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Method Validation Reports on Rules Proposals for the International Rules for Seed Testing 2019 Edition

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Validation study of seed germination test of *Felicia heterophylla* into the ISTA Rules to support B.1.2.

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Summary

The object of this validation study is to introduce a seed germination method for *Felicia heterophylla*, a new species to be introduced in the ISTA Rules. The new method is intended to be included into Chapter 5, Table 5A, part 3 “Detailed methods for germination tests. Flower, spice, herb and medicinal species”. *Felicia heterophylla* is a new species aimed to be included in the ISTA Rules. Therefore, standard ISTA methods are not available.
Five ISTA laboratories from five different countries participated in the study. Three seed lots were distributed, together with the test plan. The following methods were included in the plan, together with the recommendation to use the light:
TP; 20 <=>30°C (first count 4-7 days; final count: 21 days). Additional treatment: NONE
TP; 20°C (first count 4-7 days; final count: 21 days). Additional treatment: NONE
The statistical analysis could be performed using all the results obtained by all the participants and showed the repeatability and reproducibility for the two methods.
Based on the results of that analysis, the germination method proposed to be included in the ISTA Rules for *Felicia heterophylla* is: Top of Paper; 20 °C; 7 – 21 days, recommendation: light.

Introduction

Felicia heterophylla is a species belonging to the family *Asteraceae*, native to South Africa. It is an annual plant, known with the common name “blue true daisy” because of the beautiful flowers, that are entirely of an electric-blue colour. It fast grows to around 30 cm tall and it is ideal for sunny areas, as edging plant as well as pot plant.
The introduction of this species in the ISTA Rules has been suggested by one ISTA member laboratory from South Africa.
This species is commercialized in some areas of the word, such as Africa and Japan, and for this reason, ISTA methods are needed.

A first validation study with the same object had been already organized in 2012 by the ISTA Flower Seed Testing Committee. At that time, five laboratories participated and four methods were included in the study (combination of TP – 20 °C and 20 <=> 30 °C, with and without additional treatments). The statistical review showed that none of the four methods fulfilled the repeatability and the reproducibility criteria. Best compromise was reached by one method (TP 20 °C – 4-7-21 days - Additional treatment: NONE). Some participants to the first study had complained about the presence of fungi on TP. This may have been one of the reason of failure of the study.

For this new validation study, three new seed samples were used. Basing on the information provided by the proposing laboratory and on the experience of the first study, the ISTA Flower Seed Testing Committee approved the proposal of the test leader to focus on two testing methods, where the temperature regime represented the only difference between the two methods.

1. TP; 20 <=>30°C (1st count 4-7 days; final count: 21 days). Additional treatment: light recommended

2. TP; 20°C (1st count 4-7 days; final count: 21 days). Additional treatment: light recommended

The validation study was performed through a multi-laboratory comparative test, which took place in 2016. Results of the laboratories are here reported anonymously.

Felicia heterophylla seeds

Material and methods

Seed material

Three samples of untreated seed of *Felicia heterophylla* were used in the study, two originated from South Africa, one from The Netherlands. The three samples were drawn from commercial lots intended for marketing.

Participant laboratories

Four ISTA accredited laboratories and one more ISTA laboratory having the accreditation process ongoing and being very experienced in seed testing for that species participated in the study: Starke Ayres (South Africa), CREA-DC (Italy), Geves-SNES (France), Naktuinbouw (The Nederlands), MGSZH (Hungary).
The test was organized by Rita Zecchinelli (CREA-DC Laboratory – Tavazzano LO, Italy).

Additional treatments

No additional treatments were used, but the participants were asked to germinate the seed in the light. This suggestion came from one reviewer of the test plan.

Germination method

For each test and sample, 400 seeds were tested in replicates of 100 seeds. “Top of Paper” (TP) was used as substrate, two temperature regimes were investigated, constant temperature of 20 °C and alternating temperature of 20 °C (for 16 hours) and 30 °C (for 8 hours).

Following the test plan, germination counts were made after 4 or 7 days (1st count) and 21 days (final count). With only one exception (laboratory 5, method 1), the 1st count took always place after 7 days. Three laboratories performed the final count after 21 days, one laboratory anticipated it after 17 days, another one after 13 days.
The participant laboratories confirmed that the substrate used in the experiments met the requirements prescribed by the ISTA Rules.
Referring to the classification in the ISTA Handbook for Seedling Evaluation, the seedling type for *Felicia heterophylla* is type "E", for dicotyledons, with epigeal germination, without epicotyl elongation (see figure 1).
Following the instructions received, the laboratories based their seedling evaluation on the seedling group A-2-1-1-1, which is used for species and genera of the *Asteraceae* family.

Figure 1. Seedling development in *Felicia heterophylla* (photo: Zita Ripka)

Results
All participants sent the results back to the test organizer in a timely manner. The comparative test involved a total of 6 germination tests completed by each participant. The data received were soon checked for completeness and accuracy and then submitted for statistical analysis (see below). Data were checked confirming that the sum of the percentages was always equal to 100%; one result (method 1, lot 3, laboratory 5) was found out of tolerance (the laboratory repeated the test and the re-test gave again out of tolerance results).

The participants also provided a description of the abnormal seedlings. The laboratories agreed that the most frequent abnormalities were the following: 00/09 (seedling decayed as a result of a primary infection); 11/03 (primary root retarded) 11/04 (primary root missing); 31/05 (cotyledons >50% discoloured or necrotic). More rarely, other abnormalities were also reported: 00/01 (seedling deformed); 11/07 (primary root trapped in the seed coat); 11/08 (primary root showing negative geotropism); 21/01 (hypocotyl short and thick).

In addition to the methods prescribed in the test plan, one participant laboratory also tested the samples with a KNO_3 additional treatment (0.2% KNO_3 solution added to the substrate at the beginning of the test), for breaking physiological dormancy whenever present. The only effect of the treatment was to anticipate the first count from the 7th day to the 4th, but no promotion of germination was observed.

Statistical analysis of the results

Completeness and accuracy of the data were confirmed.

Germination results by seed lots

For the three seed samples, the figure 2 presents the overall data of the percentage of normal seedlings obtained for all methods, by all laboratories.

As shown by the median value in the side-by side boxplots, sample 1 gives 83% of normal seedlings, sample 2 gives 87% of normal seedlings and the sample 3 gives 86.5% of normal seedlings. The overall average percentage of normal seedlings is 82,3%, 85,1%, 84,8% respectively.

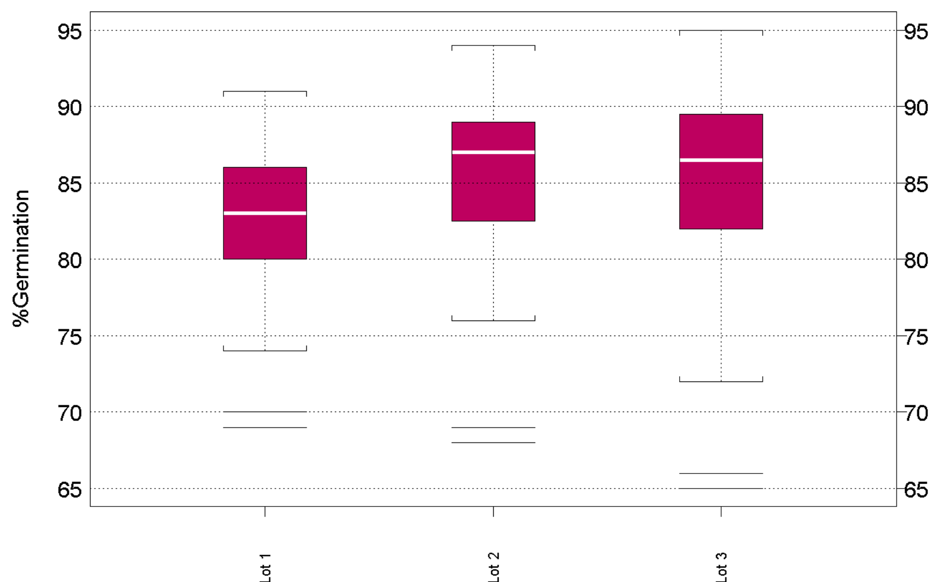

Figure 2. Percentage of normal seedlings for all methods and all laboratories, per sample.

Germination results by laboratory

The figure 3 shows the data of the percentage of normal seedlings for all samples and all methods, by each laboratory. The data show a certain variability between laboratories. However, this does not lead to the exclusion of any result for further analysis.

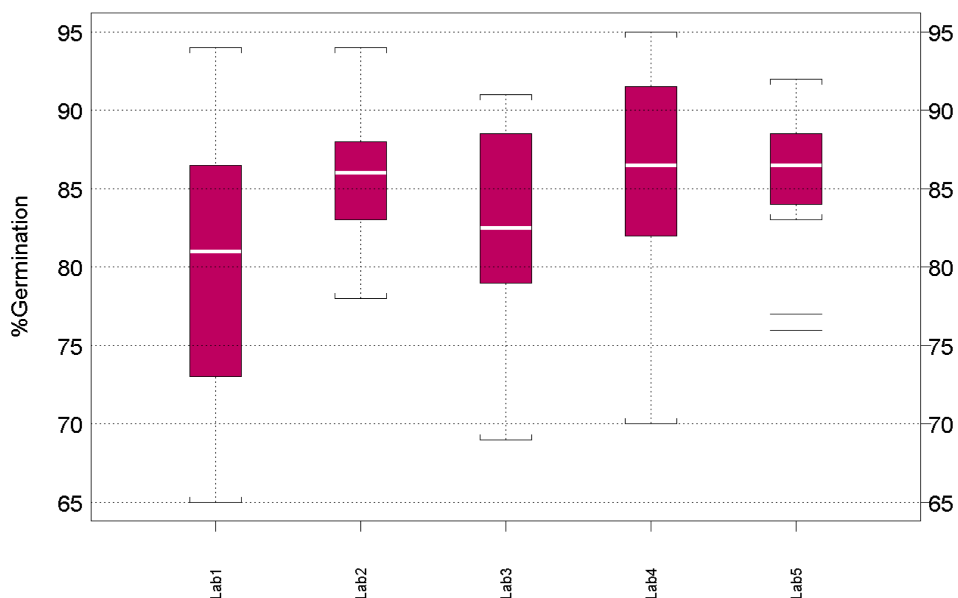

Figure 3. Percentage of normal seedlings for all samples and all methods, per each laboratory.

