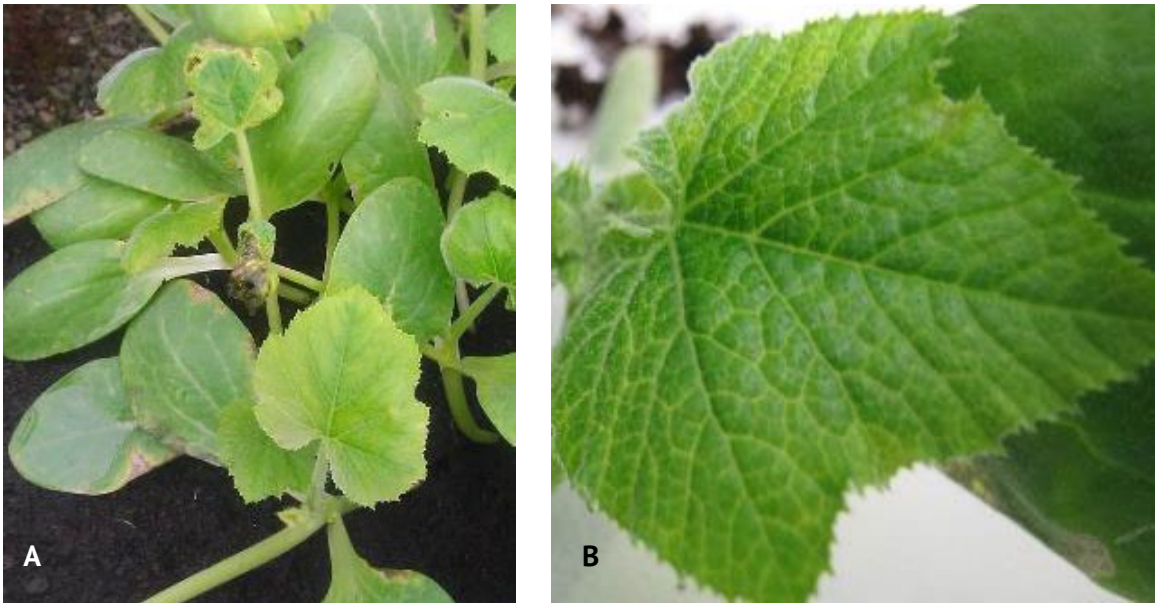

Figure A.1. Typical symptoms of *P. syringae* in a grow-out. In a greenhouse (A) and growth chamber (B).

2. Isolation of the bacterium

2.1. Harvest plants showing symptoms individually in plastic bags: choose preferably plants showing typical symptoms. Harvest at least two suspect plants per subsample if over two plants show symptoms.

Note: When no plants with symptoms are found, the test result can be considered negative but only if the NPC and PPC give expected results. The PPC should give plants with symptoms, and the NPC should give no symptoms.

2.2. Put a droplet of sterile NaCl solution in an empty Petri dish.

2.3. Briefly disinfect the surface of the symptomatic leaf or cotyledon with 70% (v/v) ethanol.

2.4. With forceps and a scalpel, excise a small piece of symptomatic tissue (marginal necrosis).

2.5. Mash the tissue in the droplet and wait for 15-30 min for bacteria to ooze from the pieces. Dip an inoculation loop in the droplet and streak on the surface of one LBC+AL plate. From the primary streak, make two more streaks on the same plate attempting to obtain individual colonies.

2.6. Incubate plates at 26-28 °C and observe after 48 hours.

2.7. Two colony types can be expected for pathogenic strains: white translucent and mucous (Figure A.2A) and opaque and drier (Figure A.2B).

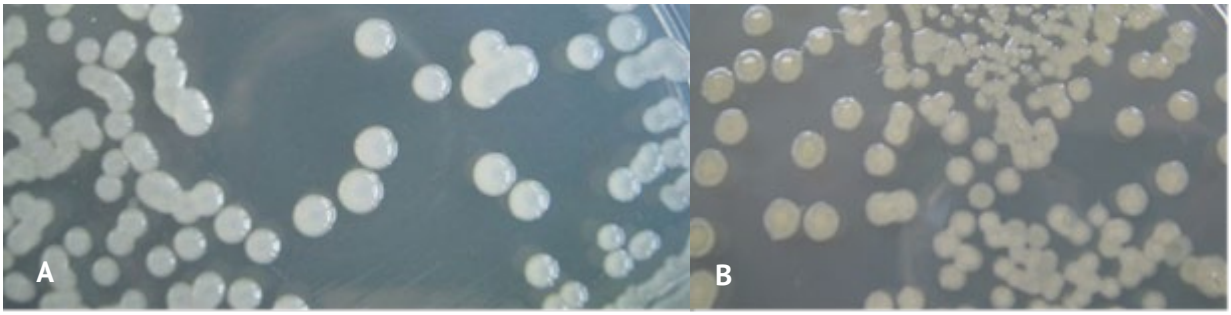

Figure A.2. *Pseudomonas syringae* colonies on LBC+AL: white, translucent and mucous (A) and opaque and drier (B).

