Interlaboratory PT detection of *Botrytis cinerea* on *Helianthus annuus* PT SH7-003

ISTA Seed Health Committee

Interlaboratory Proficiency Test
Detection of *Botrytis cinerea* on *Helianthus annuus*
PT SH7-003

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INTRODUCTION

The aim of this Proficiency Test is to verify the ability of laboratories to detect *Botrytis cinerea* on *Helianthus annuus* seeds by ISTA Seed Health Method 2014 7-003.

At first, 24 laboratories registered to participate in the proficiency test. 11 of them were accredited for Method 7-003 and 13 were not accredited for this method. 3 laboratories did not receive samples due to problems related to official documents requested and one laboratory did not return results due to experimental problems.

PT organization

- **Pretest**

A healthy lot has been selected and because it had not been possible to find naturally contaminated seeds an artificial contamination has been done to obtain 3 levels. 2 samples of 400 seeds from each level have been tested by ISTA method 7-003 to check the percentage of contamination.

- Healthy (lot A) = 0% of *Botrytis cinerea*
- Low level (lot B) = 5% of *Botrytis cinerea*
- Medium level (lot C) = 10% of *Botrytis cinerea*

- **Homogeneity Test**

After sampling and packaging, 10 extra samples of 400 seeds have been tested from each lot, by the ISTA method 7-003 to check homogeneity.

⇨ **Results of healthy lot (A)**

The 10 extra samples obtained 0% of contaminated seeds, there were no positive.

⇨ **Results low level (lot B)**

Results of the ten extra samples for lot B are given in table 1. The lot B was homogenous.

Table 1: Homogeneity Test results (lot B).

Homogeneity Test - seed health											
Sample size	400										
Subsample	1	2	3	4	5	6	7	8	9	10	
Nb infected seeds	8.06	10	4	12	8.02	8	11.1	6	5.01	8.04	Mean
Percentage of non infected seed	97.985	97.5	99	97	97.995	98	97.236	98.5	98.747	97.99	98
Sample mean					98						
Tolerance					1.41						
H value					0.16						
Homogeneity check					OK						

⇨ **Results medium level (lot C)**

Results of the ten extra samples for lot C are given in table 2. The lot C was homogenous.

Table 2: Homogeneity Test results (lot C).

Homogeneity Test - seed health											
Sample size	400										
Subsample	1	2	3	4	5	6	7	8	9	10	
Nb infected seeds	19	12	12	12	13	12	18	12	20	19	Mean
Percentage of non infected seed	95.25	97	97	97	96.73	96.99	95.49	97	95	95.25	96.27
Sample mean					96.27						
Tolerance					1.41						
H value					1.29						
Homogeneity check					OK						

⇨ **Conclusion of Homogeneity Test**

The homogeneity of the lots has been confirmed:

- For lot A, we obtained 0% of *Botrytis cinerea*

- For lot B, we obtained a mean of 2% of *Botrytis cinerea* corresponding to a mean of 98 % of non contaminated seeds which is lower than in pre test (5%) but not unusual.

- For lot C, we obtained a mean of 3.73 % of *Botrytis cinerea* corresponding to a mean on 96.27% of non contaminated seeds which is lower than in pre test (10%), but not unusual.

- **Stability Test**

3 extra samples of 400 seeds have been kept at 4°C and have been tested from each lot by ISTA method 7-003, after reception of all laboratories proficiency test results, to check the stability of contamination level.

⇨ **Results of healthy lot (A)**

All samples obtained 0% of contaminated seeds, there were no positive (Table 3).

⇨ **Results of low level (lot B) and medium level (lot C)**

Results are given in the Table 3.

Table 3: Stability test results (mean on 3 samples of 400 seeds).

Lot A		Lot B		Lot C	
Nb of contaminated seeds	% of contaminated seeds	Nb of contaminated seeds	% of contaminated seeds	Nb of contaminated seeds	% of contaminated seeds
0	0 %	6	1.5%	13.67	3.42%

⇨ **Conclusion of the stability test**

Stability of the lots has been confirmed:

- For the lot A, we obtained 0% of *Botrytis cinerea* which is the same result than homogeneity test.

- For the lot B, we obtained a mean of 1.5% of *Botrytis cinerea*, which is slightly lower but close to homogeneity test (2%).

- For lot C, we obtained a mean of 3.4% of *Botrytis cinerea*, which is slightly lower but close to homogeneity test (3.73%).

- **Seed samples**

Each lab received a total of 9 coded samples, corresponding to 3 replicates of each contamination level. Each sample contained 400 seeds.