Germination results by method

The figure 4 shows the data of the percentage of normal seedlings for all samples and all laboratories, by each method. The same data - but separated also by sample - are presented in figure 5.

As graphically shown by the median value in the side-by side boxplots, method 1 gives 82,5% of normal seedlings, method 2 gives 86,6% of normal seedlings. It is also evident the variation smaller for method 2 than for method 1 (smaller standard deviation).

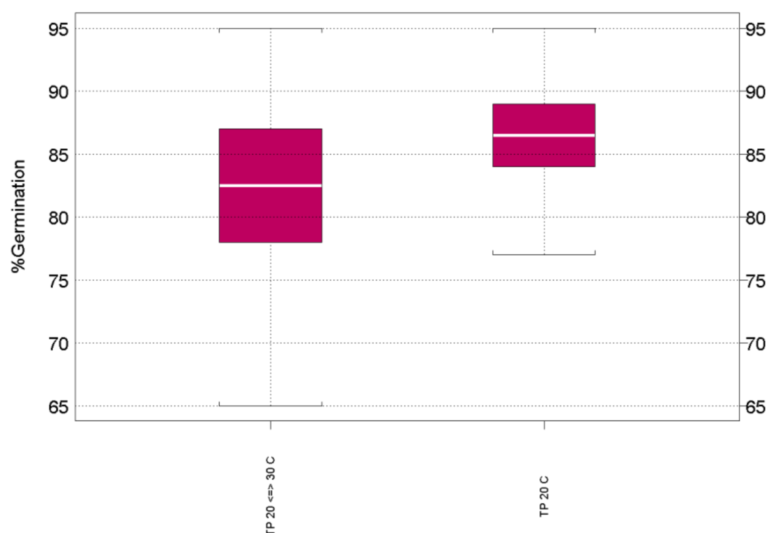

Figure 4. Percentage of normal seedlings for all samples and all laboratories, per method.

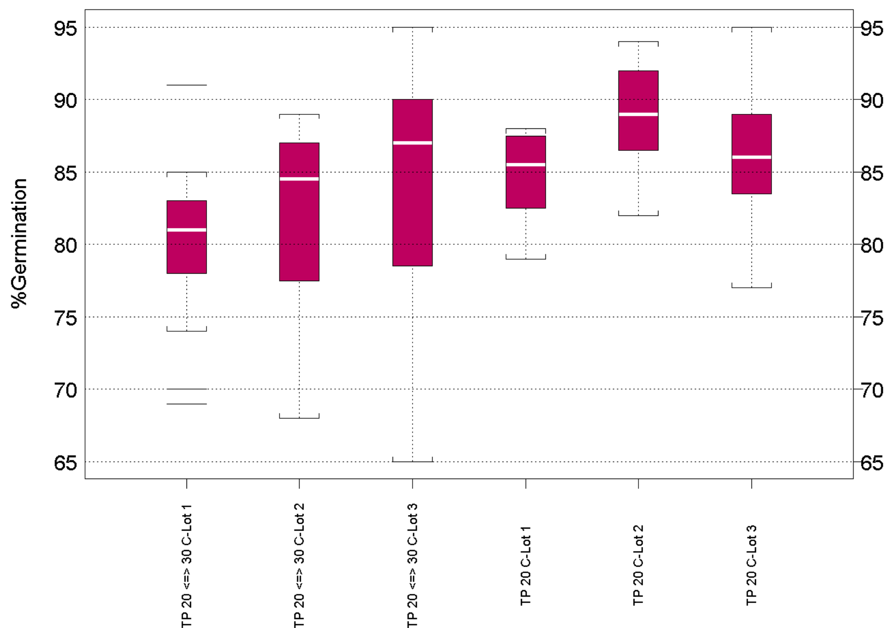

Figure 5. Percentage of normal seedlings for all laboratories, per method and sample

Germination results by laboratory

The figure 6 shows the data grouped across methods. The variability between laboratories shown in figure 3 is confirmed. As said, this does not lead to the exclusion of any result for further analysis.

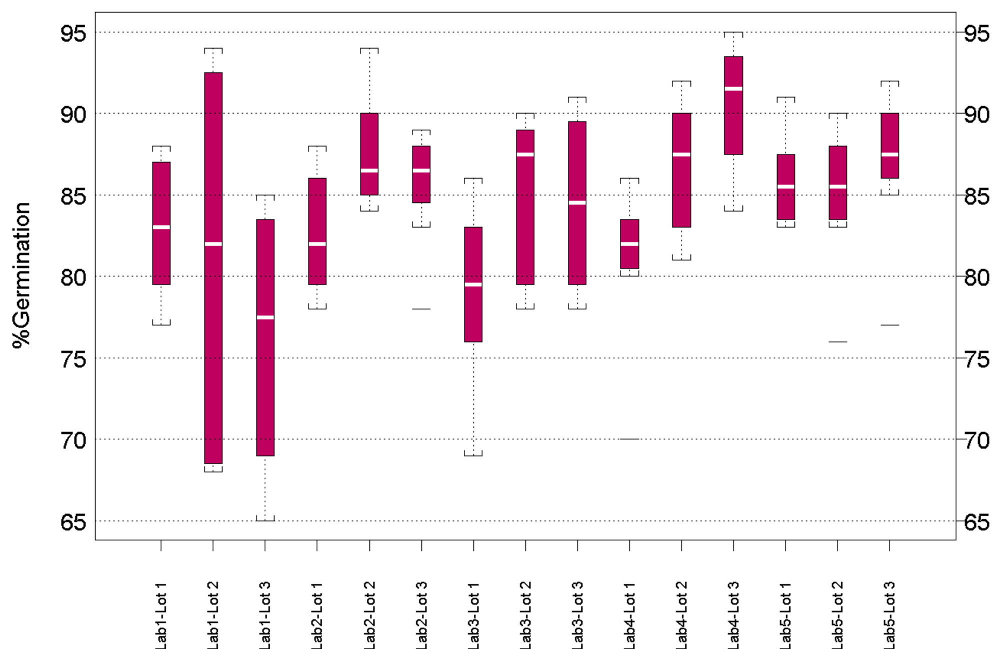

Figure 6. Percentage of normal seedlings for the two methods, per sample and laboratory

Results of data checking

Data checking has been performed according to ISTA rules by computing tolerances for germination test replicates. The results are shown in the tables below.

Method 1. TP 20 <=> 30 C

Lot 1	Lab1	Lab2	Lab3	Lab4	Lab5
Max Tol range	16	15	17	16	14
Obs range	4	6	11	13	8
Mean	79.25	80.75	75.25	79.00	85.75

Lot 2	Lab1	Lab2	Lab3	Lab4	Lab5
Max Tol range	18	14	15	14	14
Obs range	9	4	9	7	13
Mean	70.50	86.50	81.00	85.25	84.00

Lot 3	Lab1	Lab2	Lab3	Lab4	Lab5

Max Tol range	18	14	15	11	13
Obs range	9	10	11	4	3
Mean	69.25	85.00	83.00	92.50	88.00

Method 2. TP 20 C

Lot 1	Lab1	Lab2	Lab3	Lab4	Lab5
Max Tol range	13	14	15	15	14
Obs range	3	9	7	6	5
Mean	86.75	84.50	82.75	83.00	86.00

Lot 2	Lab1	Lab2	Lab3	Lab4	Lab5
Max Tol range	11	12	12	13	14
Obs range	7	10	2	10	7
Mean	91.50	88.75	89.00	88.25	86.00

Lot 3	Lab1	Lab2	Lab3	Lab4	Lab5
Max Tol range	15	14	14	13	14
Obs range	4	6	13	11	15
Mean	83.25	86.25	86.00	88.50	86.00

One result is out of tolerance.

Repeatability/Reproducibility

For each method, the following linear mixed model has been fitted:

$$y_{ijk} = \mu + \alpha_i + b_j + (\alpha b)_{ij} + e_{ijk}$$

in which:

. y_{ijk} is the observed percent of normal seedlings in rep *k* of lot *i* and lab *j*.

. □□is the intercept.

. α_i is the fixed effect of lot *i*.

. b_j is the random effect of lab *j*. b_j ~ i.i.d. $N(0,\sigma^2_{Lab})$.

. $(\alpha b)_{ij}$ is the random interaction effect between lot *i* and lab *j*.

$(\alpha b)_{ij}$ ~ i.i.d. $N(0,\sigma^2_{Lot\times Lab})$.

. e_{ijk} are the residuals. e_{ijk} ~ i.i.d. $N(0,\sigma^2)$.

Repeatability standard-deviation is then given by $S_r = \sqrt{\hat{\sigma}^2}$ and reproducibility standard-deviation by $S_R = \sqrt{\hat{\sigma}^2 + \hat{\sigma}^2_{Lab} + \hat{\sigma}^2_{Lot\times Lab}}$.

The dispersion factor is calculated as $f_r = \sqrt{\dfrac{m\,\hat{\sigma}^2}{\overline{p}_{...}(100-\overline{p}_{...})}}$ where $\overline{p}_{...}$ □is the overall average percentage of normal seedlings and *m* is the number of seeds per rep (*m* = 100 in this study). If f_r > 1 one speaks of overdispersion because the data have larger variance than expected under the assumption of a binomial distribution.

Repeatability of the results:

TP 20 <=> 30 C

$\overline{p}_{...}$	S_r	f_r
81.67	3.98	1.03

TP 20 C

$\overline{p}_{...}$	S_r	f_r
86.43	3.77	1.10

Repeatability standard deviations are acceptable for the two methods (f_r close to 1).

Reproducibility of the results:

TP 20 <=> 30 C **TP 20 C**

$\bar{p}_{...}$	S_R	$\hat{\sigma}^2_{Lab}$	$\hat{\sigma}^2_{Lot\times Lab}$
81.67	7.49	4.70	4.26

$\bar{p}_{...}$	S_R	$\hat{\sigma}^2_{Lab}$	$\hat{\sigma}^2_{Lot\times Lab}$	
86.43	3.77	0.00	0.00	

The inter-lab variability is small for TP 20 C compared to TP 20 < = > 30 C.

Conclusions

TP 20° C has the lowest reproducibility standard-deviation and also the highest general mean, compared to TP 20 <=> 30° C. Therefore, the recommendation from the ISTA Flower Seed Testing Committee is to include in the ISTA Rules the following germination method for *Felicia heterophylla*:

TP; 20°C (1st count 4-7 days; final count: 21 days). Additional treatment: light recommended

Acknowledgments

Thanks to Starke Ayres (South Africa) and to Naktuinbouw (The Netherlands) for providing the seed samples used in the validation study.

