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Detection of *Acidovorax citrulli* associated with melon seeds by grow-out in sweat boxes

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ISHI-Veg International Technical Group Cucurbits - H. Lybeert (HM-Clause, France), C. Sahuguede, G. Orgeur (GEVES, France), C. Ponzio, J.H.C. Woudenberg (ISF, Switzerland)

CONTENTS

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1. INTRODUCTION

Acidovorax citrulli, (formerly *A. avenae* subsp. *citrulli*) (Ac) (Willems *et al.*, 1992) is a Gram-negative, obligately aerobic, and motile with a single polar flagellum, biotrophic bacterium that causes seedling blight and bacterial fruit blotch (BFB) of melon and watermelon.

The bacterium was originally isolated from water-soaked lesions on cotyledons of watermelon seedlings from accessions in the USA plant introduction (PIs) collection (Webb and Goth, 1965). It was phenotypically similar to *Pseudomonas pseudoalcaligenes* but differed in that it was pathogenic to watermelon, cantaloupe, cucumber and squash (Schaad *et al.*, 1978). Therefore, this new bacterium was named *P. pseudoalcaligenes* subsp. *citrulli*. Hu *et al.* (1991) found a close similarity between this bacterium and *Pseudomonas avenae* and it was subsequently renamed *P. avenae* subsp. *citrulli*. The watermelon bacterium and other subspecies of *P. avenae* constitute a separate rRNA branch within the family *Comamonadaceae*. Comparing them phenotypically, Willems *et al.* (1992) found the members of this rRNA branch to be most closely related to the genus *Acidovorax* and the watermelon fruit blotch bacterium was renamed *Acidovorax avenae* subsp. *citrulli*. The current preferred name for this pathogen is *Acidovorax citrulli*.

BFB is a sporadic disease but under favourable environment, it becomes devastating and may cause 100% loss of marketable fruit. Infected seeds and seedlings are the most important primary sources of inoculum in commercial fruit production field. However, there may be other endemic sources of inoculum like debris from infected fruit or foliage tissue, volunteer seedlings, or cucurbitaceous weeds (Black *et al.*, 1994; Latin and Hopkins, 1995; Isakeit *et al.*, 1998). In the field, BFB development is heavily dependent on rainfall and relative humidity. Secondary dispersal of *A. citrulli* is by wind-driven rain or over-head irrigation. When *A. citrulli* lands on healthy leaves, it migrates through open stomata into the sub-stomatal intercellular spaces where it multiplies and induces water-soaked lesions.

The recommended test to detect *A. citrulli* is a seedling grow-out test conducted under favourable conditions for symptom development. Seeds are planted in a sterile potting mix in a greenhouse free from other sources of *A. citrulli*, usually a greenhouse dedicated to seed testing. Seedlings are watered by overhead irrigation to promote foci of symptomatic plants that are easily visible. Relative humidity in the greenhouse is maintained above 55% and temperature is maintained between 24 and 38°C (Hopkins, 1994; Latin and Hopkins, 1995). After 16-21 days, each seedling is carefully inspected for symptoms. Isolations are made from seedlings showing any symptoms of disease and *A. citrulli* is identified using biochemical, DNA and biological assay. This method takes 30-35 days to complete.

Elaborate precautions must be taken to ensure that cross-contamination of seedlings does not occur in test procedures. A detailed step-by-step seedling grow-out method that describes such precautions and is standardised by the USA National Seed Health System (NSHS) can be accessed at the website www.seedhealth.org.

2. OBJECTIVES

The objective was to validate an internationally accepted method for the detection of *Acidovorax citrulli* on melon seeds using a grow-out test in sweat boxes and confirmation of symptoms by a biological assay (annex A). In the inter-laboratory comparative test, laboratories also had the option to use ELISA and PCR for confirmation but for their own purposes, only the results of the bioassay trial were analysed. The method was validated according to the ISHI-Veg guidelines for the Validation of Seed Health Tests (Version 2, May 2020).

This sweat box grow-out method is faster, takes less space and, as performed in climate chamber, the environmental conditions are easier to control than in the grow-out test in a greenhouse. It could be an alternative to the validated greenhouse grow-out method and an option to confirm a positive result obtained after the Seed Extract q-PCR (SE-qPCR) pre-screening method (Method for the Detection of *Acidovorax citrulli* in seed of Cucurbit crops, August 2018). See Figure 1 for the method process flow.

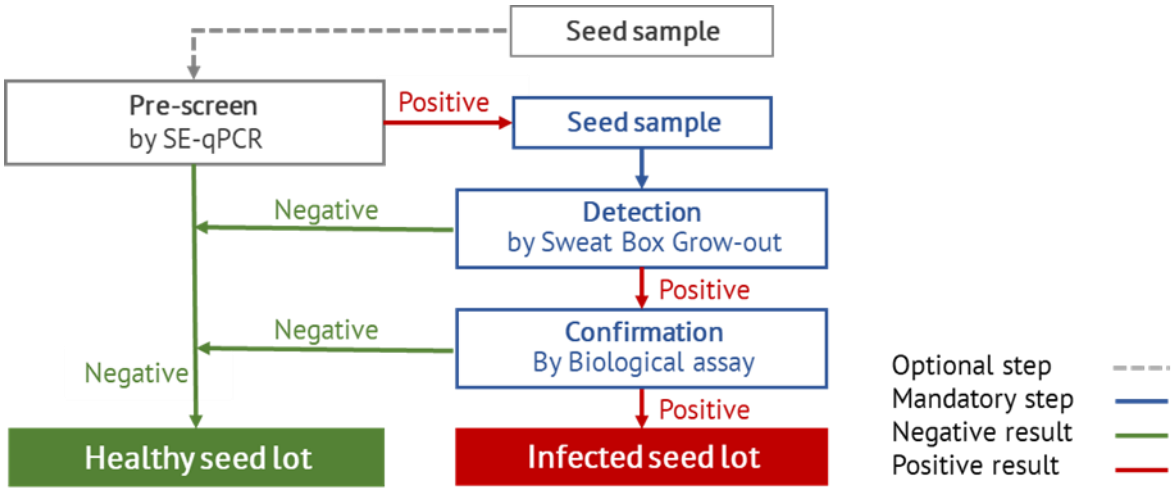


Figure 1. Method process flow

The validation was performed on melon seeds only due to the unavailability of infected watermelon seed. As watermelon is susceptible to the bacterium and growing conditions of the two crops are similar the test can be expected to be suitable for watermelon. However, it is the user's responsibility to validate the results obtained in this study prior to the test being used for watermelon seed.

The sweat box protocol is described in Annex A.
The comparative test is described in the Annex B.
The data from the comparative test can be found in Annex C.

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 sweat box method, observed symptoms were identified as being caused by *Acidovorax citrulli* via the use of a bioassay as the confirmation test. Therefore, the specificity of the sweat box method is evaluated based on the specificity of the bioassay.

The requirements for analytical specificity will be met when all tested *A. citrulli* isolates will be detected with the bioassay, and the non *A. citrulli* isolates will give a negative bioassay test result.

Experimental approach

The specificity of the method was evaluated during a project followed by European EUPHRESCO network (Consensus Detection and Identification Protocol for *Acidovorax citrulli* in cucurbit seeds, project started in 2016, (DIP-ACIT)).

Two separate experiments were performed, one on pure strains and one on plantlet extracts. In experiment one, 11 presumed *A. citrulli* strains (based on morphological identification) and 11 saprophyte strains were tested (collected from two naturally contaminated seed lots, coming from China and Thailand). In experiment two, 16 plantlets showing typical symptoms from *A. citrulli* in a grow-out sweat box assay and three plants showing atypical symptoms in the grow-out assay were tested.

See annex A for the bioassay protocol.

Results

The bioassay results on pure strains and on samples collected directly after a grow-out test are presented in Table 1.

Table 1. Bioassay results for the specificity experiments. Aac = presumed *Acidovorax citrulli* (for plantlet extract = typical symptoms); Sapro = Saprophyte (for plantlet extracts = atypical symptoms).