Samples have been tested to detect *Botrytis cinerea*, according to the ISTA method 7-003.

- **Notation of results**

All results have been recorded as number of *Botrytis cinerea* contaminated seeds by plate in the notation sheets provided. The calculation of % contaminated seeds has been calculated automatically.

- **Statistical analysis**

Homogeneity test and stability test have been analyzed with GEVES statistical software (based on H test) published on the Seed Health Committee's home page

Statistical analysis of results has been realized with the Hampel's method (Hampel *et al*, 1986) to determine outliers.

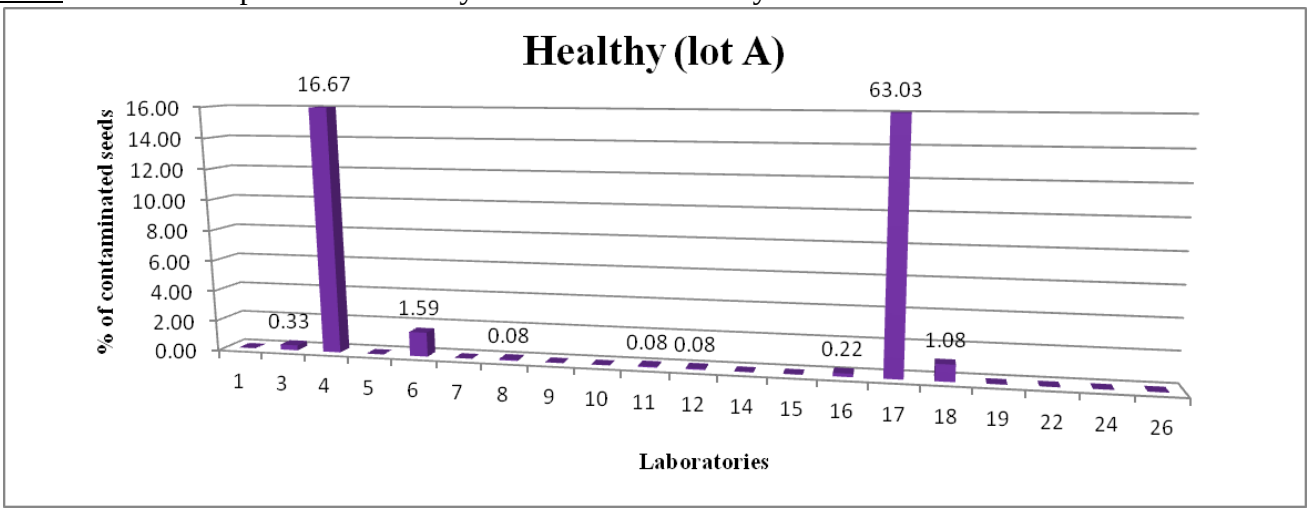
PROFICIENCY TEST RESULTS

Accredited and non accredited participants were analyzed together. Rough data of all laboratories are given in appendix (Table 4).

- **Results of healthy lot (A)**

Mean of the 3 replicates for laboratories are given in figure 1.

Figure 1: mean of 3 replicates of healthy lot for each laboratory.



Several laboratories obtained positive results for healthy samples.
As no contamination was observed during pre-test, homogeneity and stability tests, a low contamination of the healthy lot can be excluded. So, contaminations observed by the laboratories are considered as false positives.
Different percentages were obtained:
Low percentages (3 for the method accredited laboratories and 1 non accredited laboratory)

- 0.08% corresponding to 1 contaminated seed in 1200 seeds
- 0.22% corresponding to 2 contaminated seed in 1200 seeds

⇨ In these cases of low percentage of detection, it could be due to cross contamination during manipulations.

High percentages (5 non accredited laboratories)

- 0.33% corresponding to 4 contaminated seed in 1200 seeds
- 1.59% corresponding to 19 contaminated seed in 1200 seeds
- 16.67% corresponding to 200 contaminated seed in 1200 seeds (lab4)
- 63.03% corresponding to 753 contaminated seed in 1200 seeds (lab17)

⇨ In these cases of high percentage of detection, it could be explained by confusion between saprophytic fungi like *Cladosporium* sp. and *Botrytis cinerea*. The fungi are shown in picture A and B in appendix.

- **Results of low level (lot B)**

Results' analysis of lot B is based on median value according to the Hampel's method.