Thanks to the staff of the participating ISTA laboratories: Starke Ayres (South Africa), CREA-DC (Italy), Geves-SNES (France), Naktuinbouw (The Netherlands), MGSZH (Hungary). Thanks, in particular to the reference persons of those laboratories: Frikkie Marais, Rita Zecchinelli, Sylvie Ducournau, Anton Grim, Zita Ripka.

Thanks to the technical reviewers Gillian McLaren and Lea Mazor.

Thanks to Nadine Ettel, TCOM coordinator at the ISTA Secretariat for her support.

Thanks to Jean-Luis Laffont and the ISTA Statistics Committee for the review of the testing plan and the statistical analysis.

References

ISTA Handbook on Seedling Evaluation, 3rd Edition, 2013.
ISTA Method Validation for Seed Testing-V1.0 (https://www.seedtest.org/en/method-validation-programme-_content---1--1254.html)
Standard Operating Procedure: Method validation -process administration (TCOM-SOP-05- Method validation -process V1.0 - https://www.seedtest.org/en/method-validation-programme-_content---1--1254.html)

Validation study of seed germination test of *Eustoma exaltatum* into the ISTA Rules to support B.1.1.

Test leaders: [1] Rita Zecchinelli - [2]Anton Grim
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Summary

The object of this validation study is to introduce a seed germination method for *Eustoma exaltatum*, a new species to be introduced in the ISTA Rules. The new method is intended to be included into Chapter 5, Table 5A, part 3 "Detailed methods for germination tests. Flower, spice, herb and medicinal species". *Eustoma exaltatum* is a new species aimed to be included in the ISTA Rules. Therefore, standard ISTA methods are not available.
Six ISTA laboratories from five different countries participated in the study. Three seed lots were distributed, together with the test plan. Three testing methods were included in the plan, together with the recommendation to use the light.

The statistical analysis could be performed using all the results obtained by all the participants and showed the repeatability and reproducibility for the three methods.
Based on the results of that analysis, the germination method proposed to be included in the ISTA Rules for *Eustoma exaltatum* is: Top of Paper; 20 <=> 30 °C; 7 – 21 days, recommendation: light.

Introduction

Eustoma exaltatum is an herbaceous annual species belonging to the family *Gentianaceae*, native to the warm areas of Americas, where it is a well-known native species. It is an annual plant, known with the common names lisianthus and prairie gentian.
The genus name derives from the Greek words eu (good) and stoma (mouth). The name reflects the wide opening of the corolla tube which is typical for lisianthus flowers. Indeed, even lisianthus comes from the ancient Greek and lissos means smooth, anthos means flower.
Lisianthus is cultivated mainly as a cut flower, but can be used as potted and bedding plant as well.

The inclusion of the validation study in the 2013-2016 working programme of the ISTA Flower Seed Testing Committee (FSC) was approved by the members and Anton Grim from the Naktuinbouw laboratory in The Netherlands accepted to organize the study as test leader.
For the validation study, three seed samples were used. Basing on the information provided by the experienced laboratories of the FSC members, the FSC approved the proposal of the test leader to focus on three following testing methods, where the temperature regime represented the only difference between the three.
TP; 20°C (first count 4-7 days; final count: 21 days). Additional treatment: Light recommended
TP; 25°C (first count 4-7 days; final count: 21 days). Additional treatment: Light recommended
TP; 20 <=>30°C (first count 4-7 days; final count: 21 days). Additional treatment: Light recommended

The validation study was performed through a multi-laboratory comparative test, which took place in 2016. Results of the laboratories are here reported anonymously.

Material and methods

Seed material

Three samples of untreated seed of *Eustoma exaltatum* were used in the study, all originated from The Netherlands. The three samples were drawn from commercial lots intended for marketing.

Participant laboratories

Six laboratories participated in the study: Sakata Seed (Japan), Starke Ayres (South Africa), CREA-DC (Italy), Vulcani Center (Israel), Naktuinbouw (The Netherlands), Takii Europe (The Netherlands).
The test was organized by Anton Grim (Naktuinbouw, The Netherlands).

Additional treatments

No additional treatments were used, but the participants were asked to germinate the seed in the light. This suggestion came from the test leader.

Germination method

For each test and sample, 400 seeds were tested in replicates of 100 or 50 seeds. “Top of Paper” (TP) was used as substrate, three temperature regimes were investigated, constant temperature of 20 °C and 25°C, alternating temperature of 20 °C (for 16 hours) and 30 °C (for 8 hours).
Following the test plan, germination counts were made after 4 or 7 days (1st count) and 21 days (final count).
Participants were asked to base the evaluation of the seedlings on seedling group A-2-1-1-1 (the same approved for *Gentiana* belonging to the same family *Gentianaceae*). The participants will be asked to provide a description of the abnormal seedlings.

Results

All participants sent the results back to the test organizer in a timely manner. The comparative test involved a total of 9 germination tests completed by each participant. The data received were soon checked for completeness and accuracy and then submitted for statistical analysis (see below). Data were checked to confirm that the sum of the percentages was always equal to 100%; for some data (method 1, lot 2, laboratory 1; method 1, lot 3, laboratory 1; method 3, lot 3, laboratory 1), this could not be confirmed and the results needed to be adjusted according to the ISTA Rules. One result (method 3, lot 2, laboratory 4) was found out of tolerance.

The participants also provided a description of the abnormal seedlings. The laboratories agreed that the most frequent abnormalities were the following: 00/09 (seedling decayed as a result of a primary infection); 11/03 (primary root retarded) 11/04 (primary root missing). More rarely, other abnormalities were also reported: 00/08 (seedling glassy); 11/01 (primary root stunted); 21/01 (hypocotyl short and thick).

Some participants wanted to underline that the germination at 20 °C was slower compared to the other temperature regimes. One laboratory suggested to prefer the first count to be performed after

14 days and not before. These comments are also associated with the seedling size which in *Eustoma* is very small.

Statistical analysis of the results

The analysis was performed by the ISTA Statistics Committee.

Germination results by seed lots

For the three seed samples, the figure 1 presents the overall data of the percentage of normal seedlings obtained for all methods, by all laboratories.

As shown by the median value in the side-by side boxplots, lot 1 gives 89% of normal seedlings, lot 2 gives 88% of normal seedlings and the lot 3 gives 89.5% of normal seedlings. The overall average percentage of normal seedlings is 88,3%, 82,6%, 84,8% respectively.

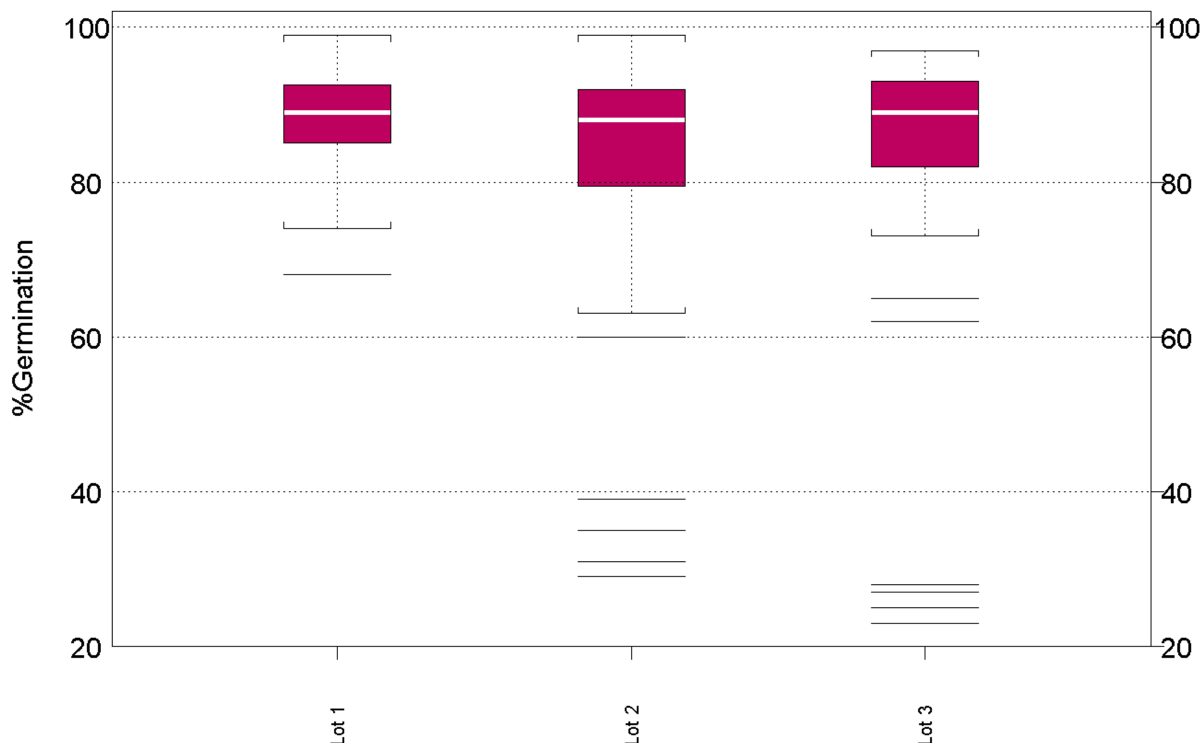

Figure 1. Percentage of normal seedlings for all methods and all laboratories, per lot.

Germination results by laboratory

The figure 2 shows the data of the percentage of normal seedlings for all samples and all methods, by each laboratory. The figure 3 shows the data of the percentage of normal seedlings for all methods,

by laboratory and lot. The data show particular low values reported by Lab 3 for lots 2 and 3. For this reason, two statistical analyses were performed, one including and one excluding Lab 3.