Strain	Type of isolate	positive/inoculated	Result
1-2 – Sapro 12	Pure strain	0+/5	Negative
2-1 – Sapro 13	Pure strain	0+/5	Negative
2-2 – Sapro 14	Pure strain	0+/5	Negative
3-2 – Sapro 15	Pure strain	0+/5	Negative
4-1 – Aac 3	Pure strain	5+/5	Positive
4-2 – Sapro 16	Pure strain	0+/5	Negative
5-1 – Sapro 17	Pure strain	0+/5	Negative
6-1 – Aac 4	Pure strain	5+/5	Positive
6-2 – Aac 5	Pure strain	4+/5	Positive
7-1 – Sapro 18	Pure strain	0+/5	Negative
7-2 – Aac 6*	Pure strain	0+/5	Negative
8-1 – Aac 7	Pure strain	5+/5	Positive
8-2 – Aac 8	Pure strain	5+/5	Positive
9-1 – Aac 9	Pure strain	5+/5	Positive
12-2 – Sapro 19	Pure strain	0+/5	Negative
13-1 – Aac 10	Pure strain	5+/5	Positive
13-2 – Aac 11	Pure strain	5+/5	Positive
14-1 – Sapro 20	Pure strain	0+/5	Negative
14-2 – Sapro 21	Pure strain	0+/5	Negative
15-2 – Sapro 22	Pure strain	0+/5	Negative
O1 – Aac 12	Pure strain	5+/5	Positive
M1 – Aac 13*	Pure strain	0+/5	Negative
Negative Control	Water	0+/5	Negative
Positive Control (PAS2020)	Pure strain	5+/5	Positive

Strain	Type of isolate	positive/inoculated	Result
1-1 – Aac 14	Plantlet extract	4+/5	Positive
1-2 – Sapro 23	Plantlet extract	0+/5	Negative
2-1 – Aac 15	Plantlet extract	4+/4	Positive
3-1 – Aac 16	Plantlet extract	4+/5	Positive
4-1 – Aac 17	Plantlet extract	4+/5	Positive
5-1 – Aac 18	Plantlet extract	5+/5	Positive
6-1 – Aac 19	Plantlet extract	5+/5	Positive
7-1 – Aac 20	Plantlet extract	4+/5	Positive
8-1 – Aac 21	Plantlet extract	5+/5	Positive
9-1 – Aac 22	Plantlet extract	5+/5	Positive
10-1 – Aac 23	Plantlet extract	2+/5	Positive
11-1 – Aac 24	Plantlet extract	5+/5	Positive
12-1 – Aac 25	Plantlet extract	4+/5	Positive
12-2 – Sapro 24	Plantlet extract	0+/5	Negative
13-1 – Aac 26	Plantlet extract	5+/5	Positive
14-1 – Aac 27	Plantlet extract	4+/5	Positive
14-2 – Sapro 25	Plantlet extract	0+/5	Negative
15-1 – Aac 28	Plantlet extract	1+/5	Positive
15-2 – Aac 29	Plantlet extract	3+/5	Positive
Negative Control	Water	0+/5	Negative
Positive Control (PAS2020)	Plantlet extract	3+/5	Positive

* Based on morphological observations isolates Aac 6 and Aac 13 were described as *A. citrulli*. However, they were consistently tested negative in pathogenicity tests (data not shown), which suggests them to be saprophytes.

Conclusion

Only *A. citrulli* strains and plantlets showing typical symptoms gave a positive bioassay result while saprophyte strains or plantlets showing atypical symptoms gave a negative result.

The specificity requirements for the bioassay have been met.

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 objective was to determine that one *A. citrulli* contaminated seed in one sub-sample could be detected by the sweat box method. The confirmation bioassay is not included in the analytical sensitivity testing, as for the *detection* of the artificially inoculated seed no *confirmation* bioassay is needed.

The analytical sensitivity requirements will be met when every tested subsample with one artificially contaminated seed mixed with 799 healthy seeds results in symptomatic plants in the sweat box grow-out assay.

Experimental approach

In 14 subsamples of 799 healthy melon seeds, one artificially contaminated seed was added (the least possible infected condition). One laboratory performed the sweat box grow-out assay as described in annex A in two rounds of seven of these subsamples at the same time.

Results

In both rounds seven positives samples were observed out of the seven expected for the least infected condition (one seed contaminated in 800 seeds). In all tests around 10% of the plantlets displayed typical symptoms on cotyledons, see Table 2.

Table 2. Analytical sensitivity test results.

		Symptomatic plantlets	Rotten plantlets	Symptomless plantlets	% of contamination	Positive/tested samples
1 infected seed in 800 1st experiment	rep1	100	0	≈700	≈12.50%	7/7
	rep2	99	0	≈700	≈12.38%	
	rep3	43	0	≈750	≈5.38%	
	rep4	111	0	≈650	≈13.88%	
	rep5	76	0	≈700	≈9.50%	
	rep6	117	0	≈650	≈14.63%	
	rep7	72	0	≈700	≈9.00%	
	total	618	0	≈5000	≈11.04%	
1 infected seed in 800 2nd experiment	rep1	80	0	≈700	≈10.00%	7/7
	rep2	69	0	≈700	≈8.63%	
	rep3	94	0	≈700	≈11.75%	
	rep4	49	0	≈750	≈6.13%	
	rep5	108	21	≈650	≈16.13%	
	rep6	85	0	≈700	≈10.63%	
	rep7	54	0	≈750	≈6.75%	
	total	539	21	≈5000	≈10.00%	
Healthy control		0	0	≈1600	0.00%	

Conclusion

One seedling from one contaminated seed mixed with 799 healthy seeds developed typical BFB symptoms and was easily detected. Neighbouring plants also became contaminated from secondary infection, which explains the percentage of contamination observed.
The sensitivity requirements for the sweat box assay have been met.

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 selectivity requirements will be met when *A. citrulli* is successfully detected by the grow-out method in melon seed from two different varieties and origins.

Experimental approach

The inter-laboratory comparative test (CT) was performed using two naturally contaminated melon seed lots:

- One was produced in 2012 in China, the other one in 2014 in Thailand.
- One lot was genetically a “yellow canari” type while the other one was a “Piel de sapo” type.

Testing of additional varieties of seeds was not deemed necessary because of the difference in production location which gives seed with a considerably different saprophytic seed background.

Furthermore, the two seed lots have a high genetic diversity between them. Finally, the study of the matrix effect is of less relevance for a biological assay compared to e.g. molecular assays since the seed background is less likely to interfere with the test result.

Eight labs tested 13 repeats of two seed lots, named a highly infected and a moderately infected seed lot, with the protocol described in Annex A. The final result depended on the biological assay only. If one extract coming from one sweat box gave a positive result in the bioassay, the sample was considered as positive.

Participants reported a qualitative (positive/negative) result for each sample. Inconclusive (no clear typical symptoms in the bioassay) or undetermined (too much growth of damping of causing saprophytes in a sweatbox) results were excluded from the statistical analysis.

See Annex B for the full comparative test description.

Results

All eight labs performed the sweat box assay but only seven performed the bioassay due to a quarantine issue for one lab. The results from the lab which did not perform the bioassay were not included in the analysis.

See Table 3 for the CT results of the two seed lots, full data of the CT can be found in annex C.

Table 3. Qualitative results for samples coming from the two seed lots.

Lab number	Number of samples (No. positive/No. tested)	
	Medium contaminated lot	Highly contaminated lot
01	8^{+}/13	12^{+}/13
02	10^{+}/13	13^{+}/13
03	12^{+}/13	13^{+}/13
04	11^{+}/12*	13^{+}/13
05	8^{+}/11*	13^{+}/13
07	2^{+}/9*	11^{+}/12*
08	3^{+}/13	13^{+}/13

* Due to the exclusion of inconclusive and undetermined results from the statistical analysis the number of tested seed lots is lower than the provided 13 repeats.

Conclusion

The 13 repetitions of 800 seeds gave around 10 000 seeds tested, the recommended sample size to detect *A. citrulli* on melon seeds. All labs found the two contaminated lots (highly and medium infection) to be positive for *A. citrulli*.