2.8. Select the most dominant type of colony for pathogenicity testing (select one colony per harvested plant)

2.9. Transfer the colony on a plate of LBC+AL.

2.10. Proceed with the pathogenicity assay for confirmation.

3. Pathogenicity assay

3.1. For each suspect colony to be tested and for the PC and NC, grow four squash seedlings until the first true leaf just starts to develop (approximately one week after sowing).

3.2. Transfer suspect colonies by streaking to a fresh plate of LBC+AL and incubate for 48 hours at 26-28 °C. Include a known pathogenic strain of *P. syringae* as a positive control.

3.3. Harvest cells with an inoculation loop and suspend in NaCl solution to a concentration of 10^6-10^8 cells/mL.

3.4. Inoculate the four assay plants by wetting the adaxial (upper) side of both cotyledons with a cotton swab soaked in the bacterial suspension.

Inoculate the NC assay plants with NaCl solution.

3.5. Incubate inoculated seedlings for 7 days at 18-24 °C and 50-85% relative humidity.

3.6. Score for pathogenic reactions (+/-), which are characterized by necrotic spots on the cotyledons (Figure A.3).

A colony is considered as positive if at least one inoculated plant (out of the 4) shows symptoms.

A seed lot is considered as infected if at least one colony obtained from the suspected plants gives a positive result in the pathogenicity assay.

Note: Test results are only valid when all included controls presented in Table A.2 obtained expected result. At least one of the four test plants inoculated with the PC and PPC should develop clear symptoms. Test plants inoculated with the NC should develop no symptoms.

Figure A.3. Results of pathogenicity assay. Symptoms on inoculated cotyledons 7 days post-inoculation of pathogenic *P. syringae* grown in greenhouse at 45-60% relative humidity (A) and climate chamber at 85-95% relative humidity (B). Note the sunken pinpoint lesions and surrounding halos in A.

Annex B: Protocol for 16S sequencing

DNA isolation from bacteria

A 10 mL overnight King's B (KB, Table C.1) suspension culture was inoculated from a single colony of bacteria plated on KB agar. The culture was incubated on a shaker at *ca.* 120 rpm at 27 °C. Subsequently, 1 mL of the culture was collected and harvested by centrifugation for 10 min at 19,000 × *g*. The supernatant was discarded and the pellet was used for DNA isolation using the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany).

16S sequencing

DNA was isolated as described above and subsequently 1,000-fold diluted in PCR-grade water. PCRs were performed as described in Table B.2 and B.3. For confirmation, PCR products were separated by gel electrophoresis using 0.8% w/v agarose. After completion the gel was stained with SERVA DNA Stain G (SERVA, Heidelberg, Germany) and exposed to UV for analysis. Products were sequenced (Eurofins Genomics, Ebersberg, Germany) and contigs were assembled using SeqMan Pro 15 (DNASTAR, Madison, Wisconsin, USA) and cropped. Resulting sequences were analysed by nucleotide blast, GenBank (NCBI, Bethesda, Maryland, USA).

Table B.1. 16S sequencing primers.

ZUP	Target	Sequence	nt	Reference
1571	16S	5'- GAA GAG TTT GAT CCT GGC TCA G – 3'	22	Based on Eden *et al*., 1991
1572	16S	5'- TAC GGC TAC CTT GTT ACG ACT T – 3'	22	

Table B.2. Amplification reaction mix for sequencing.

Compound	Final concentration	Volume (µL)
PCR grade water		6.5
Forward primer 1 (20 pmol/µL)	0.4 µM	0.5
Reverse primer 1 (20 pmol/µL)	0.4 µM	0.5
PerfeCTa qPCR ToughMix (Quanta- Bio)	1 x	12.5
Template		5

Table B.3. PCR program for 16S sequencing.

Temperature	Time [min:sec]	
95 °C	10:00	
95 °C	0:30	repeat 35 times
57 °C	0:30	
72 °C	1:30	
72 °C	10:00	

References

Eden, P.A., Schmidt, T.M., Blakemore, R.P. and Pace, N.R. (1991) Phylogenetic analysis of *Aquaspirillum magnetotacticum* using polymerase chain reaction-amplified 16S rRNA-specific DNA. *International Journal of Systematic and Evolutionary Microbiology*, **41**, 324-325.