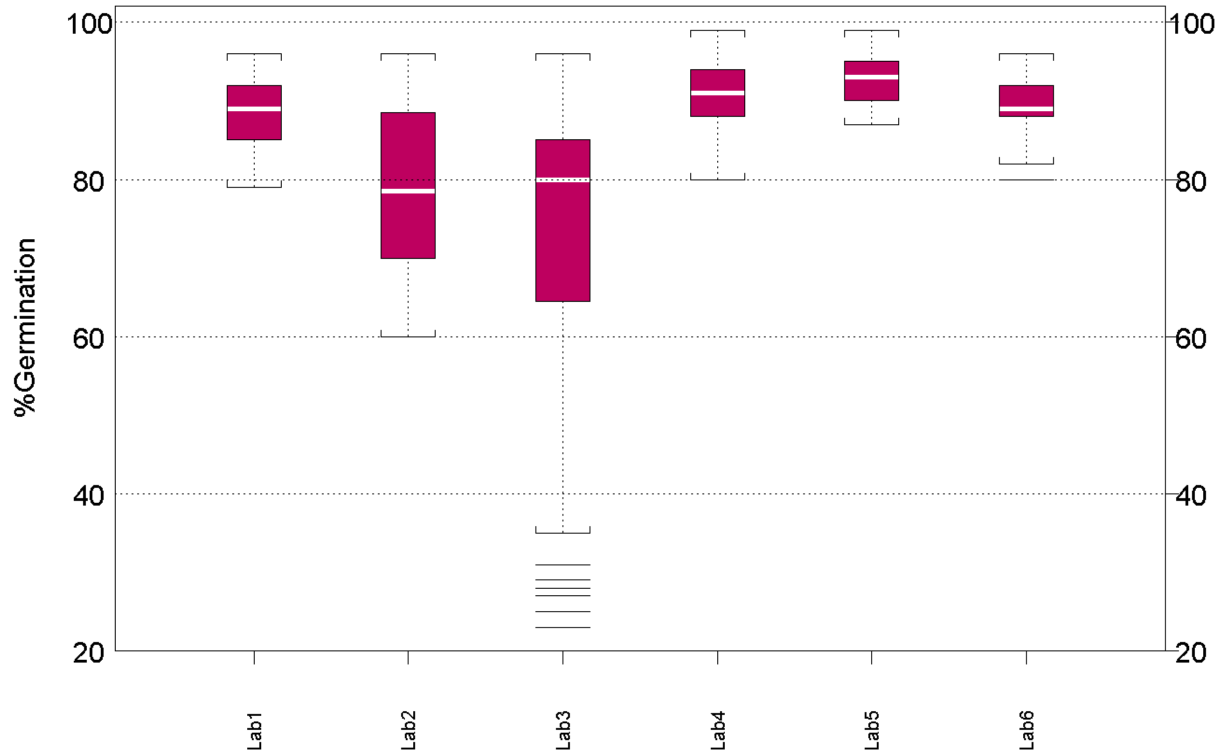

Figure 2. Percentage of normal seedlings for all lots and all methods, per each laboratory.

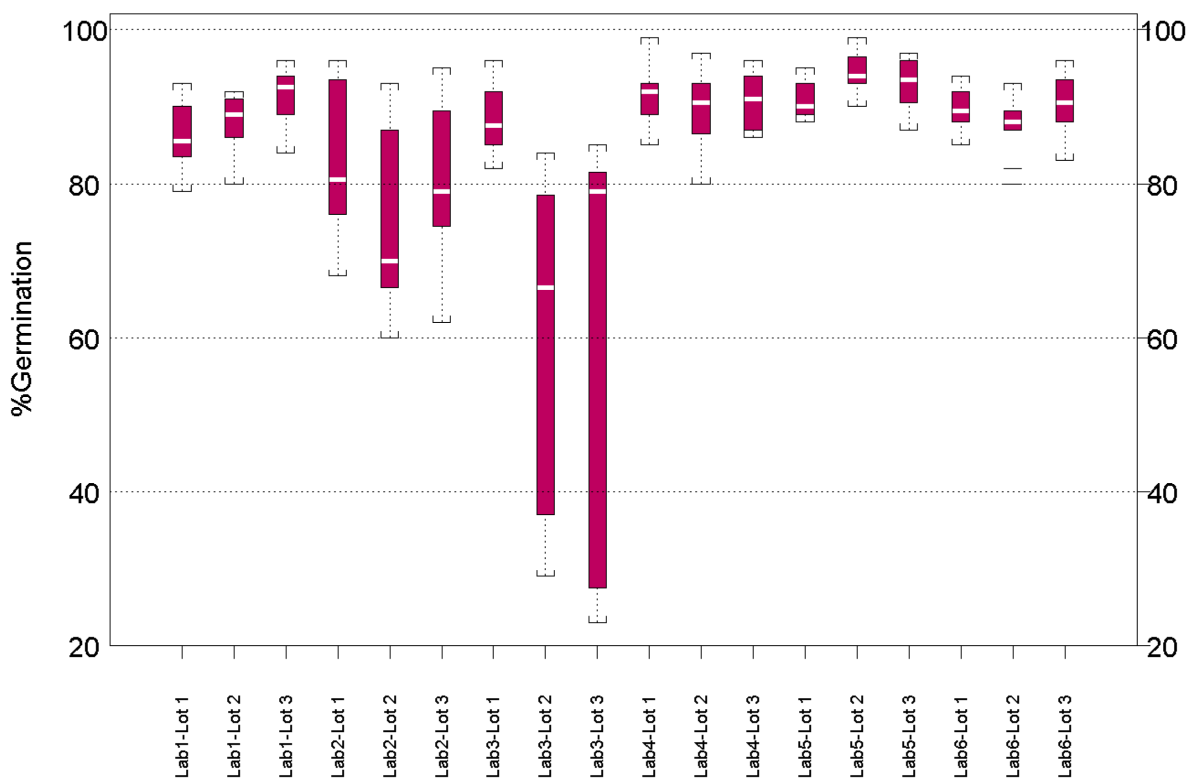

Figure 3. Percentage of normal seedlings for all methods, per each laboratory and lot.

Germination results by method

The figure 4 shows the data of the percentage of normal seedlings for all lots and all laboratories, by each method. The same data - but separated also by lot - are presented in figure 5.

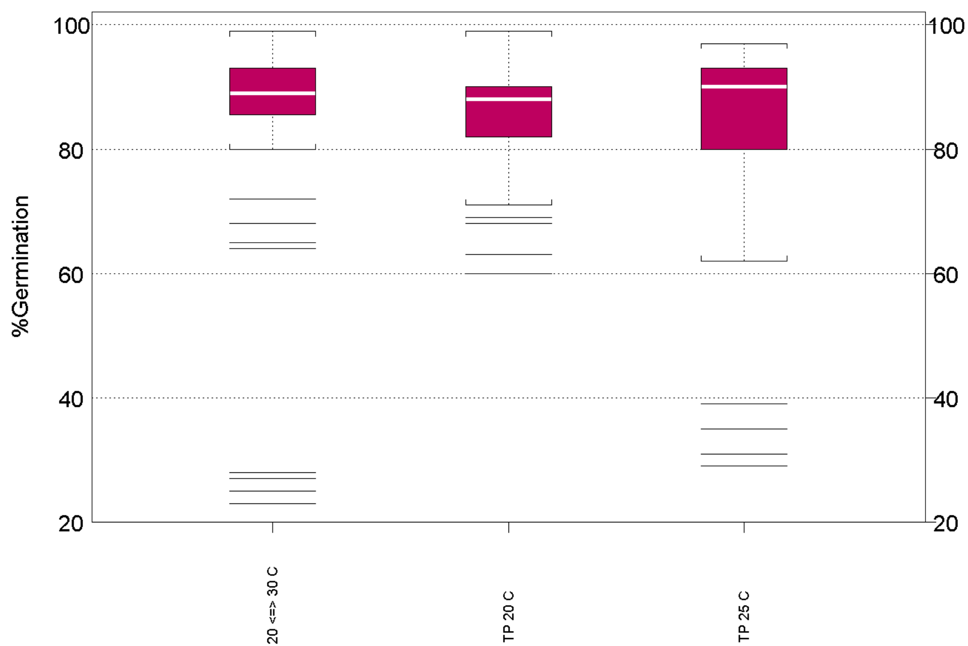

Figure 4. Percentage of normal seedlings for all samples and all laboratories, per method.

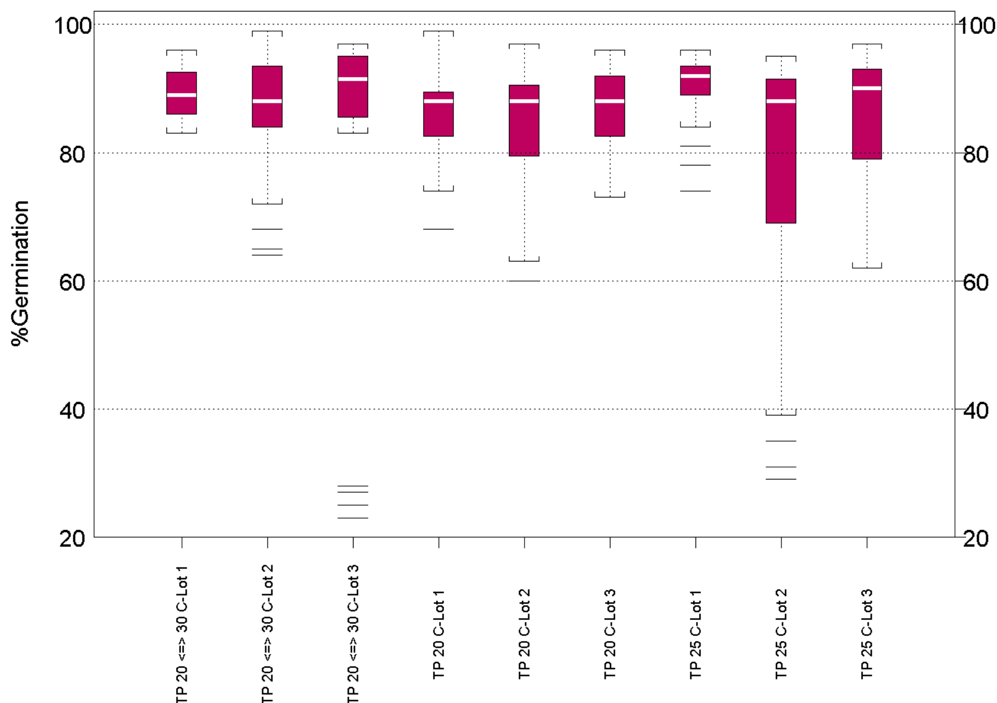

Figure 5. Percentage of normal seedlings for all laboratories, per method and sample

Results of data checking

Data checking has been performed according to ISTA rules by computing tolerances for germination test replicates.