The selectivity requirements for the method have been met.

NOTE: This CT was performed on melon seeds. Because watermelon is also susceptible to *A. citrulli* and growing conditions of the two crops are similar the test can be expected to be suitable for watermelon as well as melon. However, it is the user's responsibility to validate the results obtained in this study prior to the test being used for watermelon seed.

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 criteria, i.e. accordance within labs is >90%.

Experimental approach

Samples for homogeneity, stability and CT were tested in one lab (the organising lab) and used to evaluate the repeatability of the method. See annex A for the protocol of the method used.

In the CT, 13 repeats of three seed lots of 800 seeds each (highly infected, medium infected and healthy seed lots) were tested.

In the homogeneity test, eight extra samples of 800 seeds representing each contamination level were tested after packaging and just before sending the samples to participating labs in the CT.

In the stability test, three extra samples of 800 seeds representing each contamination level were tested after receiving the confirmation of all other participating labs in the CT that they started the test.

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

Results

The homogeneity and stability results together with the CT results from the lab performing the homogeneity and stability tests are presented in Table 4.

Table 4. Repeatability data.

Lot	Homogeneity results	Comparative test results	Stability results
Healthy	8 negative/8	13 negative/13	3 negative/3
Medium	3 positive samples/8	8 positive samples/11	1 positive sample/3
High	8 positive samples/8	13 positive samples/13	3 positive samples/3

In the CT and stability tests, all healthy seed samples gave a negative result, and all high infected seed samples gave a positive result which corresponds with the results from the homogeneity test (Table 4).

The Langton analysis gave a 100% accordance for the healthy and high contaminated seed samples.

For the medium contaminated seed lot, the homogeneity test results showed that not all samples contained infected seeds, so that no accordance can be calculated for these seed samples.

However for the medium infected lot, the results from the CT and the stability test should fall within the expected number of contaminated samples as calculated based on the percentage of infection obtained from the homogeneity test. The percentage of infection for the medium contaminated samples is calculated with the Seedcalc8 software (the computed % in sample at 95% confident, provided in the STATCOM ISTA webpage). The percentage of infection, corresponding to 3 positive samples out of 8 totals, was 0.06% (Figure 2). This percentage 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" provided on the SHC ISTA webpage.

Impurity Estimation & Confidence Intervals (Assay measures impurity characteristic)

(Number of seed sampled should not exceed 10% of total number in population)

# of Seed Pools	8	Computed % in sample	0.06 %
# of Seeds per Pool	800		
Total Seeds Tested	6400	*Measured property on seed pools*	
# Deviants Pools	3		
		Desired Confidence Level	95 %
Upper Bound of True % Impurity		0.15	
		(95% confident that the lot impurity is below 0.15%.)	
2-sided CI for True % Impurity		*0.01 to 0.18*	
Lower Bound of True % Purity		99.85	
		(95% confident that the lot purity is above 99.85%.)	
2-sided CI for True % Purity		*99.82 to 99.99*	

Figure 2. Results of medium level with the Seedcalc8 software (provided in the STATCOM ISTA webpage

Considering each value with a probability higher than 5%, the stability test should give from 0 to 3 positives on the 3 samples tested (Figure 3) and the comparative test should give from 2 to 7 positives on the 11 samples tested (Figure 4).

# of seeds per sample (m)	800	k	Prob (%) of k positive out of n
True contamination rate (π)	0,0600%	0	23,6825%
Total # of samples (n)	3	1	43,7872%
		2	26,9863%
		3	5,5440%
		4	
		5	
		6	
		7	
		8	
		9	
		10	
		11	
		12	
		13	
		14	
		15	

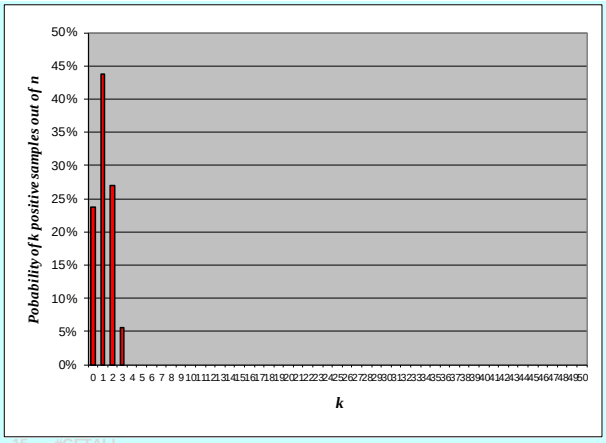


Figure 3. Expected number of contaminated samples for the stability test according to infection rate with the "probability of k positive samples out of n" provided on the SHC ISTA webpage

# of seeds per sample (m)	800	k	Prob (%) of k positive out of n
True contamination rate (π)	0,0600%	0	0,5084%
Total # of samples (n)	11	1	3,4469%
		2	10,6217%
		3	19,6387%
		4	24,2069%
		5	20,8865%
		6	12,8725%
		7	5,6667%
		8	1,7462%
		9	0,3587%
		10	0,0442%
		11	0,0025%
		12	
		13	
		14	
		15	

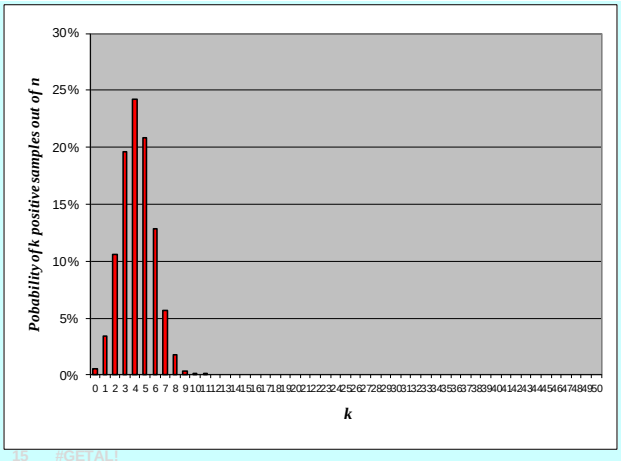


Figure 4. Expected number of contaminated samples for the comparative test according to infection rate with the "probability of k positive samples out of n" provided on the SHC ISTA webpage

The observed one positive sample in the stability test for the medium contaminated seed lot fall within the expected range. In contrast, the eight positive results obtained in the CT are above the expected range of two to seven (Figure 4). However, this difference was found not to be statistically significant when taking into account the overall CT data (see the '*statistical significance of results obtained with medium contaminated seed lot*' part in section 3.5).

Conclusion

Accordance for the high and healthy seed lot is well above 90%. Furthermore, the results for the medium contaminated seed lot was considered appropriate when taking into account the statistical significance of the CT results, see reproducibility section 3.5. Therefore, the repeatability is considered to be fit for purpose.

3.5. Reproducibility

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

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

Experimental approach

Eight laboratories participated in the CT. All performed this method previously but with more or less experience.
They were randomly allocated a number, so that the results remained anonymous. Each participating laboratory received 43 coded samples of 800 seeds each. 39 of them represented 13 repeats of three seed lots (highly infected, medium infected and healthy seed lots). The remaining non-coded seed samples (four) represented two repeats of 800 seeds each of the healthy and artificially contaminated seed lot and served as negative and positive process controls.

Typical or doubtful BFB symptoms on plants were confirmed by the labs using a bioassay. In addition, labs could perform ELISA and PCR for their own purposes.

The final result depended on the biological assay only. If one extract coming from one sweat box gave a positive bioassay, the sample was considered as positive.

Participants reported a qualitative (positive/negative) result for each sample. Inconclusive (no clear typical symptoms in the bioassay) or undetermined (too much growth of damping of causing saprophytes in a sweatbox) results were excluded from the statistical analysis.

See Annex B for the complete inter-laboratory comparative test plan.

The reproducibility (concordance) of the sweat box test was calculated using the method developed by Langton et al. (2002).