Annex C: Protocol for seed spot inoculation assay

The following protocol from Syngenta is used to produce artificially infected squash seed.

Materials

- *Pseudomonas syringae* strain(s)
- King's B (KB) medium (Table C.1)
- Inoculation loop
- Sterile NaCl solution (Table C.2)
- Petri dishes
- Squash seeds
- Lab disposables

Table C.1. King's B (KB) medium (King *et al*. 1954).

Compound	Amount / L
Proteose peptone (e.g. #3 Difco)	20.0 g
K_2HPO_4	1.5 g
$MgSO_4.7H_2O$	1.5 g
Glycerol	15.0 mL
Agar	15.0 g
De-ionized water up to a volume of	1 L

Note: Autoclave at 121°C, 15 psi for 15 min.

Table C.2. NaCl solution.

Compound	Amount / L
Sodium chloride (NaCl)	8.5 g
De-ionized water up to a volume of	1 L

Note: Autoclave at 121°C, 15 psi for 15 min.

Seed spot inoculation

1. Plate the *P. syringae* strain on KB medium and incubate for 2 to 4 days at 26-28°C.
2. Collect bacteria from the plate using a sterile inoculation loop and re-suspend in sterile NaCl to obtain a bacterial suspension. Dilute in NaCl solution to obtain the desired concentration (if required, confirm concentration by dilution plating).
3. Distribute the required amount of squash seeds (100) in sterile, empty Petri dishes.
4. Spot 10 µL of the bacterial suspension onto each seed.
5. Let the droplet air dry (e.g. in a flow hood, ca 30 min).
6. After drying, seeds can be sown.
7. Germinate and grow under the same conditions used in the grow-out (see Annex A).
8. Do not store the seeds, use on the day of inoculation.

References

King, E.O., Ward, M.K., and Raney, D.E. (1954). Two simple media for the demonstration of pyocyanin and fluorescin. *Journal of Laboratory and Clinical Medicine*, **44**, 301-307.

Annex D. Test plan for the detection and confirmation of pathogenetic *Pseudomonas* on *Cucurbita pepo* (squash) seeds

1. Organisation and design

1.1 Test Organiser

Hubert Lybeert
HM-Clause
Rue Louis Saillant
BP 83
26802 Portes-lès-Valence cedex, France

1.2 Criteria required

Laboratories experienced in seed health testing and having airconditioned greenhouse or growth chamber means.

1.3 Timeline

Time	Action	Person
August 2018	Homogeneity test	Test organiser
September 2018	CT sample sending	Test organiser
October 2018	CT	Labs participant
November 2018	Stability test	Test organiser

2. Introduction and objective of the comparative test

2.1 Background

A *Pseudomonas* (named here Psp) was isolated from very young, stunted plants of squash.

Depending on the climatic conditions, symptoms could be strong (necrosis of cotyledons, necrosis of leaves, vein clearing of leaves, plants stopping to grow) or absent from the same source of seeds.

Cool temperatures and humidity were the most favourable conditions for the expression of the disease.

Artificial inoculation on plants and on different cucurbits showed that systemic symptoms were only observed on *Cucurbita pepo*. Genetic analysis ranked this bacterium in the group 1 of the *Pseudomonas syringae*.

Research projects helped to develop a specific selective media for this bacterium.

2.2 Aim and objective of the comparative test

This comparative test is based on a grow-out assay followed by isolating the pathogen in pure culture and demonstrating its pathogenicity on plants. The grow-out can be performed in a greenhouse or growth chamber.

3. Materials and methods

3.1 Seed subsamples and samples

Each participating laboratory will analyse three seed samples of 1,000 seeds with one healthy, one moderately infected and one highly infected sample. The samples will be coded, and their identity will only be known by the test organizer. Each sample will be sub-divided into 10 subsamples of 100 seeds. An artificially infected seed sample will also be used as a positive control.

3.2 Statistical analysis

The data will be analysed on a qualitative level per sample (final positive or negative result). The ISO 16140 (AFNOR, 2003) will be followed to evaluate the performance criteria of sensitivity, specificity and accuracy for the presented method.

The method of Langton *et al*. (2002) will be used to evaluate accordance (repeatability of qualitative data) and concordance (reproducibility of qualitative data) of the method for different contamination levels.

3.3 Method

See Annex A for the protocol.

3.3.1 Materials needed to perform the test provided by the Test Organizer

- ✓ Psp isolate to serve as a positive control in the pathogenicity test and to produce the artificially infected sample (see section 3.3.2)
- ✓ Squash seeds for the pathogenicity assay

3.3.2. Preparation of the Positive Process Control

Prepare the artificially infected sample (50 seeds) following the procedure in Appendix 1: Squash seed inoculation procedure to generate PPC with *Pseudomonas syringae*

3.3.3. Note on cross contamination

As this comparative test is a blind test, cross contamination could occur between subsamples. It is essential to avoid any contact between plants coming from different subsamples.