Results:

TP 20 <=> 30 C

Lot 1	Lab1	Lab2	Lab3	Lab4	Lab5	Lab6
Max Tol range	14	10	13	12	11	13
Obs range	6	3	3	7	6	4
Mean	85.00	94.50	86.75	88.75	92.00	87.50

Lot 2	Lab1	Lab2	Lab3	Lab4	Lab5	Lab6

Max Tol range	13	12	18	11	7	14
Obs range	6	6	8	10	5	13
Mean	88.50	89.00	67.25	92.50	96.75	85.50

Lot 3	Lab1	Lab2	Lab3	Lab4	Lab5	Lab6
Max Tol range	10	11	17	11	10	14
Obs range	4	7	5	9	5	5
Mean	94.00	91.75	25.75	92.50	94.25	85.75

TP 20 C

Lot 1	Lab1	Lab2	Lab3	Lab4	Lab5	Lab6
Max Tol range	14	17	14	10	13	13
Obs range	11	12	6	10	1	4
Mean	84.75	75.25	84.50	93.75	88.50	88.50

Lot 2	Lab1	Lab2	Lab3	Lab4	Lab5	Lab6
Max Tol range	13	19	15	13	10	12
Obs range	12	11	6	8	7	5
Mean	87.75	65.75	80.75	88.00	93.00	89.00

Lot 3	Lab1	Lab2	Lab3	Lab4	Lab5	Lab6
Max Tol range	12	16	15	12	11	10
Obs range	10	9	7	5	7	6
Mean	89.00	78.25	82.00	88.75	91.00	93.25

TP 25 C

Lot 1	Lab1	Lab2	Lab3	Lab4	Lab5	Lab6

Max Tol range	12	16	10	11	11	10
Obs range	9	12	7	4	5	2
Mean	90.00	79.75	93.75	92.00	91.75	92.75

Lot 2	Lab1	Lab2	Lab3	Lab4	Lab5	Lab6
Max Tol range	13	18	18	13	10	12
Obs range	8	14	10	15	3	2
Mean	88.50	70.00	33.50	88.00	93.75	88.75

Lot 3	Lab1	Lab2	Lab3	Lab4	Lab5	Lab6
Max Tol range	11	18	16	10	10	11
Obs range	6	16	2	5	7	6
Mean	91.50	70.25	79.50	92.00	93.75	92.00

One result is out of tolerance.

Repeatability/Reproducibility

For each method, the following linear mixed model has been fitted:

$$y_{ijk} = \mu + \alpha_i + b_j + (\alpha b)_{ij} + e_{ijk}$$

in which:

. y_{ijk} is the observed percent of normal seedlings in rep *k* of lot *i* and lab *j*.

. μ is the intercept.

. α_i is the fixed effect of lot *i*.

. b_j is the random effect of lab *j*. b_j ~ i.i.d. $N(0, \sigma^2_{Lab})$.

. $(\alpha b)_{ij}$ is the random interaction effect between lot *i* and lab *j*.

$(\alpha b)_{ij}$ ~ i.i.d. $N(0, \sigma^2_{Lot \times Lab})$.

. e_{ijk} are the residuals. e_{ijk} ~ i.i.d. $N(0,\sigma^2)$.

Repeatability standard-deviation is then given by $S_r = \sqrt{\hat{\sigma}^2}$ and reproducibility standard-deviation by $S_R = \sqrt{\hat{\sigma}^2 + \hat{\sigma}^2_{Lab} + \hat{\sigma}^2_{Lot \times Lab}}$.

The dispersion factor is calculated as $f_r = \sqrt{\dfrac{m\,\hat{\sigma}^2}{\overline{p}_{...}(100 - \overline{p}_{...})}}$ where $\overline{p}_{...}$ is the overall average percentage of normal seedlings and *m* is the number of seeds per rep (*m* = 100 in this study). If f_r > 1 one speaks of over dispersion because the data have larger variance than expected under the assumption of a binomial distribution.

Repeatability of the results *(calculated with and without Lab 3):*

With Lab 3:

TP 20 <=> 30 C

$\overline{p}_{...}$	S_r	f_r
85.44	3.01	0.85

TP 20 C

$\overline{p}_{...}$	S_r	f_r
85.65	3.64	1.04

TP 25 C

$\overline{p}_{...}$	S_r	f_r
84.42	3.89	1.07

Without Lab 3:

TP 20 <=> 30 C

$\overline{p}_{...}$	S_r	f_r
90.55	3.09	1.06

TP 20 C

$\overline{p}_{...}$	S_r	f_r
86.3	3.78	1.1

TP 25 C

$\overline{p}_{...}$	S_r	f_r
87.58	4.02	1.22

Repeatability standard deviations are acceptable for the three methods (f_r close to 1), except for TP 25°C when the analysis is performed without Lab 3.

Reproducibility of the results *(calculated with and without Lab 3):*

TP 20 <=> 30 C - With Lab 3

$\bar{p}_{...}$	S_R	$\hat{\sigma}^2_{Lab}$	$\hat{\sigma}^2_{Lot\times Lab}$
85.44	17.04	10.19	13.32

TP 20 <=> 30 C - Without Lab 3

$\bar{p}_{...}$	S_R	$\hat{\sigma}^2_{Lab}$	$\hat{\sigma}^2_{Lot\times Lab}$
90.55	4.69	2.51	2.48

TP 20 C - With Lab 3

$\bar{p}_{...}$	S_R	$\hat{\sigma}^2_{Lab}$	$\hat{\sigma}^2_{Lot\times Lab}$
85.65	8.1	6.63	2.91

TP 20 C - Without Lab 3

$\bar{p}_{...}$	S_R	$\hat{\sigma}^2_{Lab}$	$\hat{\sigma}^2_{Lot\times Lab}$
86.3	8.75	7.22	3.19

TP 25 C - With Lab 3

$\bar{p}_{...}$	S_R	$\hat{\sigma}^2_{Lab}$	$\hat{\sigma}^2_{Lot\times Lab}$
84.42	14.99	7.72	12.24

TP 25 C - Without Lab 3

$\bar{p}_{...}$	S_R	$\hat{\sigma}^2_{Lab}$	$\hat{\sigma}^2_{Lot\times Lab}$
87.58	9.07	7.93	1.82

The inter-laboratory variability is small for TP 20 <=> 30° C and TP 25° C compared to TP 20, when Lab 3 is not taken into account in the analysis. When Lab 3 is considered in the calculation, the comparison gives the opposite results, but the differences are much larger.

Conclusions

The results of the study suggest to remove Lab 3 from the analysis, due to inter-laboratory variability that results from its inclusion in the study.

Method 3 (TP 25 °C) did not provide an acceptable repeatability standard deviation.

Method 2 (TP 20 °C) showed a large inter-laboratory variability compared to other methods. This is also understandable looking to the comments submitted my several participants: it is their opinion that seedling evaluation is more challenging when seeds are germinating at 20 °C.

The recommendation from the ISTA Flower Seed Testing Committee is to include in the ISTA Rules the following germination method for *Eustoma exaltatum*:

TP; 20 <=> 30 °C (1st count 4-7 days; final count: 21 days).
Additional treatment: light recommended

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ISTA Method Validation for Seed Testing-V1.0 (https://www.seedtest.org/en/method-validation-programme-_content---1--1254.html)
Standard Operating Procedure: Method validation -process administration (TCOM-SOP-05- Method validation -process V1.0 - https://www.seedtest.org/en/method-validation-programme-_content---1--1254.html)

ISHI-Veg validation report for pre-screening untreated cabbage seeds by bio-PCR and seed extract PCR for the detection of Xanthomonas campestris pv. Campestris to support C.7.1.

Maaike Bruinsma (Naktuinbouw); Thomas Baldwin (GEVES); Camille Ponzio (Naktuinbouw).

1. Introduction

Xanthomonas campestris pv. *campestris* (Xcc) (Pammel, 1895; Dowson, 1939) is a Gram-negative bacterium and is the causal agent of black rot in *Brassica oleracea* and other crucifers. The disease was first reported in the USA in the late 1880s. Since then, it has spread worldwide and is of high economic importance (CABI, 2014).

Xcc survives by overwintering in soil and seeds (Schaad, 1982; Schaad and White, 1974) and in cruciferous weeds near cabbage fields (Schaad and Dianese, 1981). Seeds are an important source of inoculum (Russel, 1898; Walker, 1952; Schultz and Gabrielson, 1986). Therefore, the use of pathogen-free seeds has been recommended for several decades (Williams, 1980; Schaad, 1988). The current reference method for the detection of Xcc on Brassica seeds is based on dilution plating (ISTA Rules 7-019a and b for untreated and treated seeds, respectively: Roberts and Koenraadt, 2014; Asma *et al*., 2014).

However, in classic dilution plating, a common problem is the growth of saprophytes. Excessive growth of saprophytes can hamper the recognition and isolation of Xcc colonies, even at high dilutions. Another disadvantage is the duration of the test to obtain the result. Therefore, several laboratories have developed molecular-based assays to detect Xcc, with the advantages of being sensitive, specific and faster. However, molecular methods do not give any information about viability of the pathogens not their pathogenicity (see ISF's Viewpoint on Indirect Seed Health Tests, http://www.worldseed.org/wp-content/uploads/2015/10/Indirect_Seed_Health_Tests_2013.pdf). Therefore, molecular methods are used as a pre-screen followed by a bio-assay or grow-out.

The aim of the molecular-based method validation study was to measure the performance of two optional pre-screening methods - seed extract PCR and bio-PCR - in the detection of Xcc in untreated cabbage seeds. A PCR pre-screen must allow for the identification of true negative (healthy) seed lots and all putatively positive seed lots must be tested for confirmation of infection. Accordingly, if the pre-screen result is negative, the seed lots are considered to be healthy. If cabbage seed lots are found to be positive in the pre-screen, they are tested again using the reference method (dilution plating) as developed by Sato et al. (2015) (ISTA Rule 7-019a). A flow chart describing the sequence of methods and decisions in testing cabbage seed to detect the presence of Xcc is presented in Appendix 1.

Performance criteria were evaluated independently by Naktuinbouw and Vilmorin to compare seed extract PCR and bio-PCR, respectively, against the reference method. Validation data for seed extract PCR (analytical sensitivity & specificity, selectivity, repeatability, reproducibility and robustness) are

covered in **Chapter 2** while data for bio-PCR (analytical sensitivity, repeatability, and reproducibility) are the subject of **Chapter 3**. In **Chapter 4** the results of the comparative test are presented. The comparative test was carried out to examine the two pre-screening methods in relation to the dilution-plating reference method (Sato et al. 2015, ISTA Rule 7-019a).