Results

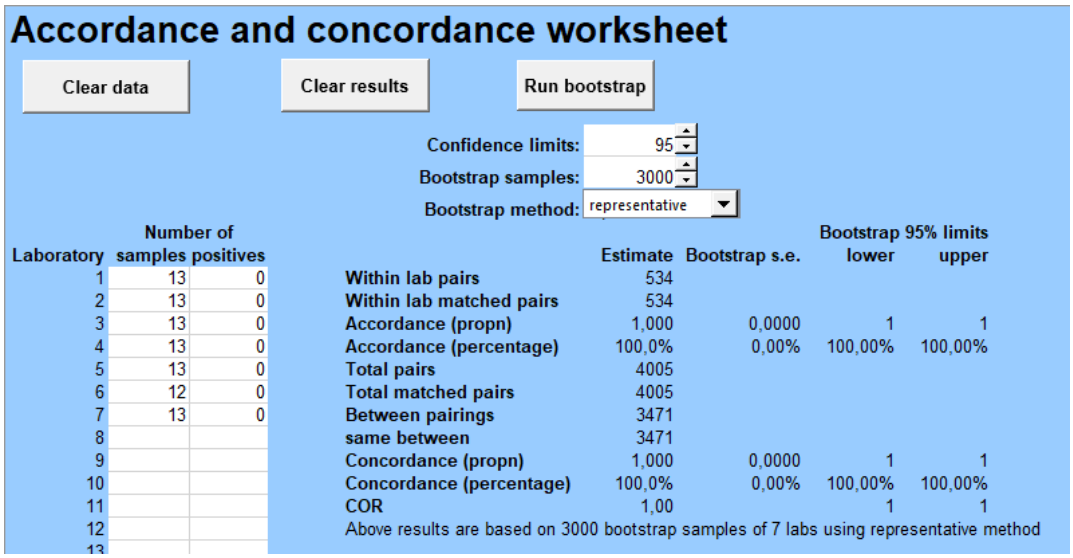
All eight labs performed the sweat box assay but only seven performed the bioassay due to a quarantine issue for one laboratory (number 6). The results from laboratory 6 were therefore excluded from the analysis.

A summary of the results of the healthy and high contaminated lots is provided in Table 5, full data of all laboratories can be found in Annex C.

Table 5. Qualitative results for samples coming from healthy and highly contaminated lots.

Lab number	Number of samples (No. positive/No. tested)	
	Healthy lot	Highly contaminated lot
01	$0^{+}/13$	$12^{+}/13$
02	$0^{+}/13$	$13^{+}/13$
03	$0^{+}/13$	$13^{+}/13$
04	$0^{+}/13$	$13^{+}/13$
05	$0^{+}/13$	$13^{+}/13$
07	$0^{+}/13$	$11^{+}/12$
08	$0^{+}/13$	$13^{+}/13$

A reproducibility (concordance) for the healthy and high contaminated seed lots of 100%, and 95,6% respectively was calculated using the method developed by Langton et al. (2002), see Figure 5 and 6.



Accordance and concordance worksheet

Clear data | Clear results | Run bootstrap

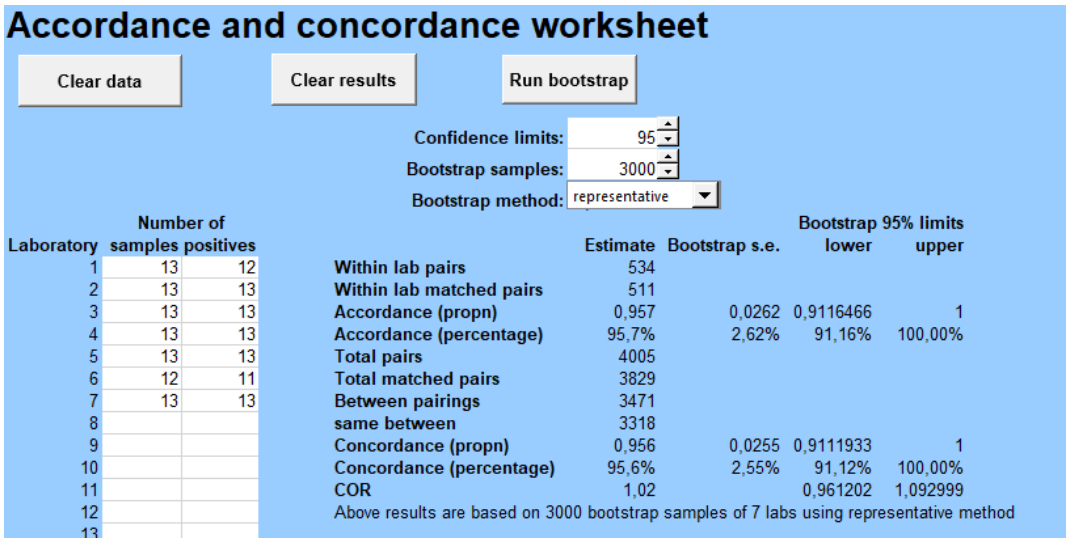
Confidence limits: 95
Bootstrap samples: 3000
Bootstrap method: representative

Laboratory	Number of samples	positives
1	13	0
2	13	0
3	13	0
4	13	0
5	13	0
6	12	0
7	13	0
8		
9		
10		
11		
12		

	Estimate	Bootstrap s.e.	Bootstrap 95% limits lower	upper
Within lab pairs	534			
Within lab matched pairs	534			
Accordance (propn)	1,000	0,0000	1	1
Accordance (percentage)	100,0%	0,00%	100,00%	100,00%
Total pairs	4005			
Total matched pairs	4005			
Between pairings	3471			
same between	3471			
Concordance (propn)	1,000	0,0000	1	1
Concordance (percentage)	100,0%	0,00%	100,00%	100,00%
COR	1,00		1	1

Above results are based on 3000 bootstrap samples of 7 labs using representative method

Figure 5. Concordance of the healthy samples using the Langton et al (2002) method.



Accordance and concordance worksheet

Clear data | Clear results | Run bootstrap

Confidence limits: 95
Bootstrap samples: 3000
Bootstrap method: representative

Laboratory	Number of samples	positives
1	13	12
2	13	13
3	13	13
4	13	13
5	13	13
6	12	11
7	13	13
8		
9		
10		
11		
12		

	Estimate	Bootstrap s.e.	Bootstrap 95% limits lower	upper
Within lab pairs	534			
Within lab matched pairs	511			
Accordance (propn)	0,957	0,0262	0,9116466	1
Accordance (percentage)	95,7%	2,62%	91,16%	100,00%
Total pairs	4005			
Total matched pairs	3829			
Between pairings	3471			
same between	3318			
Concordance (propn)	0,956	0,0255	0,9111933	1
Concordance (percentage)	95,6%	2,55%	91,12%	100,00%
COR	1,02		0,961202	1,092999

Above results are based on 3000 bootstrap samples of 7 labs using representative method

Figure 6. Concordance of the high contaminated samples using the Langton et al (2002) method.

For the medium infected samples, concordance could not be calculated due to the homogeneity test results. Here the expected number of positives samples according to infection rate is calculated per lab with the "probability of k positive samples out of n" (SHC ISTA webpage). The infection rate comes from the homogeneity test results, see section 3.4. The actual detected samples are compared to the calculated probabilities. Considering each value with a probability higher than 5% the labs testing 13 medium contaminated lots should detect between 2 to 8 positives, see Figure 7.