3.3.4. Expected time for completion of the CT by each participating laboratory

Steps	Action	Time needed
Sowing	Distribute seed samples for sowing	3 hours
Observing	Inspect seedlings for symptoms Collect tissues for bacterial isolation	5 hours
Isolating	Isolating bacteria on Petri Dishes	4 hours
Confirmation	Performing pathogenicity tests	4 hours

4. References

AFNOR (2003). NF EN ISO 16140. Microbiology of food and animal feeding stuffs — Protocol for the validation of alternative methods. AFNOR eds.

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.

Appendix 1: Squash seed inoculation procedure to generate PPC with *Pseudomonas syringae*

The following procedure from Syngenta is used to produce the artificially infected sample using a known pathogenic strain provided by the Test Organizer.

PURPOSE

Prepare seeds inoculated with viable *Pseudomonas syringae* to be used as positive control.

Materials

- Healthy squash seeds (provided by the Test Organizer)
- A *Pseudomonas syringae* strain (provided by the Test Organizer)
- Photometer (Absorption/optical density at 600 nm)
- Sterile work bench (laminar flow or similar)
- Photometer cuvettes (Semi-micro, 10 mm path length)
- Petri dishes
- Sterile inoculation loops
- King's B (KB) agar (recipe see Table C.1) or LBC+ AL (recipe see Table A.3)
- NaCl solution (recipe see Table A.1)

Method

1. Plate the *Pseudomonas syringae* strain on medium and incubate for 2 to 4 days at 26-28°C.
2. Collect bacteria from the plate using a sterile inoculation loop and re-suspend in sterile NaCl solution to obtain a bacterial suspension of an optical density at 600 nm = 0.1
3. Distribute the required amount of squash seeds (50) in sterile, empty Petri dishes.
4. Spot 10 µL of the bacterial suspension onto each seed.
5. Let the droplet air dry (e.g. in a flow hood, ca 30 min).
6. After drying the seed can be sown.
7. Do not store the seeds, use on the day of inoculation.

Annex E: Raw data from the inter-laboratory comparative test.

LABORATORY 1: Greenhouse

Lab. 1 results 21 days after sowing					Confirmation tests results					Final score (+ / -)	Comments
Seed sub-samples		Number of plants observed	Number of typical and doubtful plants per sample of 100 seeds sown	% of plants showing symptoms*	Number of harvested plants showing symptoms (0, 1 or 2)	Number of suspected selected colonies (the most predominant in number, on the dish)		Pathogenicity test result (number of colonies found positive) (0, 1 or 2)			
						A	B	A	B		
Highly contaminated seed lot	1	95	3	3.16	2	0	3	N/A	1/1	+	
	2	100	8	8.00	2	3	0	1/1	N/A	+	
	3	90	15	16.67	2	3	3	1/1	1/1	+	
	4	99	20	20.20	2	3	3	1/1	1/1	+	Aggressive
	5	100	13	13.00	2	3	3	1/1	1/1	+	
	6	90	16	17.78	2	3	3	1/1	1/1	+	
	7	99	16	16.16	2	3	3	1/1	1/1	+	
	8	97	12	12.37	2	3	3	1/1	1/1	+	
	9	90	18	20.00	2	3	3	1/1	1/1	+	
	10	95	30	31.58	2	3	3	1/1	1/1	+	Aggressive
Medium contaminated seed lot	11	104	24	23.08	2	3	3	1/1	1/1	+	
	12	100	25	25.00	2	3	0	1/1	N/A	+	
	13	100	28	28.00	2	3	3	0/1	1/1	+	
	14	100	19	19.00	2	3	0	1/1	N/A	+	
	15	100	23	23.00	2	3	3	1/1	1/1	+	
	16	99	21	21.21	2	0	3	N/A	1/1	+	
	17	99	20	20.20	2	3	3	1/1	1/1	+	
	18	90	12	13.33	2	3	0	1/1	N/A	+	
	19	100	5	5.00	2	0	3	N/A	1/1	+	
	20	98	5	5.10	2	3	3	1/1	1/1	+	