It is important to note that the work in Chapters 2, 3 and 4 were carried out in different laboratories and in different years. The validation studies on SE-PCR and bio-PCR were not done in the perspective of the comparative test, and thus there can be discrepancies between the methodologies presented within these studies and the method for which ISTA approval is sought via this report. The validation of the SE-PCR method was performed at Naktuinbouw as part of the EU-TESTA project (full title: TESTA- Seed Health: Development of Seed Treatment Methods, Evidence for Seed Transmission and Assessement of Seed Health) and the report was completed in 2015. The bio-PCR method was developed and internally validated at Vilmorin SA laboratories, in the period 2010-2011. The comparative test for ISTA was completed at a later date, with the test plan submitted and accepted mid-2015. The CT samples were sent to participants in February 2016. Addition of the validation data produced by Naktuinbouw and Vilmorin SA relevant to the CT was included to make the report more complete.

2. Validation of seed extract PCR by Naktuinbouw for detection of Xanthomonas campestris pv. campestris (Xcc) on untreated cabbage seeds (Brassica oleracea)

The method validated is a seed extract PCR based on two complementary Taqman assays, one specific for Xcc developed by Köhl *et al*. (2011) and the other detecting both Xcc and *X.c.* pv. *raphani*, *X.c.* pv. *barbarae*, *X.c.* pv. *incanae* and *X.c*. pv. *aberrans*, the causal agent of leaf spot disease of cruciferous and solanaceous hosts, as developed by Berg *et al*. (2006). Seed extract PCR detects pathogens directly in seed extract, without a culture step. Detection is in this case with real-time Taqman PCR. Because no culture step for bacteria is present in this method, it does not discriminate between viable and dead cells. The bacterium *Acidovorax cattleyae* (Acat) was used as an internal extraction control (IEC) in the seed extract PCR. The IEC was added as a spike to the seed wash prior further processing and was used to monitor the extraction of DNA and amplification in PCR. The protocol is described in the method proposal.

The following performance characteristics will be determined: analytical sensitivity, analytical specificity, selectivity, repeatability and reproducibility, trueness and robustness.

2.1. ANALYTICAL SENSITIVITY

2.1.1. Introduction

Analystical sensitivity is defined as 'The lowest value, in a laboratory sample, of the target pathogen or pest, which can still be determined with a certain degree of reliability' (Anonymous, 2010).

Since the new assay should be as sensitive as dilution plating, testing by dilution plating is incorporated in this experiment. The requirements for this performance characteristic is the detection

of at least the lowest concentration detected by dilution plating on the CS20ABN and mFS semi-selective media.

2.1.2. Materials and methods

2.1.2.1. Analytical sensitivity

The analytical sensitivity of the assay was determined by three dilution series, tested in triplicates. Negative seed extract (ZZB700) was spiked with Xcc (NBC278). The positive seed extracts were diluted with negative seed extract in ten-fold dilution series. The extracts were tested according to the protocol in the method proposal.

2.1.2.2. Calibration curve

In order to determine the number of cell per mL detected in the PCR reaction, a calibration curve was prepared. Cell cultures of Xcc (NBC278) were grown for 72 h in three separate tubes containing 4 mL of liquid medium (TSB). Each series of three 4 mLtubes was pooled in a 50 mL Greiner tube. The bacterial suspension was centrifuged at 3,000 × *g* for 10 min and resuspended with 10 mL of 0.05 M sPBS twice. Using a spectrophotometer the suspension was then adjusted to an OD_{620} of 1.0. DNA of three replicates of 500 µL of this bacterial suspension was isolated and purified using the DNeasy blood and tissue kit (Qiagen), according to the manufacturer's recommendation for Gram positive bacteria. The amount of DNA was determined using a Nanodrop. Based on the yield obtained and the genome size of 5.1 Mb (Da Silva et al, 2002; Qian et al, 2005), the number of cells was calculated according using the following formula:

number of copies = (amount * 6.022×10^{23}) / (length * 1×10^{9} * 660) in units: number = (ng * number/mole) / (bp * ng/g * g/mole of bp) (Staroscik, 2015)

Using this DNA, three independent ten-fold dilution series were prepared in TE containing the Acat DNA with a Ct-value of approximately 27 (tested prior to use). Each dilution series was pooled to correct for dilution errors. A triplicate PCR reaction of the obtained dilution series was performed for each dilution. Based on the obtained Ct-values a calibration curve was prepared for both the FAM- and VIC-labeled probes. To determine the number of colony forming units (CFU)/mL, the dilution series (without Xcc or Acat) were also plated in duplicate onto GF, CS20ABN and mFS medium.

2.1.3. Results

To determine the analytical sensitivity, in total of nine replicates were tested per dilution. Nine seed extract subsamples Xcc was detected in dilutions up to 10^{7} for both Xcc and Xcr (FAM) and Xcc (VIC), and subsequent dilutions were mostly negative (Table 1A and 1B). A curve was fitted to the results for both FAM and VIC (figure 1). This results in a detection limit of 46 cells/mL for FAM and 39 cells/mL for VIC at a 95% probability.

Using the DNA calibration curves, it was determined which Ct-value corresponds to these detection limits (figure 2). For FAM the calibration curve was calculated to be Ct = -1.423 ln(conc) + 43.815,

therefore the Ct-value corresponding to 46 cells/mL is 38.4. For VIC the calibration curve was calculated to be Ct = -1.427 * ln(conc) + 44.046, therefore the Ct-value at 39 cells/mL is 38.6.

On semi-selective media, all six samples were detected in dilutions up to a theoretical concentration of 103 cells/mL (at least one of the media was detected as positive), subsequent dilutions were mostly negative (table 2). Therefore, the detection limit of dilution plating in seed extract was around 100 CFU/mL. For dilution plating on GF, a non-selective medium, Xcc spiked in buffer could be detected up to dilutions of 10^8 on four out of six plates.

Table 1A. Three dilution series of spiked Xcc in negative seed extract in triplicate for Xcc/Xcr (FAM). Ct-values per dilution (cells/mL).

Sample		1.03E+06	1.03E+05	1.03E+04	1.03E+03	103.00	10.30	1.03	0.10
1	A	20.22	23.27	26.75	29.89	34.04	34.43	35.04	>40
	B	20.34	23.14	26.45	30.6	33.29	>40	36.04	>40
	C	20.12	23.33	26.83	30.04	34.6	>40	>40	36.9
2	A	20.1	23.29	26.68	29.94	32.36	35.13	>40	>40
	B	20.25	23.09	26.65	30.47	33.68	35.05	>40	>40
	C	20.11	23.21	26.74	30.06	33.48	>40	>40	>40
3	A	20.19	23.41	26.64	29.85	34.06	35.1	>40	>40
	B	20.26	23.04	26.75	31.01	33.86	34.63	>40	>40
	C	20.11	23.29	26.72	30.13	33.3	>40	>40	>40
Positive reactions		9	9	9	9	9	5	2	1

Table 1B. Three dilution series of spiked Xcc in negative seed extract in triplicate for Xcc primerset) labelled VIC). Ct-values per dilution (cells/mL).

Sample		1.03E+06	1.03E+05	1.03E+04	1.03E+03	103.00	10.30	1.03	0.10
1	A	20.88	23.93	27.23	30.33	34.37	>40	>40	>40
	B	21.02	23.72	27.14	31.21	35.45	>40	>40	>40
	C	20.65	24.1	27.54	30.97	35.06	>40	>40	>40
2	A	20.62	24.11	27.25	30.75	35.09	>40	>40	>40
	B	21.1	23.59	27.16	31.34	33.29	>40	>40	>40
	C	20.62	24.1	27.36	30.88	35.08	>40	>40	>40
3	A	20.81	24.32	27.26	30.66	33.78	35.59	>40	>40
	B	21.11	23.61	27.29	31.32	35.45	35.17	>40	>40
	C	20.58	24.21	27.39	30.97	34.41	>40	>40	>40
Positive		9	9	9	9	9	2	0	0

reactions	

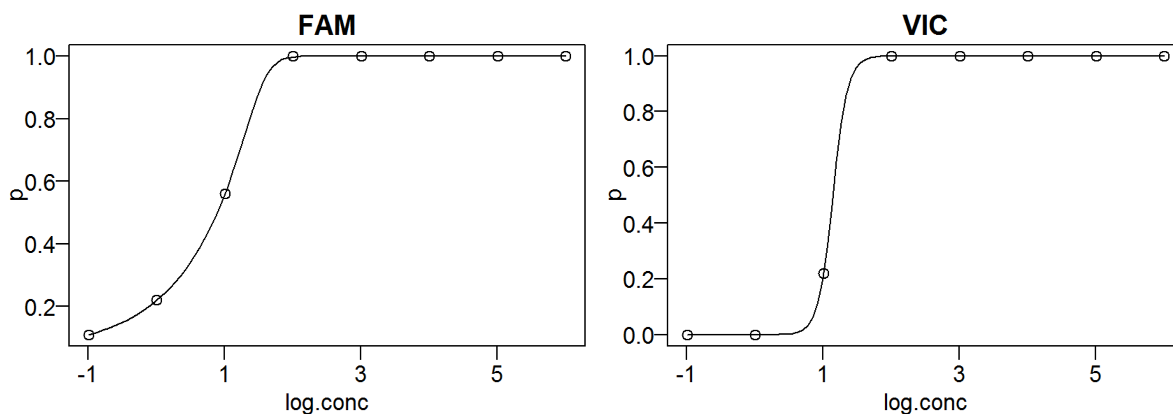

Figure 1. Fitted models for the analytical sensitivity of two *Xanthomonas campestris* pv. *campestris* Taqman PCRs, log-linear models A) for Köhl Taqman and B) for Berg Taqman.

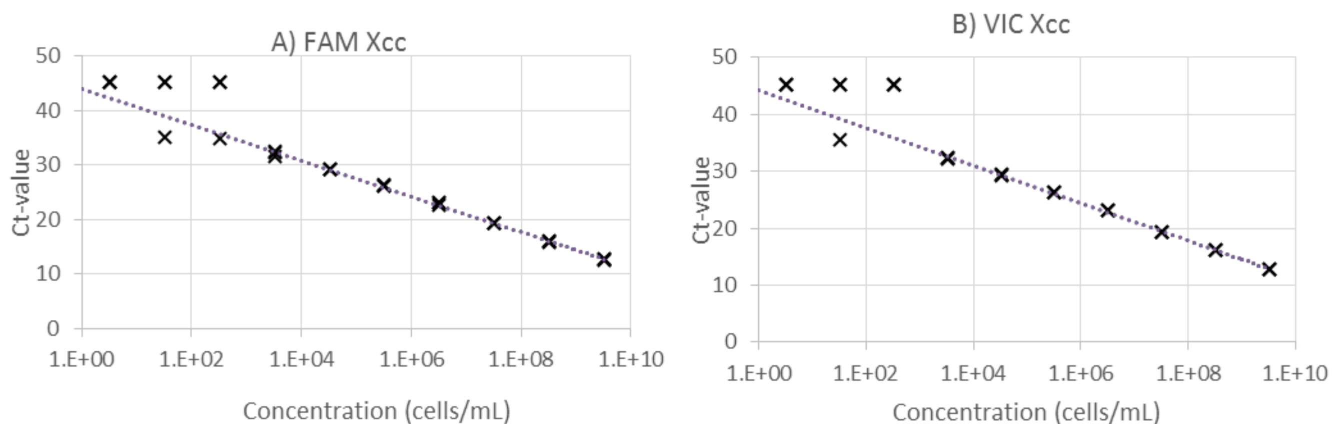

Figure 2. Calibration curves for *Xanthomonas campestris* pv. *campestris*.
Results obtained with primer sets for A) Köhl Taqman and B) Berg Taqman are presented.