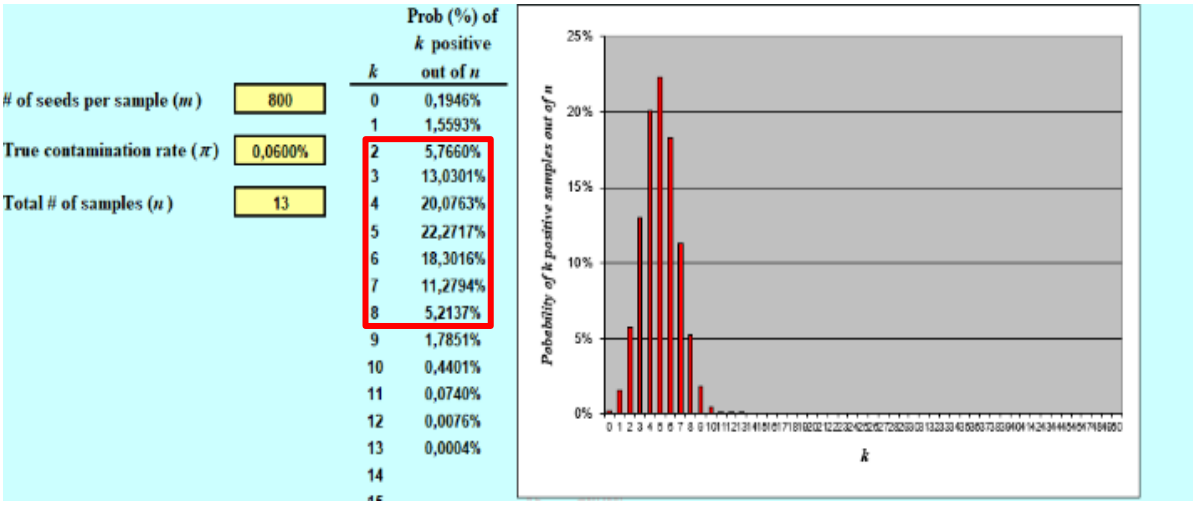


Figure 7. Expected number of contaminated samples for the medium contaminated seed lot according to infection rate calculated for 13 samples.

Similar calculations were performed for the different numbers of medium infected seed samples tested / included in the analysis (due to the exclusion of inconclusive and undetermined results). The actual test results together with the expected number of positives are summarised in Table 6.

Table 6. Analysis of results of laboratories for the medium contaminated samples.

Lab. number	N° of samples tested	N° of positive samples expected[1]	N° of positive samples obtained
01	13	2 to 8	8
02	13	2 to 8	10
03	13	2 to 8	12
04	12	2 to 7	11
05	11	2 to 7	8
07	9	1 to 6	2
08	13	2 to 8	3

[1] calculated with the "probability of k positive samples out of n" provided on the SHC ISTA webpage

Three labs reported positive results for the medium contaminated seed samples which were within the expected range, while four labs reported more positive samples as expected.

Statistical significance of results obtained with medium contaminated seed lot

The calculation of the expected number of positive samples for the medium infection level is based on the results of the homogeneity test where 3 out of 8 samples were found positive. This result gives an estimated infection level of 0.06% based on the Seedcalc8 software (see section 3.4, Figure 2). Using this infection level, the range of expected number of positive samples for the CT has been calculated using the "probability of k positive samples out of n" program.

The observed number of positive samples lies outside the expected range for 4 out of the 7 labs (Table 6), suggesting that the results of the CT do not match the expected range and hence, not the estimated level of infection. To test this hypothesis, the results of the CT were used to estimate the probability of a positive sample (of 800 seeds) and this estimate was then used to calculate the probability of finding 3 positive samples out of 8, the result of the homogeneity test.

The estimate of the probability of finding a positive sample using all laboratories is 0.643 (54 positive samples out of 84 samples tested). Using this estimate, the probability of the result of the homogeneity test was calculated using a binomial distribution. This probability was found to be 0.084 (8.4%). As this probability is higher than 5%, a frequently used significance level, it is considered to be not significantly different from the expected results based on the CT test results.

In addition, the infection level has been estimated using the CT data as input to the Seedcalc8 program. The estimated level using all laboratories was found to be 0.13%, which is well within the 95% confidence interval for the 0.06% infection level calculated for the homogeneity test which ranged from 0.01 to 0.18% (Figure 2).

In conclusion, the results of the CT were found to be not statistically different from those obtained for the homogeneity test.

Conclusion

With a concordance of 100% for the healthy seed lots and 95.6% for the high contaminated seed lots the reproducibility requirements are met for the healthy and high contaminated seed lots.

For the medium contaminated samples, four out of seven labs detected more positives samples of the medium contaminated seed lot as expected. However, these results were considered appropriate when taking into account their statistical significance and the confidence intervals of the expected ranges. Therefore, the reproducibility is considered to be fit for purpose.

3.6. Diagnostic performance

Definition ISHI-Veg guidelines: *The ability of the method to detect target pathogens in known infected seed samples while excluding non-target organisms in known healthy seed samples.*

The diagnostic performance requirements will be met when diagnostic sensitivity and specificity are > 95%.

Experimental approach

The diagnostic performance was calculated based on the CT data, see Annex B for the CT plan.

Analysis of results was carried out according to the Norm NF EN ISO 16140 suitable to results expressed as positive / negative. Inconclusive (no clear typical symptoms in the bioassay) or undetermined (too much growth of damping of causing saprophytes in a sweatbox) results were excluded from the statistical analysis. The final result depended on the biological assay only. If one extract coming from one sweat box gave a positive bioassay, the sample was considered as positive.

Inclusivity (diagnostic sensitivity), exclusivity (diagnostic specificity) and accuracy of the assay were calculated according to the following mathematical formulas:

Inclusivity = ΣPA / (ΣPA+ΣND) x 100

Exclusivity = ΣNA / (ΣNA+ΣPD) x 100

Accuracy = (ΣNA+ΣPA) / (ΣPA+ΣNA+ΣPD+ΣND) x 100

PA = positive agreement, ND = negative deviation, NA = negative agreement and PD = positive deviation.

Results

All eight labs performed the sweat box assay but only seven performed the bioassay due to a quarantine issue for one lab. The results from the lab that did not perform the bioassay were excluded from the analysis.

Analysis of the CT results for the healthy and highly contaminated lots from the seven labs combined are presented in Table 7. All CT data can be found in Annex C.

Table 7. Analysis of qualitative results for the healthy and highly contaminated seed lots

	Expected + result	Expected - result	Inclusivity = Diagnostic Sensitivity	Exclusivity = Diagnostic Specificity	Accuracy
Obtained + result	88 (PA)	0 (PD)	97.78%	100.00%	98.90%
Obtained - result	2 (ND)	91 (NA)			
Total	90	91			

The diagnostic sensitivity, specificity and accuracy for the healthy and highly contaminated seed lots are > 95% (Table 7).

For the medium infected samples, the diagnostic sensitivity and specificity cannot be calculated due to the homogeneity test results. Here the expected number of positives samples according to infection rate is calculated with the "probability of k positive samples out of n" (SHC ISTA webpage). The infection rate comes from the homogeneity test results, see section 3.4. The actual detected samples are compared to the calculated probabilities. Analysis of the results for the medium contaminated lots are presented in Table 8.

Table 8. Analysis of qualitative results for the medium contaminated seed lots

N° of samples tested	N° of positive samples expected[1]	N° of positive samples obtained
84	28-36	54

[1] calculated with the "probability of k positive samples out of n" provided on the SHC ISTA webpage

Considering each value with a probability higher than 5%, the CT should give from 28 to 36 positives for the medium contaminated seed lot tested. The 54 positive results from the 84 medium contaminated seed lots included in the CT, fall outside the expected range. This correlates with the repeatability and reproducibility results (section 3.4 and 3.5 respectively), which showed that four labs detected more positive samples from the medium contaminated seed lot as expected. However, as shown in the '*statistical significance of results obtained with medium contaminated seed lot*' part in the reproducibility section 3.5, this was found not to be statistically significant.

Conclusion

The diagnostic sensitivity (percentage of samples correctly identified as positives) and the diagnostic specificity (percentage of samples correctly identified as being negative) of the method for the healthy and highly contaminated seed lots are above 95%. The method, therefore, confirmed healthy samples and detected highly contaminated ones.

The results observed for the medium contaminated seed samples fell outside the expected range of detection. However, although a higher number of positives than expected were observed, the difference was found not to be statistically significant.

The diagnostic performance is therefore considered to be fit for purpose.

4. CONCLUSION

The performance criteria measured during method validation confirm that the sweat box grow-out method for the detection of *A. citrulli* from melon seeds is suitable to detect contaminated seed lots with viable and infectious *A. citrulli* bacteria in melon.