Lab. 1 results 21 days after sowing					Confirmation tests results					Final score (+ / -)	Comments
Seed sub-samples		Number of plants observed	Number of typical and doubtful plants per sample of 100 seeds sown	% of plants showing symptoms*	Number of harvested plants showing symptoms (0, 1 or 2)	Number of suspected selected colonies (the most predominant in number, on the dish)		Pathogenicity test result (number of colonies found positive) (0, 1 or 2)			
						A	B	A	B		
Healthy seed lot	21	99	3	3.03	2	3	3	1/1	1/1	+	
	22	100	4	4.00	2	0	3	N/A	1/1	+	
	23	85	6	7.06	2	3	0	1/1	N/A	+	
	24	100	4	4.00	2	3	0	1/1	N/A	+	
	25	100	7	7.00	2	3	0	0/1	N/A	-	the symptoms were only curly edges
	26	96	22	22.92	2	0	0	N/A	N/A	-	the symptoms were only curly edges
	27	92	7	7.61	2	3	0	1/1	N/A	+	
	28	96	2	2.08	2	0	3	N/A	1/1	+	
	29	98	5	5.10	2	0	3	N/A	1/1	+	
	30	95	8	8.42	2	3	0	0/1	N/A	-	
Artificially contaminated lot		50	7	14.00	2	3	3	0/1	0/1	-	the symptoms were observed directly on the leaves. The cotyledons were healthy
Pathogenicity test strain control		NA	NA	NA	NA	NA		4/4	4/4	+	
Pathogenicity test NaCl solution		NA	NA	NA	NA	NA		0/1	0/1	-	
Observations		- Was difficult to detect symptoms because all the plants were showing curly damaged edges. - From all the samples, two plants with symptoms were harvested and individually plated (A and B). - Only one kind of suspected colony was observed per plate. 3 colonies were selected, polled and replated for the pathogenicity assay. - For the pathogenicity assay, one strain from HM Clause and four strains from Enza Zaden were included as positive controls. All of them were positive.									

* (%) Number of typical and doubtful plants / number of plants observed.

LABORATORY 2: Growth chamber

Lab. 2 results 14 days after sowing					Confirmation tests results			Final score (+ / -)	Comments: TaqMan PCR results using primers/probe ZUP2536, ZUP2537, ZUP254
Seed sub-samples		Number of plants observed	Number of typical and doubtful plants per sample of 100 seeds sown	% of plants showing symptoms*	Number of harvested plants showing symptoms (0, 1 or 2)	Number of suspected selected colonies (the most predominant in number, on the dish)	Pathogenicity test result (number of colonies found positive) (0, 1 or 2)		
Highly contaminated seed lot	1	99	21	21.2	2	2	2	+	PCR: +
	2	100	28	28.0	2	2	2	+	PCR: +
	3	99	26	26.3	2	2	2	+	PCR: +
	4	98	25	25.5	2	2	1	+	PCR: +
	5	98	22	22.4	2	2	2	+	PCR: +
	6	100	27	27.0	2	2	2	+	PCR: +
	7	100	20	20.0	2	2	2	+	PCR: +
	8	100	20	20.0	2	2	2	+	PCR: +
	9	100	27	27.0	2	2	1	+	PCR: +
	10	98	21	21.4	2	2	2	+	PCR: +
Medium contaminated seed lot	11	95	0	0.0	0			-	
	12	97	5	5.2	2	2	0	-	PCR: -
	13	99	10	10.1	2	2	1	+	PCR: ?
	14	96	4	4.2	2	2	0	-	PCR: -
	15	96	16	16.7	2	2	1	+	PCR: +
	16	97	15	15.5	2	2	1	+	PCR: +
	17	97	0	0.0	0			-	
	18	99	1	1.0	1	1	0	-	PCR: -
	19	99	15	15.2	2	2	0	-	PCR: -
	20	97	4	4.1	2	2	0	-	PCR: -

Lab. 2 results 14 days after sowing					Confirmation tests results			Final score (+ / -)	Comments: TaqMan PCR results using primers/probe ZUP2536, ZUP2537, ZUP254
Seed sub-samples		Number of plants observed	Number of typical and doubtful plants per sample of 100 seeds sown	% of plants showing symptoms*	Number of harvested plants showing symptoms (0, 1 or 2)	Number of suspected selected colonies (the most predominant in number, on the dish)	Pathogenicity test result (number of colonies found positive) (0, 1 or 2)		
Healthy seed lot	21	91	10	11.0	2	2	0	-	PCR: -
	22	95	10	10.5	2	2	0	-	PCR: -
	23	96	17	17.7	2	2	0	-	PCR: -
	24	97	10	10.3	2	1	0	-	PCR: -
	25	100	12	12.0	2	2	0	-	PCR: -
	26	99	17	17.2	2	2	0	-	PCR: -
	27	97	20	20.6	2	1	0	-	PCR: -
	28	95	9	9.5	2	2	0	-	PCR: -
	29	93	11	11.8	2	2	0	-	PCR: -
	30	99	8	8.1	2	2	0	-	PCR: -
Artificially contaminated lot		50	32	64.0	2	2	2	+	PCR: +
Pathogenicity test strain control							2	+	PCR: +
Pathogenicity test NaCl solution							0	-	PCR: -

* (%) Number of typical and doubtful plants / number of plants observed.