Table 2. Dilution plating results for *Xanthomonas campestris* pv. *campestris*. Numbers per plate per dilution.

A medium	replicate	1.03E+06	1.03E+05	1.03E+04	1.03E+03	103.00	10.30	1.03	0.10
CS20ABN	1A	∞	∞	∞	60/16	40/3	25/2	0	0
	1B	∞	∞	∞	50/20	40/6	30/0	0	0
	1C	∞	∞	∞	70/14	45/1	40/0	0	0
	2A	∞	∞	∞	70/28	50/3	40/0	0	0
	2B	∞	∞	∞	60/25	45/2	35/0	0	0
	2B	∞	∞	∞	60/18	50/3	50/0	0	0
mFS	1A	∞	∞	∞	50/18	9/5	4/0	0	0
	1B	∞	∞	∞	30/18	8/4	7/0	0	0
	1C	∞	∞	∞	25/21	20/5	4/1	0	0
	2A	∞	∞	∞	30/20	10/1	9/0	0	0
	2B	∞	∞	∞	30/20	5/2	7/0	0	0
	2B	∞	∞	∞	35/30	6/0	10/0	0	0
Positive replicates		12	12	12	12	11	2	0	0

B medium	replicate	1.03E+06	1.03E+05	1.03E+04	1.03E+03	103.00	10.30	1.03
GF	1A	∞	∞	∞	90	10	3	0
	1B	∞	∞	∞	100	8	0	0
	1C	∞	∞	∞	80	10	2	0
	2A	∞	∞	∞	90	11	5	0
	2B	∞	∞	∞	80	7	2	0
	2B	∞	∞	∞	90	11	0	0
Positive replicates		6	6	6	6	6	4	0

Numbers represent the number of saprophytes over confirmed Xcc. A) seed extract on two selective media, CS20ABN and mFS, and B) in buffer one non-selective medium, GF. Header row:Theoretical concentration in cells/ml.

2.1.4. Discussion

The limit of detection at 95% probability was 46 cells/mL for the Köhl Taqman and 39 cells/mL for the Berg Taqman. These results reveal that the detection limits of both Taqman PCRs are slightly lower than the detection limit of the dilution plating method using CS20ABN and mFS, therefore the requirement for analytical sensitivity is obtained. This measurement is based on spiked samples with freshly prepared Xcc culture. In naturally contaminated samples, it is expected that samples with viable Xcc also carry some dead Xcc that will be detected by the PCR method, and that this will therefore increase the sensitivity of the PCR method.

2.2. ANALYTICAL SPECIFICITY

2.2.1. Introduction

Analytical specificity represents 'The ability of a method to distinguish the target organism (pathogen) from other organisms, whether related or not, and the extent to which the analysis can distinguish (known) variants of the organism' (Anonymous, 2010).

For this assay the requirement is that the two primer sets for Xcc together do not miss any isolates and few false-positives are acceptable. Therefore the diagnostic sensitivity - the probability of a positive test result given that the sample is positive - should reach 1. The diagnostic specificity- the probability of a negative test result given that the sample is not contaminated - for the two primer sets is required to be above 95%.

2.2.2. Materials and methods

A collection of 70 Xcc and look-alike Xcc isolates from Naktuinbouw, selected based on genetic and geographic variation and previously characterized by AFLP, were tested with the triplex Taqman PCR to determine the specificity of the primer sets (for sequences see Table 3). The DNA was isolated from an extract with approximately 10^6 cells/mL. In literature, the Köhl and Berg primer sets for Xcc and Xca have already been tested. Data are presented in Appendix 6.

Table 3. Primer sets triplex Taqman PCR *Xanthomonas campestris* pv. *campestris (Xcc), Xanthomonas campestris pv. raphani (Xcr) and Acidovorax cattleyae (Acat)*.

Primer name	Target	Developed by	Sequence
XCC F	Xcc	Köhl et al, 2011	5' CGG ATG CAG AGC GTC TTA CA 3'
XCC R			5' GTG CAT AGG CCA CGA TGT TG 3'
XCC Pr			5' FAM-CAA GCG ATG TAC TGC GGC CGT G-NFQ-MGB 3'
DLH153-F	Xcc/Xcr	Berg et al, 2006	5' GTA ATT GAT ACC GCA CTG CAA 3'
DLH154-R			5' CAC CGC TCC AGC CAT ATT 3'
P7			5' VIC-ATG CCG GCG AGT TTC CAC G-BHQ1 3'
Acat – F	Acat	Naktuinbouw,	5' TGTAGCGATCCTTCACAAG
Acat – R		elongated primer set	5' TGTCGATAGATGCTCACAAT
Acat – P			5' Texas Red-CTT GCT CTG CTT CTC TAT CAC G-BHQ2 3'

Acat: *Acidovorax cattleyae*.

2.2.3. Results

Of the 70 isolates tested by AFLP, 15 isolates were out of the tree and 55 in the tree. Most isolates responded as expected with both Taqmans. However, seven isolates were positive for one or both

Taqmans for isolates out of the tree and some were negative when they were in the tree. In these cases a pathogenicity assay was performed to confirm results obained. The results confirmed that all isolates positive with the Berg Taqman were indeed pathogenic Xcc or Xca. For the Köhl Taqman, if the Köhl Taqman but not the Berg Taqman was positive, the isolates were not pathogenic (see table 4and appendix 7).

Table 4. Xcc isolates and lookalikes and their response in AFLP, Köhl Taqman and Berg Taqman. (In: based on AFLP study isolate is Xcc or Xcr; out: based on AFLP study isolate is not Xcc or Xcr)

No. of isolates	AFLP	Köhl	Berg	Remarks
38	In	Yes	Yes	
12	In	No	Yes	Xca
2	In	Yes	No	Both negative in pathogenicity assay
3	In	No	No	All 3 negative in pathogenicity assay
13	Out	No	No	
1	Out	Yes	No	Negative in pathogenicity assay
1	Out	Yes	Yes	Pathogenicity assay: Xcc

2.2.4. Discussion

Results revealed that the specificity is acceptable. No pathogenic Xcc isolates were missed using both Taqman assays. Two isolates were detected as false-positives based on the AFLP data. However, the pathogenicity assay showed that one was indeed a pathogenic isolate. Three isolates obtained Köhl Taqman positive and Berg Taqman negative results and are likely non-pathogenic.

2.3. SELECTIVITY

2.3.1. Introduction

Selectivity is 'The ability of a method to distinguish the target organism (Xcc) from other components in the sample'.(Anonymous, 2010). In this case, another matrix, radish seeds instead of *Brassica* seeds, was used to determine if Xcc could still be detected. The requirement is that dilution plating positive seed lots are also identified as positive by the direct Taqman PCR method.

2.3.2. Materials and methods

Due to the limited availability of (Xcc-tested) radish seed lots, three lots potentially Xcc-contaminated were selected. The three seed lots tested were ZZB472, ZZB701 and ZZB704. As Brassica seeds are usually tested in subsamples of 10,000 seeds and radish in subsamples of 2,500 only, seeds were tested using both subsample sizes to compare the detection and potential inhibition due to contaminants in large sample sizes. The size of the ZZB472 lot did not allow for testing of a 10,000 seed subsample, so seeds from the ZZB703 lot (radish seed lot negative for *Pseudomonas syringae* pv.

maculicola and Xcc) were used to complement. For each sample size of 2,500 and 10,000 seeds three replicates were tested with both seed extract PCR and dilution plating.

2.3.3. Results

The results for 2,500 and 10,000 seeds were similar (table 5). For the seed lot ZZB701, the Ct value obtained in both sample sizes was around 22, and in dilution plating 6-60 suspect colonies were detected in the 100x dilution. Seed lots ZZB472 and ZZB704 were found to be negative in dilution plating, but had Ct-values between 30 and 35.

Table 5. Detection of *Xanthomonas campestris* pv. *campestris* on seeds of radish in subsamples of 2,500 or 10,000 seeds.

No. of seeds	Seed lot	Subsample	Xcc (FAM)	Xcc/Xcr (VIC)	Acat (Texas Red)	Dilution plating result
2500	ZZB 472	A	31.35	31.4	25.74	Neg
		B	31.64	31.91	26.14	Neg
		C	31.92	32.05	26.06	Neg
	ZZB 701	A	22.23	22.63	25.78	Pos
		B	22.68	23.21	25.79	Pos
		C	22.99	23.29	25.98	Pos
	ZZB 704	A	30.58	31.09	26.34	Neg
		B	30.47	31.75	26.55	Neg
		C	30.53	31.16	26.24	Neg
10000	ZZB 472 (1000) + ZZB 703 (9000)	A	35.12	34.17	26.16	Neg
		B	35.79	33.05	26.41	Neg
		C	33.35	34.17	26.34	Neg
	ZZB 701	A	22.07	22.35	26.63	Pos
		B	22.05	22.35	26.61	Pos
		C	21.13	21.43	26.28	Pos
	ZZB 704	A	30.49	30.55	27.04	Neg
		B	30.33	30.53	26.67	Neg
		C	30.06	30.27	26.25	Neg

2.3.4. Discussion

Since no difference was observed in results for the two subsample sizes, it was decided to use subsamples of 2,500 seeds for radish and 10,000 seeds for *Brassica*. Unfortunately, only seed lot, ZZB701, was detected as infected using both dilution plating and seed extract PCR assays. For the other two seed lots, it was not possible to determine whether these are seed extract PCR false-

positives (e.g. caused by dead cells) or dilution plating false-negatives. However, the three seed lots were detected as positive by seed extract PCR, showing that this method meets the requirements set for this performance criteria. It is recommend to first perform parallel testing to identify other positive seed lots, and thus determine with certainty that no positive seed lots will be missed with the new assay.