All labs found the healthy lot to be healthy and the two contaminated lots (highly and medium infection) to be positive for *A. citrulli*. The 13 repetitions of 800 seeds gave around 10,000 seeds tested, the recommended sample size to detect *A. citrulli* on melon seeds.

The grow-out in sweat boxes is a good alternative to the grow-out in a greenhouse because it is faster, less expensive and uses less space while giving less climate variation risks because of the fixed environment used in growth chamber.

Training on the method before routine use is recommended.

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6. ANNEXES

Annex A: Protocol for the detection of *Acidovorax citrulli* associated with melon seeds by grow-out in sweat boxes

MATERIALS

- Ac isolate to serve as positive control for PCR/ELISA confirmation provided by the Test Organizer
- Plastic sweat box (35 x 25 x 20 cm ± 5 cm difference) with a lid (e.g. Rotho: Ref 4045, 10 L or equivalent)
- 70% alcohol
- Thiram fungicide solution

  Recipe:
  Prepare extemporaneously a Thiram solution for irrigation; 40 mg of Thiram per L water or 48 mg of Thiram (commercial product of 80 %) per L (treating the seed to replace irrigation solution by water is possible; 1.4 g of Thiram per kg seeds)

- Growth chamber
- 0.85% sterile saline
  Preparation:
  Saline should be autoclaved at 121°C, 115 psi for 15 min.

Compound	g/L
Sodium chloride (NaCl)	8.5
De-ionised water to a final volume of	1000 mL

- Small grinding plastic bags and a press grinder (or equivalent)
- De-ionized water
- Sterile micro tubes (1.5 mL; 0.2 mL)
- Laminar airflow cabinet
- Microliter pipettes (e.g. Gilson or Finn) with sterile filtered tips (1 µL – 1 000 µL)
- Filter plastic bags

1. SWEAT BOX GROW-OUT

1.1. Clean, disinfect (with 70% alcohol) and label the plastic sweat boxes with the appropriate code number.

1.2. Add 1000 mL of potting soil to each sweat box.

1.3. Spread 800 seeds of each subsample evenly in the sweat boxes.

1.4. Add 2 L of clean and fresh vermiculite in the sweat boxes carefully and spread evenly.

1.5. Add 1 L of the irrigation solution of Thiram into each sweat box

1.6. Close the sweat boxes and incubate at 25 – 28 °C with a minimum of 14 hour light per day.

NOTE: These are the regulation temperatures. A comparison made earlier showed no difference in the expression of disease symptoms between 25°C and 28°C in the box.

1.7. Fourteen (14) days after sowing, inspect seedlings for typical disease symptoms (water soaked lesions on cotyledons and leaves, see Figure A1: suspect seedlings) or showing necrosis (doubtful symptoms).

Figure A1. Symptoms of *Acidovorax citrulli* on leaves of melon seedlings.

1.8. Pick and place suspect seedlings (cotyledons only and not the stems or roots) in plastic bags. Each plastic bag should contain no more than 5 seedlings. Seedlings with doubtful symptoms must be treated in the same manner. Do not mix suspect and doubtful seedlings in a bag. Pick no more than a maximum of 20 (to form a maximum of 4 pools) suspect and doubtful seedlings per sweat box favouring suspect ones.

For example:

If in a sweat box there are 15 suspect and 7 doubtful seedlings, they would make

- 3 pools of 5 suspect seedlings + 1 pool of 5 doubtful seedlings

If in a sweat box there are 24 suspect and 15 doubtful seedlings, they would make

- 4 pools of 5 suspect seedlings

If in a sweat box there are 13 suspect and 3 doubtful seedlings, they would make

- 2 pools of 5 suspect, 1 pool of 3 suspect, 1 pool of 3 doubtful seedlings

1.9. Take only 2 plastic bags of 5 plants each for the negative and positive controls (suspect seedlings) from the healthy and highly contaminated seed lots.

1.10. *Optional*: Take one plastic bag of 5 plants from the healthy seed lot which will be used for the PCR spike positive control

1.11. Add 5 mL of 0.85% sterile saline to each plastic bag containing the infected cotyledons from the test and control samples. Grind them with a press grinder or equivalent.

1.12. Proceed with the bioassay for confirmation.

NOTE: Samples can be stored at 4 °C for a maximum of 48 hours before the confirmation steps.

2. CONFIRMATION STEPS

2.1. PCR (OPTIONAL)

2.1.1. Transfer 1mL of the cotyledon extract to an Eppendorf tube (keep those at -20 °C or proceed directly with the DNA extraction).

2.1.2. In a separate Eppendorf tube transfer 1 mL of 0.85% sterile saline to use as extraction control.

2.1.3. Perform the DNA extraction by NaOH: Centrifuge the extracts including the process control for 5 min at 6000–10 000 g. Discard the supernatant and re-suspend the pellet with 200 µL 0.5N NaOH. Incubate for 10 min at 65 °C by shaking at 1 000 rpm. Dilute 5 µL of solution into 495 µL of 20 mM Tris-HCl, pH 8 and vortex. Suspensions can be stored at –20 °C until identification.

2.1.4. Add a PCR positive control (DNA extract of *Acidovorax citrulli* strain).
Add a PCR negative control (deionized water).
Add a positive process control: spike a non-symptomatic cotyledon extract with an *Acidovorax citrulli* positive control isolate at an approximate concentration of 10^6 CFU/mL.

2.1.5. Use the primer sets Contig 21 (Table A1) and Zup (Table A2)

Table A1. Contig 21 primers and probe sequences.

Name	Target	Label	Sequence 5'→ 3'
Ac F1	Ac	-	ACC gAA CAg AgA gTA ATT CTC AAA gAC
Ac R1	Ac	-	gAg CgT gAT ggC CAA TgC
Ac P1	Ac	6FAM	CAT CgC TTg AgC AA

Table A2. Zup primers and probe sequences.

Name	Target	Label	Sequence 5'→ 3'
2549	Ac	-	gAg TCT CAC gAg gTT
2550	Ac	-	gAC CCT ACg AAA gCT CAg
2551	Ac	6FAM	TgC AgC CCT TCA TTg ACg g

2.1.6. Prepare the master mixes (Table A3 and A4).

Table A3. Contig 21 master mix.

Products	Volume (µl) per well	Final concentration
water	6.988	
F contig 21 (100 µmol/L)	0.225	0.9 µmol/L
R contig 21 (100 µmol/L)	0.225	0.9 µmol/L
Master Mix (2x)	12.5	1x
Probe contig 21 (100 µmol/L)	0.062	0.25 µmol/L
Volume mix (µL)	**20**	
DNA extract	5	
Total volume (µL)	**25**	

Table A4. ZUP master mix.

Products	Volume (µl) per well	Final concentration
water	6.25	
Oligo Zup 2549 (20 µmol/L)	0.5	0.4 µmol/L
Oligo Zup 2550 (20 µmol/L)	0.5	0.4 µmol/L
Master Mix (2x)	12.5	1x
probe Zup 2551 (20 µmol/L)	0.25	0.2 µmol/L
Volume mix (µL)	**20**	
DNA extract	5	
Total volume (µL)	**25**	

2.1.7. Run the qPCR at the conditions listed in Table A5 and A6.

NOTE: A DNA extract from a pool is considered as positive when the PCR result gives a Cq value ⩽ 35 for at least one of the two primer sets.

Table A5. PCR program contig 21

program			
1	95 °C	10'	
2	95 °C	15"	40 cycles
3	60 °C	1' + plate read	
4	40 °C	10"	

Table A6. PCR program Zup.

program			
1	95 °C	10'	
2	95 °C	15"	40 cycles
3	58 °C	45'' + plate read	

2.2. ELISA (OPTIONAL)

2.2.1. The ELISA Agdia kit is recommended: Agdia SRA14800.

2.2.2. Transfer 1 mL of the cotyledon extract in 4 mL of the ELISA extraction buffer.

2.2.3. Follow the serum provider protocol.

2.2.4. Add controls from the antiserum provider.

2.2.5. Use the “Best Practices for ELISA assays in Seed Health tests” from ISHI to define the positive wells

2.3. BIOASSAY

2.3.1. Sow seeds from the healthy lot 7 days after the sowing date of the sweat box assay and incubate at 23 °C with 16-20 hours light.