LABORATORY 3: Greenhouse

Lab. 3 results 21 days after sowing						Confirmation tests results			Final score (+ / -)	Comments
Seed sub-samples		Pools	Number of plants observed	Number of typical and doubtful plants per sample of 100 seeds sown	% of plants showing symptoms*	Number of harvested plants showing symptoms (0, 1 or 2)	Number of suspected selected colonies (the most predominant in number, on the dish)	Pathogenicity test result (number of colonies found positive) (0, 1 or 2)		
Highly contaminated seed lot	1	1	91	50	54.95	2	1	1/1	+	
		2					1	1/1		
	2	1	92	40	43.48	2	1	1/1	+	
		2					1	1/1		
	3	1	97	40	41.24	2	1	1/1	+	
		2					1	1/1		
	4	1	100	40	40.00	2	1	1/1	+	
		2					1	1/1		
	5	1	98	60	61.22	2	1	1/1	+	
		2					1	1/1		
	6	1	100	30	30.00	2	1	1/1	+	
		2					1	1/1		
	7	1	100	30	30.00	2	1	1/1	+	
		2					1	1/1		
	8	1	94	30	31.91	2	1	1/1	+	
		2					1	1/1		
	9	1	92	30	32.61	2	1	1/1	+	
		2					1	1/1		
	10	1	100	30	30.00	2	1	1/1	+	
		2					1	1/1		
Me diu	11	1	95	30	31.58	2	1	1/1	+	
		2					1	0/1		

Lab. 3 results 21 days after sowing						Confirmation tests results			Final score (+ / -)	Comments
Seed sub-samples		Pools	Number of plants observed	Number of typical and doubtful plants per sample of 100 seeds sown	% of plants showing symptoms*	Number of harvested plants showing symptoms (0, 1 or 2)	Number of suspected selected colonies (the most predominant in number, on the dish)	Pathogenicity test result (number of colonies found positive) (0, 1 or 2)		
	12	1	92	30	32.61	2	1	1/1	+	
		2					1	1/1		
	13	1	100	3	3.00	2	1	1/1	+	
		2					1	0/1		
	14	1	94	30	31.91	2	1	1/1	+	
		2					1	1/1		
	15	1	95	30	31.58	2	1	1/1	+	
		2					1	1/1		
	16	1	99	30	30.30	2	1	1/1	+	
		2					1	0/1		
	17	1	100	30	30.00	2	1	1/1	+	
		2					1	1/1		
	18	1	92	30	32.61	2	1	1/1	+	
		2					1	1/1		
	19	1	95	7	7.37	2	1	1/1	+	
		2					1	1/1		
	20	1	95	6	6.32	2	1	1/1	+	
		2					1	0/1		
Healthy seed lot	21	1	100						-	
		2								
	22	1	97	2	2.06	2	1	0/1	-	
		2					1	0/1		
	23	1	100	2	2.00	2	1	0/1	-	
		2					1	0/1		

Lab. 3 results 21 days after sowing						Confirmation tests results			Final score (+ / -)	Comments
Seed sub-samples		Pools	Number of plants observed	Number of typical and doubtful plants per sample of 100 seeds sown	% of plants showing symptoms*	Number of harvested plants showing symptoms (0, 1 or 2)	Number of suspected selected colonies (the most predominant in number, on the dish)	Pathogenicity test result (number of colonies found positive) (0, 1 or 2)		
	24	1	100	2	2.00	2	1	0/1	-	
		2					1	0/1		
	25	1	95						-	
		2								
	26	1	94						-	
		2								
	27	1	100	2	2.00	2	1	0/1	-	
		2					1	0/1		
	28	1	97	1	1.03	2	1	0/1	-	
		2					1	0/1		
	29	1	90	6	6.67	2	1	0/1	-	
		2					1	0/1		
	30	1	94	2	2.13	2	1	0/1	-	
		2					1	0/1		
Artificially contaminated lot			50	24	48.00	1	1	1/1	+	
Pathogenicity test strain control									+	
Pathogenicity test NaCl solution									-	

* (%) Number of typical and doubtful plants / number of plants observed.