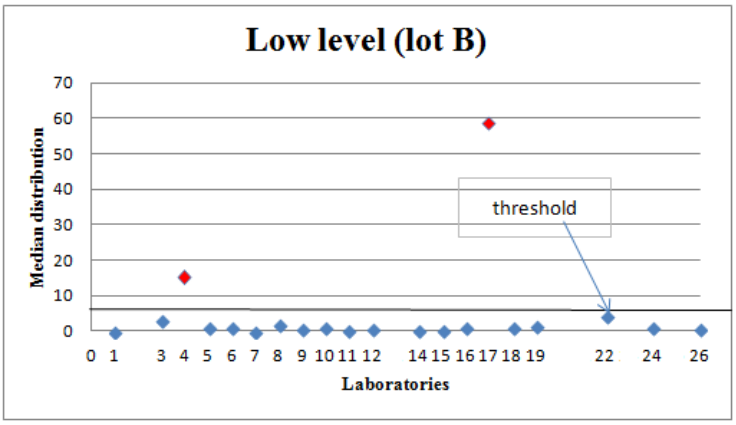
Results distribution of all laboratories and outliers detection is given in table 5 and figure 2. Outliers correspond to red diamond-shape in figure 2. Two laboratories were identified as outliers, laboratories 4 and 17. These laboratories are not accredited for the Method 7-003 and participate as volunteers.

Table 5: Results analysis according to the Hampel's method for low level (lot B).

Low level			
Lab	Lab Values (Xi)	| Xi - M|	Status
1	2,17	0,042	OK
3	5,01	2,888	OK
4	17,17	15,042	Outlier
5	1,17	0,952	OK
6	3,08	0,958	OK
7	2,08	0,042	OK
8	0,08	2,042	OK
9	1,67	0,457	OK
10	1,09	1,035	OK
11	1,83	0,292	OK
12	1,58	0,542	OK
14	2,18	0,051	OK
15	1,83	0,290	OK
16	3,17	1,042	OK
17	60,72	58,599	Outlier
18	3,17	1,042	OK
19	0,83	1,292	OK
22	6,23	4,104	OK
24	1,25	0,875	OK
26	2,75	0,625	OK

Median (M):	**2,125**
MAD:	**0,955**
5.2 X MAD	**4,968**

Figure 2: Results distribution for low level for all laboratories. Outliers are in red diamond-shape.



- **Results of medium level (lot C)**

Results analysis of lot C is based on median value according to the Hampel's method.

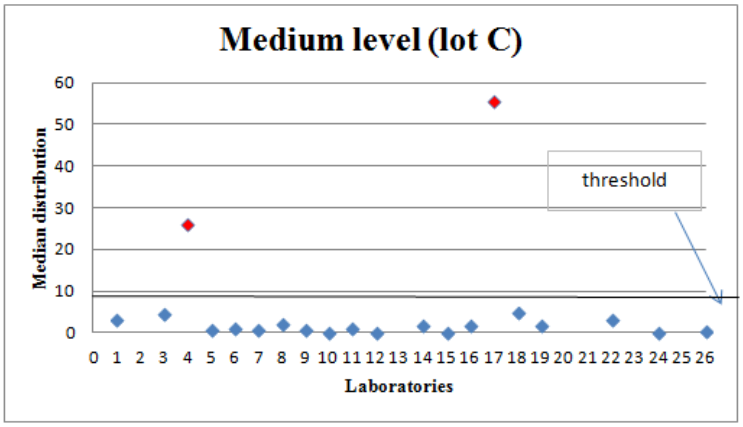
Results distribution of all laboratories and outliers detection is given in table 6 and figure 3. Outliers correspond to red diamond-shape in figure 2. Two laboratories were identified as outliers, laboratories 4 and 17. These laboratories are not accredited for the Method 7-003 and participate as volunteers.

Table 6: Results analysis according to the Hampel's method for medium level (lot C).

High level			
Lab	Lab Values (Xi)	| Xi - M|	Status
1	6,67	3,207	OK
3	8,01	4,553	OK
4	29,33	25,874	Outlier
5	2,67	0,788	OK
6	2,25	1,210	OK
7	2,75	0,710	OK
8	1,25	2,208	OK
9	2,51	0,947	OK
10	3,33	0,126	OK
11	4,67	1,207	OK
12	3,34	0,121	OK
14	5,53	2,068	OK
15	3,50	0,043	OK
16	5,33	1,874	OK
17	58,98	55,521	Outlier
18	8,33	4,874	OK
19	1,42	2,043	OK
22	6,89	3,433	OK
24	3,42	0,043	OK
26	2,83	0,625	OK

Median (M):	**3,460**
MAD:	**1,542**
5.2 X MAD	**8,017**

Figure 3: Results distribution for medium level for all laboratories. Outliers are in red diamond-shape.