2.3.2. Sow ten (10) seeds per pot and keep only five (5) seedlings that are at the right physiological stage for the biological assay. For the assay at least 150 pots of 5 plants at the right physiological stage are required.

NOTES: The right physiological age of the seedlings is important; keep only those seedlings where just the tip of the developing first true leave can be observed (7 to 10 days, depending on the speed of development, see Figure A2.)

In case the plants are not at the right physiological age for the biological assay keep the extracts in the refrigerator at 4°C but no longer than 2 days.

Figure A2. Five healthy melon seedlings at the optimum stage for a biological assay.

2.3.3. Place a droplet of 10 µL from each sample extract between the cotyledon and the stem of five (5) seedlings in the same pot. Stab the spot on the seedlings where the droplets have been placed with a toothpick. Label the pot with the sample-extract number.

2.3.4. Follow the same procedure to inoculate five (5) seedlings with saline solution and five (5) more with 10^5-10^8 CFU of the reference *A. citrulli* strain. These will serve as negative and positive control plants, respectively.

2.3.5. Place the pots with inoculated seedlings in trays and put the trays in a bigger container / sweat box with some water on the bottom and close firmly with a lid. Place the containers / sweat boxes in the climate chamber at 28 °C; with 16 hours light, 8 hours dark and high humidity.

2.3.6. Evaluate the plants after 7 days. Compare the symptoms of the test plants to the positive and negative control plants (see Figure A3). A biological assay is considered as positive if at least one plantlet gives symptoms as the positive control.

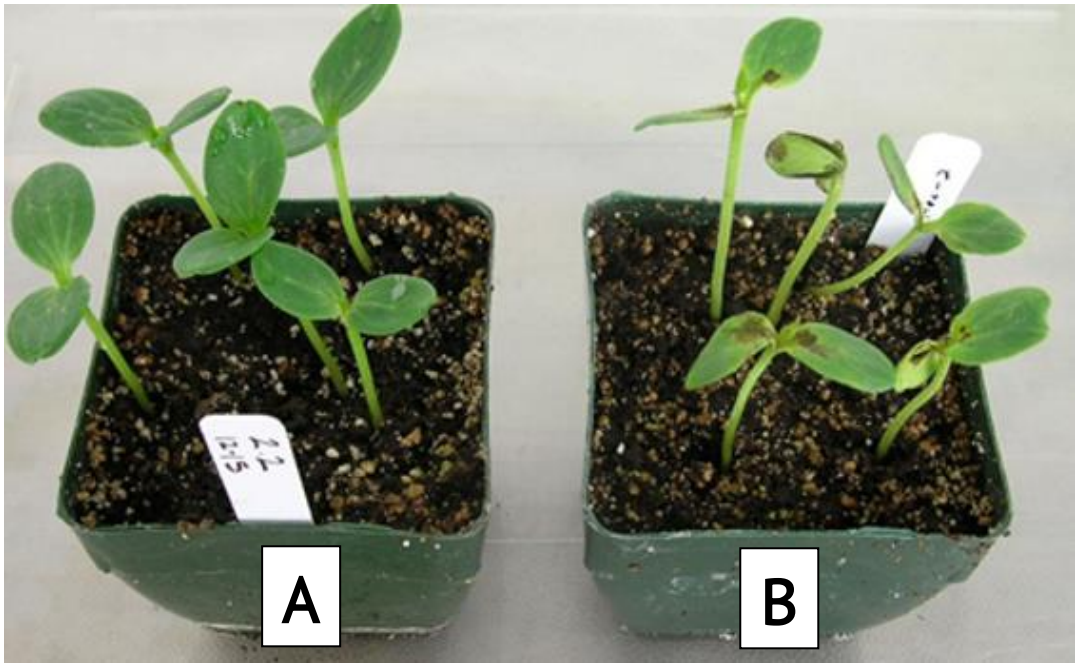

Figure A3. (A) Negative control plants at 7 days post inoculation with sterile saline. No developed symptoms. (B) Positive control plants at 7 days post inoculation. Development of clear symptoms.

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Annex B: Test plan for the detection and confirmation of *Acidovorax citrulli* in melon seeds

1. Organisation and design

1.1 Test Organiser

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

1.2 Pathogen

Acidovorax citrulli

1.3 Crops

Cucumis melo (melon)

1.4 Participating laboratories and contact persons

Laboratories	Contact persons
HM-Clause, USA	Geeta Sanjeev
Syngenta, NL	Bert Woudt
NAKT, NL	Harrie Koenraadt
BAYER, NL	Bart Geraats
Rijk Zwaan, NL	Marjolein Spiekerman
Hazera, IL	Smadar Kleiman
GEVES-SNES, FR	Valérie Grimault
HM-Clause, FR	Hubert Lybeert

Criteria required: Experienced laboratory on sweat box, ELISA and PCR testing

2. Introduction and objective of the method

2.1 Background

Acidovorax citrulli, (formerly *A. avenae* subsp. *citrulli*) (Ac) (Willems *et al.*, 1992) is a Gram-negative, obligately aerobic, and motile with a single polar flagellum, biotrophic bacterium that causes seedling blight and bacterial fruit blotch (BFB) of melon and watermelon.

The bacterium was originally isolated from water-soaked lesions on cotyledons of watermelon seedlings from accessions in the USA plant introduction (PIs) collection (Webb and Goth, 1965). It was phenotypically similar to *Pseudomonas pseudoalcaligenes* but differed in that it was pathogenic to watermelon, cantaloupe, cucumber and squash (Schaad *et al.*, 1978). Therefore, this new bacterium was named *P. pseudoalcaligenes* subsp. *citrulli*. Hu *et al.* (1991) found a close similarity between this bacterium and *Pseudomonas avenae* and was renamed *P. avenae* subsp. *citrulli*. The watermelon bacterium and other subspecies of *P. avenae* constitute a separate rRNA branch within the family *Comamonadaceae*. Comparing them phenotypically, Willems *et al.* (1992) found the members of this rRNA branch to be most closely related to the genus *Acidovorax*

and the watermelon fruit blotch bacterium was renamed *Acidovorax avenae* subsp. *citrulli*. The current preferred name for this pathogen is *Acidovorax citrulli*.

BFB is a sporadic disease but under favourable environment, it becomes devastating and may cause 100% loss of marketable fruit. Infected seeds and seedlings are the most important primary sources of inoculum in commercial fruit production field. However, there may be other endemic sources of inoculum like debris from infected fruit or foliage tissue, volunteer seedlings, or cucurbitaceous weeds (Black *et al.*, 1994; Latin and Hopkins, 1995; Isakeit *et al.*, 1998). In the field, BFB development is heavily dependent on rainfall and relative humidity. Secondary dispersal of *A. citrulli* is by wind-driven rain or over-head irrigation. When *A. citrulli* lands on healthy leaves, it migrates through open stomata into the sub-stomatal intercellular spaces where it multiplies and induces water-soaked lesions.

The recommended test to detect *A. citrulli* is a seedling grow-out test conducted under favourable conditions for symptom development. The watermelon seed industry currently tests seed lots in a grow-out of 10,000 to 50,000 seedlings per seed lot. Seeds are planted in a sterile potting mix in a greenhouse free from other sources of *A. citrulli*, usually a greenhouse dedicated to seed testing. Seedlings are watered by overhead irrigation to promote foci of symptomatic plants that are easily visible. Relative humidity in the greenhouse is maintained above 55% and temperature is maintained between 24 and 38°C (Hopkins, 1994; Latin *et al.*, 1995). After 16-21 days, each seedling is carefully inspected for symptoms. Isolations are made from seedlings showing any symptoms of disease and *A. citrulli* is identified using biochemical, DNA and biological assay. This method takes 30-35 days to complete. There is zero tolerance for contaminated seed and any infested seed lot is rejected.