LABORATORY 4: Growth chamber

Lab. 4 results 21 days after sowing					Confirmation tests results			Final score (+ / -)	Comments	qPCR on colonies
Seed sub-samples		Number of plants observed	Number of typical and doubtful plants per sample of 100 seeds sown	% of plants showing symptoms*	Number of harvested plants showing symptoms (0, 1 or 2)	Number of suspected selected colonies (the most predominant in number, on the dish)	Pathogenicity test result (number of colonies found positive) (0, 1 or 2)			
Highly contaminated seed lot	1	100	5	5.00	2	2	1	+		+
	2	100	10	10.00	2	0	0	-	Failed, due to bad PPC	-
	3	100	1	1.00	1	1	1	+		+
	4	100	11	11.00	2	1	0	-	Failed, due to bad PPC	-
	5	100	5	5.00	2	1	1	+		+
	6	100	8	8.00	2	1	1	+		+
	7	100	1	1.00	1	1	0	-	Failed, due to bad PPC	-
	8	100	8	8.00	2	0	0	-	Failed, due to bad PPC	-
	9	100	3	3.00	2	1	1	+		+
	10	100	1	1.00	1	0	0	-	Failed, due to bad PPC	-
Medium contaminated seed lot	11	100	11	11.00	2	0	0	-	Failed, due to bad PPC	-
	12	100	21	21.00	2	0	0	-	Failed, due to bad PPC	-
	13	100	6	6.00	2	2	0	-	Failed, due to bad PPC	-
	14	97	3	3.09	2	0	0	-	Failed, due to bad PPC	-
	15	100	10	10.00	2	1	0	-	Failed, due to bad PPC	-
	16	98	4	4.08	2	2	0	-	Failed, due to bad PPC	-
	17	100	11	11.00	2	0	0	-	Failed, due to bad PPC	-
	18	100	8	8.00	2	2	1	+		+
	19	100	9	9.00	2	0	0	-	Failed, due to bad PPC	-
	20	99	4	4.04	2	0	0	-	Failed, due to bad PPC	-

Lab. 4 results 21 days after sowing					Confirmation tests results			Final score (+ / -)	Comments	qPCR on colonies
Seed sub-samples		Number of plants observed	Number of typical and doubtful plants per sample of 100 seeds sown	% of plants showing symptoms*	Number of harvested plants showing symptoms (0, 1 or 2)	Number of suspected selected colonies (the most predominant in number, on the dish)	Pathogenicity test result (number of colonies found positive) (0, 1 or 2)			
Healthy seed lot	21	100	5	5.00	2	1	0	-	Failed, due to bad PPC	-
	22	100	2	2.00	2	0	0	-	Failed, due to bad PPC	-
	23	100	7	7.00	2	0	0	-	Failed, due to bad PPC	-
	24	100	12	12.00	2	2	0	-	Failed, due to bad PPC	-
	25	100	7	7.00	2	1	0	-	Failed, due to bad PPC	-
	26	100	15	15.00	2	1	0	-	Failed, due to bad PPC	-
	27	100	7	7.00	2	1	0	-	Failed, due to bad PPC	-
	28	100	9	9.00	2	1	0	-	Failed, due to bad PPC	-
	29	100	6	6.00	2	1	0	-	Failed, due to bad PPC	-
	30	100	9	9.00	2	1	0	-	Failed, due to bad PPC	-
Artificially contaminated lot		50	8	16.00	2	1	1	+	This PPC did not show the symptoms that we seen before. Something went wrong but we do not know what. Conditions were perfect.	+
Pathogenicity test strain control		NA	NA	NA	NA	NA	NA	+	test strains seems less severe than the isolates collected from the growout subsamples	+
Pathogenicity test NaCl solution		NA	NA	NA	NA	NA	NA	-		-

* (%) Number of typical and doubtful plants / number of plants observed..

LABORATORY 5: Growth chamber

Lab. 5 results 14 days after sowing					Confirmation tests results			Final score (+ / -)	Comments
Seed sub-samples		Number of plants observed	Number of typical and doubtful plants per sample of 100 seeds sown*	% of plants showing symptoms**	Number of harvested plants showing symptoms (0, 1 or 2)***	Number of suspected selected colonies (the most predominant in number, on the dish)	Pathogenicity test result (number of colonies found positive) (0, 1 or 2)		
Highly contaminated seed lot	1	100	4	4.00%	4	Did not quantify, but overall there were few suspect colonies	1	+	
	2	100	1	1.00%	1		2	+	
	3	100	2	2.00%	2		4	+	
	4	100	0	0.00%	0		0	-	
	5	100	0	0.00%	0		0	-	
	6	100	0	0.00%	0		0	-	
	7	100	8	8.00%	4		0	-	
	8	100	2	2.00%	2		4	+	
	9	100	2	2.00%	2		1	+	
	10	100	1	1.00%	1		0	-	
Medium contaminated seed lot	11	100	1	1.00%	1		0	-	
	12	100	0	0.00%	0		0	-	
	13	100	2	2.00%	2		0	-	
	14	100	7	7.00%	4		0	-	
	15	100	5	5.00%	4		0	-	
	16	100	10	10.00%	4		1	+	
	17	100	8	8.00%	4		0	-	
	18	100	7	7.00%	4		0	-	
	19	100	2	2.00%	2		0	-	
	20	100	7	7.00%	4		0	-	