- **<u>Conclusion of results lots B and C</u>**

Laboratories 4 and 17, which were not accredited for the method overestimate the percentage of contamination for lot B and lot C. In these cases of high percentage of detection, it could be explained by confusion between saprophytic fungi like *Cladosporium* sp. and *Botrytis cinerea*. The fungi are shown in picture A and B in appendix.
The results of the others laboratories showed a small variability between laboratories.

Z-SCORES COMPUTATIONS AND RATING SYSTEM

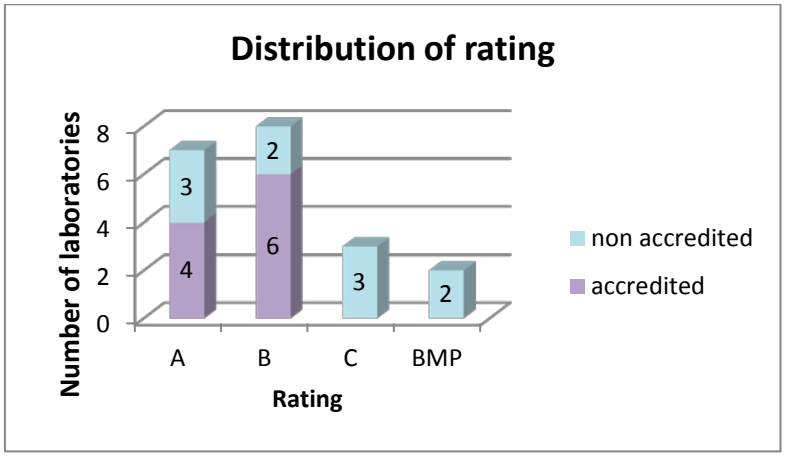
(For information only)

The Z-scores computations and rating system are under development and these results are given for information only.
The calculation of z-scores and the rating were done with the Excel file provided by the Statistical committee.
For each lot, detection of outliers is automatically detected according to the Hampel's method. Results are shown in table 7 and figure 4.
The missing laboratories correspond to those which did not return results to ISTA Seed Health Committee (4 laboratories).
Table7: Computations of laboratories z-scores and rating.

	Max abs(z-score) for the Healthy lot		Max abs(z-score) for the Medium lot		Max abs(z-score) for the High lot
Minimum requirements for A rating	0.08	and	0.67	and	0.67
Minimum requirements for B rating	1.5	and	1.5	and	1.5
Minimum requirements for C rating	2.33	and	2.33	and	2.33

Lab	abs(z-score) for the Healthy lot	abs(z-score) for the Medium lot	abs(z-score) for the High lot	Rating	
1	0.00	0.09	1.16	B	
3	0.33	1.79	1.78	C	
4	16.67	9.50	11.60	BMP	
5	0.00	0.64	0.68	B	
6	1.59	0.57	0.88	C	
7	0.00	0.07	0.64	A	
8	0.08	1.33	1.34	B	
9	0.00	0.33	0.75	B	
10	0.00	0.70	0.38	B	
11	0.08	0.22	0.24	A	
12	0.08	0.38	0.37	A	
14	0.00	0.01	0.63	A	
15	0.00	0.22	0.30	A	
16	0.22	0.62	0.54	B	
17	63.03	37.12	25.25	BMP	
18	1.08	0.62	1.93	C	
19	0.00	0.86	1.26	B	
22	0.00	1.49	1.26	B	rep 1 excluded
24	0.00	0.59	0.34	A	
26	0.00	0.36	0.61	A	

Figure 4: Distribution of rating



This figure shows the rating distribution for accredited (purple) and non accredited laboratories for the method 7-003.

For the laboratory 22 one of the three repetitions showed higher value, this value was excluded before calculation. This laboratory obtained a B rating whereas it would have obtained a BMP rating. This different value could be due to cross contamination during manipulations or bad record of results on the Excel file.