Elaborate precautions must be taken to ensure that cross-contamination of seedlings does not occur in test procedures. A detailed step-by-step seedling grow-out method that describes such precautions and is standardized by the USA National Seed Health System (NSHS) can be accessed at the website www.seedhealth.org.

2.2 Objective

The objective of this comparative test is to develop an internationally accepted method for the detection of *Acidovorax citrulli* on melon seeds using a grow-out test in sweat boxes and confirmation by a bioassay. Labs may use confirmation step using ELISA and PCR in addition to the biological assay.

3. 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 used to evaluate the diagnostic sensitivity, diagnostic specificity and accuracy performance criteria of the presented method.

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

4. Materials and methods

This comparative test will be performed only on melon seeds due to the unavailability of infected watermelon seed. As watermelon is susceptible to the bacterium and growing conditions of the two crops are similar the test can be expected to be suitable for watermelon. However, it is the user's responsibility to validate the results obtained in this study prior to the test being used for watermelon seed.

4.1 Seed subsamples and samples

Seed lots used in the inter-laboratory comparative test (CT) will be characterized as per the recommendation made by ISTA (2012) in its guidelines for organizing and analysing proficiency and comparative tests. A homogeneity and stability test will be also performed by the Test Organizer before and after the CT. Eight (8) subsamples of 800 seeds will be tested in the homogeneity test and 3 subsamples of 800 seeds for the stability test.

Each participating laboratory will receive 43 coded subsamples samples of 800 seeds. 39 of them will represent 13 repeats of 3 seed lots (highly infected, moderately infected and healthy seed lots). The remaining non-coded seed subsamples (4) will represent 2 repeats of 800 seeds each of the healthy and high artificially contaminated seed lot and will serve as negative and positive reference controls. The contamination level of seed lots will be determined by the Test Organizer.

4.2 Sweat box grow-out protocol

See Annex A.

5. Results

In the excel data record sheet provided:

- Indicate the level of saprophytic growth developed in each sweat box by following a 0 to 3 scale with 0 = no growth and 3 = abundant growth.
- Indicate the results of the different confirmation tests per pool (+/-)
- Indicate the seed subsamples that are found to be positive upon confirmation by writing (+) or (-) on "Final score" column. A seed subsample is considered positive if at least one sample containing cotyledons is found positive by the bioassay.

6. Expected time of work for each participating laboratory

The time needed to perform the test depends on the experience of participating laboratories and the number of people working. An estimation of the time per day is given in the table below.

Day	Action	Time needed
1	Prepare boxes and sow the 43 subsamples	6 h
7	Sow the seeds for biological assay	2 h
14	Reading time and sampling diseased plants	13 h (total, i.e. 6.5 h for two persons)
15	Run bioassay	4 h
15	Run ELISA test	5 h
15	Run PCR test	5 h
22	Reading and recording results of bioassays	2 h

7. Safety precautions

Thiram

Wear a mask, gloves and lab coat when weighing the amount of the fungicide Thiram needed per sample. Thiram is harmful if inhaled or swallowed. It is irritating to eyes and skin. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

It is very toxic to aquatic organisms and may cause long term adverse effects in the aquatic environment. Thiram and its container must be disposed of as hazardous waste. Avoid release to the environment. Refer to special instructions/safety data sheets (http://nj.gov/health/eoh/rtkweb/documents/fs/1854.pdf).

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Annex C. Data from the inter-laboratory comparative test

		Homogeneity test lab	Laboratory			
			1	2	3	4
Test performance date		10/08/16	31/10/16	15/11/16	16/11/16	18/11/16
Healthy seed lot	1		Negative	Negative	Negative	Negative
	2		Negative	Negative	Negative	Negative
	3		Negative	Negative	Negative	Negative
	4		Negative	Negative	Negative	Negative
	5		Negative	Negative	Negative	Negative
	6		Negative	Negative	Negative	Negative
	7		Negative	Negative	Negative	Negative
	8		Negative	Negative	Negative	Negative
	9		Negative	Negative	Negative	Negative
	10		Negative	Negative	Negative	Negative
	11		Negative	Negative	Negative	Negative
	12		Negative	Negative	Negative	Negative
	13		Negative	Negative	Negative	Negative
	Total	0+/8	0+/13	0+/13	0+/13	0+/13
Medium contaminated seed lot	1		Negative	Positive	Positive	Negative
	2		Negative	Positive	Positive	Positive
	3		Negative	Positive	Positive	Positive
	4		Negative	Positive	Positive	Positive
	5		Positive	Positive	Positive	Positive
	6		Positive	Negative	Positive	Positive
	7		Positive	Positive	Positive	Positive
	8		Positive	Negative	Positive	Positive
	9		Positive	Positive	Negative	Positive
	10		Positive	Positive	Positive	Positive
	11		Positive	Negative	Positive	Positive
	12		Positive	Positive	Positive	Undetermined
	13		Negative	Positive	Positive	Positive
	Total	3+/8	8+/13	10+/13	12+/13	11+/12
Heavy contaminated seed lot	1		Negative	Positive	Positive	Positive
	2		Positive	Positive	Positive	Positive
	3		Positive	Positive	Positive	Positive
	4		Positive	Positive	Positive	Positive
	5		Positive	Positive	Positive	Positive
	6		Positive	Positive	Positive	Positive
	7		Positive	Positive	Positive	Positive
	8		Positive	Positive	Positive	Positive
	9		Positive	Positive	Positive	Positive
	10		Positive	Positive	Positive	Positive
	11		Positive	Positive	Positive	Positive
	12		Positive	Positive	Positive	Positive
	13		Positive	Positive	Positive	Positive
	Total	8+/8	12+/13	13+/13	13+/13	13+/13

Contd.

Annex C continued

		Laboratory				Stability test lab
		5	6	7	8	
Test performance date		28/11/16	8/11/16 10/11/16	15/11/16	11/09/16	13/02/17
Healthy seed lot	1	Negative	Negative	Negative	Negative	
	2	Negative	Negative	Negative	Negative	
	3	Negative	Negative	Negative	Negative	
	4	Negative	Negative	Negative	Negative	
	5	Negative	Negative	Negative	Negative	
	6	Negative	Negative	Negative	Negative	
	7	Negative	Negative	Negative	Negative	
	8	Negative	Negative	Negative	Negative	
	9	Negative	Negative	Negative	Negative	
	10	Negative	Negative	Negative	Negative	
	11	Negative	Negative	Negative	Negative	
	12	Negative	Negative	Negative	Negative	
	13	Negative	Negative	Negative	Negative	
	Total	0+/13	0+/13	0+/13	0+/13	0+/3
Medium contaminated seed lot	1	Negative	Positive	Undetermined	Negative	
	2	Positive	Positive	Undetermined	Negative	
	3	Positive	Positive	Undetermined	Negative	
	4	Inconclusive	Positive	Negative	Positive	
	5	Positive	Positive	Negative	Negative	
	6	Negative	Positive	Negative	Positive	
	7	Inconclusive	Positive	Positive	Negative	
	8	Positive	Positive	Negative	Negative	
	9	Positive	Positive	Negative	Negative	
	10	Positive	Positive	Positive	Negative	
	11	Negative	Positive	Inconclusive	Positive	
	12	Positive	Positive	Negative	Negative	
	13	Positive	Positive	Negative	Negative	
	Total	8+/11	13+/13	2+/9	3+/13	1+/3
Heavy contaminated seed lot	1	Positive	Positive	Negative	Positive	
	2	Positive	Positive	Inconclusive	Positive	
	3	Positive	Positive	Positive	Positive	
	4	Positive	Positive	Positive	Positive	
	5	Positive	Positive	Positive	Positive	
	6	Positive	Positive	Positive	Positive	
	7	Positive	Positive	Positive	Positive	
	8	Positive	Positive	Positive	Positive	
	9	Positive	Positive	Positive	Positive	
	10	Positive	Positive	Positive	Positive	
	11	Positive	Positive	Positive	Positive	
	12	Positive	Positive	Positive	Positive	
	13	Positive	Positive	Positive	Positive	
	Total	13+/13	13+/13	11+/12	13+/13	3+/3