Lab. 5 results 14 days after sowing					Confirmation tests results			Final score (+ / -)	Comments
Seed sub-samples		Number of plants observed	Number of typical and doubtful plants per sample of 100 seeds sown	% of plants showing symptoms*	Number of harvested plants showing symptoms (0, 1 or 2)	Number of suspected selected colonies (the most predominant in number, on the dish)	Pathogenicity test result (number of colonies found positive) (0, 1 or 2)		
Healthy seed lot	21	100	10	10.00%	4	Did not quantify, but overall there were few suspect colonies	0	-	
	22	100	5	5.00%	4		0	-	
	23	100	5	5.00%	4		0	-	
	24	100	0	0.00%	0		0	-	
	25	100	10	10.00%	4		0	-	
	26	100	10	10.00%	4		0	-	
	27	100	5	5.00%	4		0	-	
	28	100	4	4.00%	4		0	-	
	29	100	7	7.00%	4		0	-	
	30	100	8	8.00%	4		0	-	
Artificially contaminated lot		100	2	2.00%	2	Did not quantify, but overall there were many suspect colonies	5	+	
Pathogenicity test strain control		NA	NA	NA	NA	NA	NA	Culture was dead by time of pathogenicity test	
Pathogenicity test NaCl solution		NA	NA	NA	NA	NA	NA	-	

* Plants with symptoms that have expanded/become more severe since first read.
** (%) Number of typical and doubtful plants / number of plants observed.
*** Harvested all symptomatic seedlings, up to 4 per flat.

LABORATORY 6: Growth chamber

Lab. 6 results 14 days after sowing					Confirmation tests results			Final score (+ / -)	Comments
Seed sub-samples		Number of plants observed	Number of typical and doubtful plants per sample of 100 seeds sown	% of plants showing symptoms*	Number of harvested plants showing symptoms (0, 1 or 2)	Number of suspected selected colonies (the most predominant in number, on the dish)	Pathogenicity test result (number of colonies found positive) (0, 1 or 2)		
Highly contaminated seed lot	1	98	14	14.29	2	2	2	+	
	2	99	18	18.18	2	2	2	+	
	3	96	43	44.79	2	2	2	+	
	4	101	10	9.90	2	2	2	+	
	5	100	21	21.00	2	2	2	+	morphology different from other colonies detected
	6	98	12	12.24	2	2	2	+	
	7	98	14	14.29	2	2	2	+	
	8	99	8	8.08	2	2	2	+	8.2 : symptoms less strong
	9	101	30	29.70	2	2	2	+	
	10	96	28	29.17	2	2	2	+	
Medium contaminated seed lot	11	94	0	0.00	0			-	
	12	92	11	11.96	1	1	1	+	mistake: just one colony taken (instead of two)
	13	88	6	6.82	2	2	2	+	
	14	95	1	1.05	1	1	1	+	
	15	81	0	0.00	0			-	
	16	93	0	0.00	0			-	
	17	97	2	2.06	2	2	2	+	
	18	86	0	0.00	0			-	
	19	97	1	1.03	1	1	0	-	
	20	95	1	1.05	1	1	1	+	

Lab. 6 results 14 days after sowing					Confirmation tests results			Final score (+ / -)	Comments
Seed sub-samples		Number of plants observed	Number of typical and doubtful plants per sample of 100 seeds sown	% of plants showing symptoms*	Number of harvested plants showing symptoms (0, 1 or 2)	Number of suspected selected colonies (the most predominant in number, on the dish)	Pathogenicity test result (number of colonies found positive) (0, 1 or 2)		
Healthy seed lot	21	97	0	0.00	0			-	
	22	95	3	3.16	1	1	0	-	
	23	92	0	0.00	0			-	
	24	95	0	0.00	0			-	
	25	91	0	0.00	0			-	
	26	96	1	1.04	1	1	0	-	
	27	91	0	0.00	0			-	
	28	91	1	1.10	1	1	0	-	
	29	91	1	1.10	1	1	0	-	
	30	91	0	0.00	0			-	
Artificially contaminated lot		48	8	16.67	2	2	2	+	
Pathogenicity test strain control		P99	NA	NA	NA	NA	1	+	
Pathogenicity test NaCl solution		NA	NA	NA	NA	NA	0	-	

* (%) Number of typical and doubtful plants / number of plants observed.