APPENDIX

lab	sample	rep	Value %	lab	sample	rep	Value %
1	Healthy	1	0	3	Healthy	1	0
1	Healthy	2	0	3	Healthy	2	0
1	Healthy	3	0	3	Healthy	3	1
1	Low	1	1.25	3	Low	1	2
1	Low	2	1.75	3	Low	2	8.77
1	Low	3	4	3	Low	3	4.26
1	Medium	1	2.25	3	Medium	1	8.25
1	Medium	2	6.75	3	Medium	2	9.77
1	Medium	3	11	3	Medium	3	6.02
4	Healthy	1	14.5	5	Healthy	1	0
4	Healthy	2	9.5	5	Healthy	2	0
4	Healthy	3	26	5	Healthy	3	0
4	Low	1	10	5	Low	1	0.75
4	Low	2	27.5	5	Low	2	1.75
4	Low	3	14	5	Low	3	1.01
4	Medium	1	40	5	Medium	1	3.5
4	Medium	2	17.5	5	Medium	2	3.02
4	Medium	3	30.5	5	Medium	3	1.5
6	Healthy	1	1.26	7	Healthy	1	0
6	Healthy	2	2.26	7	Healthy	2	0
6	Healty	3	1.26	7	Healthy	3	0
6	Low	1	3	7	Low	1	1
6	Low	2	2	7	Low	2	2
6	Low	3	4.25	7	Low	3	3.25
6	Medium	1	2.5	7	Medium	1	2.75
6	Medium	2	2.25	7	Medium	2	2.75
6	Medium	3	2	7	Medium	3	2.75
8	Healthy	1	0	9	Healthy	1	0

8	Healthy	2	0	9	Healthy	2	0
8	Healthy	3	0.25	9	Healthy	3	0
8	Low	1	0	9	Low	1	1.25
8	Low	2	0	9	Low	2	1.75
8	Low	3	0.25	9	Low	3	2
8	Medium	1	2	9	Medium	1	1.75
8	Medium	2	0.25	9	Medium	2	3.27
8	Medium	3	1.5	9	Medium	3	2.51
10	Healthy	1	0	11	Healthy	1	0
10	Healthy	2	0	11	Healthy	2	0.25
10	Healthy	3	0	11	Healthy	3	0
10	Low	1	1.01	11	Low	1	2.25
10	Low	2	1.26	11	Low	2	1.5
10	Low	3	1	11	Low	3	1.75
10	Medium	1	3	11	Medium	1	5
10	Medium	2	3.25	11	Medium	2	5
10	Medium	3	3.75	11	Medium	3	4
12	Healthy	1	0	14	Healthy	1	0
12	Healthy	2	0	14	Healthy	2	0
12	Healthy	3	0.25	14	Healthy	3	0
12	Low	1	0.75	14	Low	1	1.75
12	Low	2	2.5	14	Low	2	1.26
12	Low	3	1.5	14	Low	3	3.52
12	Medium	1	3.25	14	Medium	1	5.28
12	Medium	2	3.75	14	Medium	2	5.03
12	Medium	3	3.02	14	Medium	3	6.28
15	Healthy	1	0	16	Healthy	1	0.67
15	Healthy	2	0	16	Healthy	2	0
15	Healthy	3	0	16	Healthy	3	0
15	Low	1	2	16	Low	1	1
15	Low	2	1.75	16	Low	2	8.5

15	Low	3	1.75	16	Low	3	0
15	Medium	1	4	16	Medium	1	3
15	Medium	2	3.26	16	Medium	2	11
15	Medium	3	3.25	16	Medium	3	2
17	Healthy	1	66.25	18	Healthy	1	2
17	Healthy	2	67.09	18	Healthy	2	1.25
17	Healthy	3	55.75	18	Healthy	3	0
17	Low	1	52.25	18	Low	1	1.5
17	Low	2	61.5	18	Low	2	5
17	Low	3	68.42	18	Low	3	3
17	Medium	1	77.19	18	Medium	1	5.5
17	Medium	2	49.75	18	Medium	2	5
17	Medium	3	50	18	Medium	3	14.5
19	Healthy	1	0	22	Healthy	1	0
19	Healthy	2	0	22	Healthy	2	0
19	Healthy	3	0	22	Healthy	3	0
19	Low	1	1.25	22	Low	1	10.61
19	Low	2	0.75	22	Low	2	3.28
19	Low	3	0.5	22	Low	3	4.80
19	Medium	1	1.5	22	Medium	1	8.59
19	Medium	2	0.75	22	Medium	2	8.08
19	Medium	3	2	22	Medium	3	4.01
24	Healthy	1	0	26	Healthy	1	0
24	Healthy	2	0	26	Healthy	2	0
24	Healthy	3	0	26	Healthy	3	0
24	Low	1	2	26	Low	1	3.5
24	Low	2	1.25	26	Low	2	3.25
24	Low	3	0.5	26	Low	3	1.5
24	Medium	1	3.75	26	Medium	1	5
24	Medium	2	4.25	26	Medium	2	1.50
24	Medium	3	2.25	26	Medium	3	2

Table 4: Proficiency Test Results

Picture 1: sporulated mycelium of *Botrytis cinerea*.

Picture 2: sporulated mycelium of *Cladosporium sp.*