2.4. REPEATABILITY AND REPRODUCIBILITY

2.4.1. Introduction

Repeatability is 'the degree of correspondence between the results of successive measurements of the same measurand performed under equal conditions' (Anonymous, 2010). Reproducibility is 'the degree of correspondence between the results of measurements of the same measurand performed under varying measurement conditions' (Anonymous, 2010). The requirement for this characteristic was set at at least 95%.

2.4.2. Materials and methods

Three samples (A,B,C) were prepared as presented in Table 6 in order to obtain 16 replicates per sample. For repeatability, eight replicates were tested on one day by one operator at the Naktuinbouw R&D department. For reproducibility, eight replicates were tested over several days by different operators at the routine laboratory of Naktuinbouw.

Table 6. Sample composition for repeatability and reproducibility.

Sample A	Extract of 10,000 seeds ZZB473 diluted 1000x in negative seed extract of ZZB547
Sample B	300 seeds ZZB695 + 9,700 seeds ZZB694
Sample C	100 seeds ZZB689 + 9,900 seeds of negative seed lot ZZB700

2.4.3. Results

The three contaminated samples were tested with eight replicates each. All replicates gave the same qualitative test result. The quantitative variation between replicates was limited (Figure 3 and Appendix 8).

2.4.4. Discussion

Repeatability and reproducibility were good with small variation between replicates , even for the low-level infected sample (sample A).

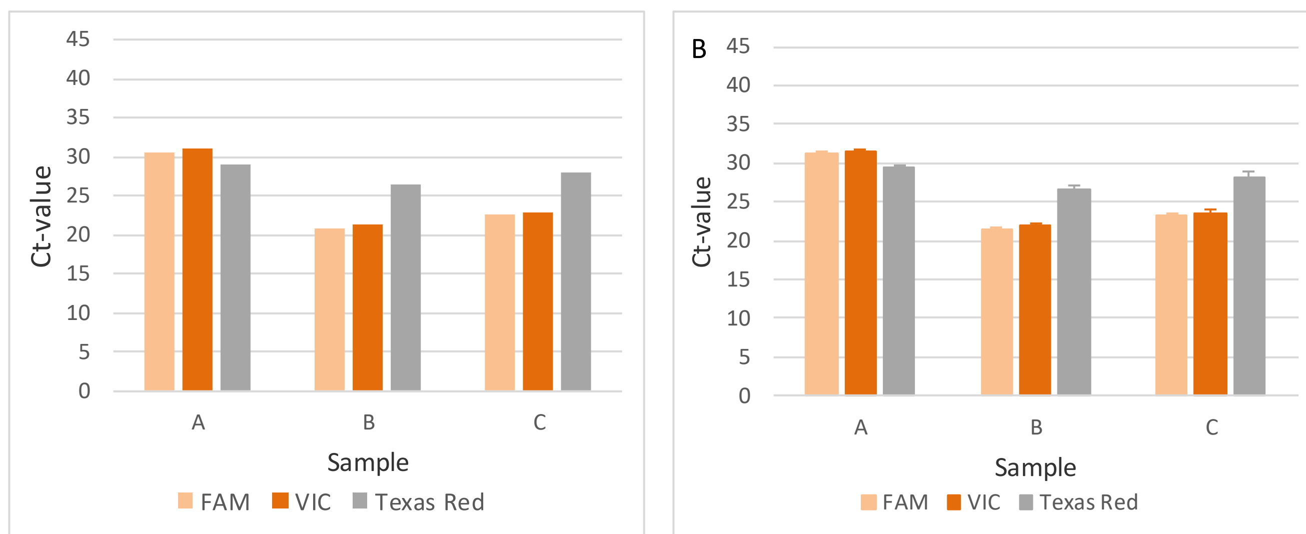

Figure 3. A) Repeatability and B) reproducibility for three samples infected with *Xanthomonas campestris* pv. *campestris* (n=8). Average + standard deviation

2.5. ROBUSTNESS

2.5.1. Introduction

Robustness represents 'The degree of insensitivity of the results of a measurement to deviations in procedure, circumstances and nature of materials like these may occur in practice' (Anonymous, 2010).

Due to the soaking time of this assay and the duration of several extraction steps, it is not possible to perform the whole assay in one day in a routine setting. Therefore, the effect of cooling or freezing the extract or pellet on several points in the protocol on the detection of Xcc was investigated. No significant deviation from treatment F (table 8) is allowed.

2.5.2. Materials and methods

Three samples were prepared that were subjected to six different treatments and tested in eightfold. The sample composition is described in table 7 and the six treatments in table 8.

Table 7. Sample composition for robustness experiment.

Sample 1	10,000 seeds ZZB473
Sample 2	100 seeds ZZB689 + 9900 seeds of negative seed lot ZZB700
Sample 3	10,000 seeds ZZB700

Table 8. Treatments for robustness experiment.

A	Make seed wash extract and freeze[1] after soaking

B	Make seed wash extract and cool[2] after soaking
C	Concentrate bacteria and freeze pellet
D	Lysis and freeze
E	Isolate DNA and freeze eluate
F	Do not freeze or cool (complete protocol on one day)

[1] freezing = -20°C, [2] cooling = 4°C

2.5.3. Results

No significant difference was observed between any of the treatments for both target primer sets (Figure 4 and Appendix 9).

2.5.4. Discussion

No significant effect of freezing or cooling steps was observed. Therefore, the protocol can be stopped and continued at a later time point without significantly affecting the results of the assay. The requirement for the robustness was met.

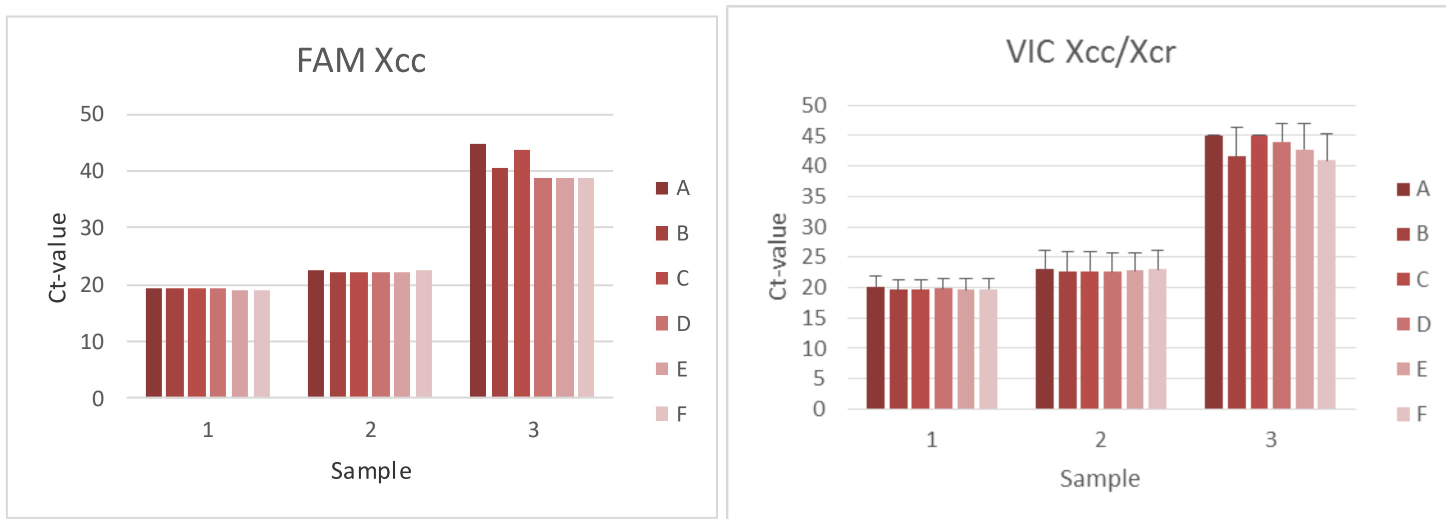

Figure 4. Average Ct-values (+ standard deviation) for three samples for six treatments (n=8). A) freeze after soaking B) cool after soaking, C) freeze pellet, D) freeze after lysis, E) freeze DNA, F) no freezing or cooling.

3. Validation of a bio-PCR method by Vilmorin SA for the detection of *Xanthomonas campestris pv. campestris and Xanthomonas campestris pv. raphani* on radish and cabbage seeds

3.1. INTRODUCTION

3.1.1. Scope of the test

A new method was developed as a pre-screen to the dilution plating method by using gel-based bio-PCR and by real-time SYBR-green based bio-PCR methods. This method was developed and internally validated in the Vilmorin laboratory (2010-2011). Bio-PCR consists of biological amplification followed by conventional gel-based (electrophoresis) PCR or real-time SYBR-green based PCR. Prior to the PCR step, bacterial cells are cultured on media, which increases method sensitivity and allows for earlier detection of target bacteria.

The same primers were retained for the gel based bio-PCR method, namely DLH120-125 primers. These primers were replaced by the DLH153-154 primers for the real-time bio-PCR method. These primers also target the *hrpF* gene, but amplify a smaller PCR product adapted for real-time PCR (Berg et al., 2006). The DLH153-154 primers have been tested on a range of Xcc and Xcr strains (see *Appendix 6)*

The performance characteristics that have been tested are: Analytical specificity, Repeatability and Reproducibility.

3.1.2. Validation material

Performance characteristic	Validation material
Analytical sensitivity	Spiked and blank laboratory samples
Repeatability/reproducibility	Naturally infected seed samples

3.2. ANALYTICAL SENSITIVITY

Seed extracts from three subsamples of 10,000 seeds were spiked with different dilutions of Xcc and Xcr control isolates. Each dilution was also plated on mFS media, which allowed for a visual quantification of the number of colony forming units (CFUs). The analytical sensitivity was identified with:

- A sample of 30,000 seeds from an uninfected untreated radish seed lot (Tables 10 and 11).
- A sample of 30,000 seeds from an uninfected disinfected radish seed lot (Tables 12 and 13).
- A sample of 30,000 seeds from an uninfected untreated cabbage seed lot (Tables14 and 15).
- A sample of 30,000 seeds from an uninfected disinfected cabbage seed lot (Tables 16 and 17)

Seed wash from each subsample of each seed lot was artificially inoculated by mixing 4.5 mL seed wash with 0.5 mL bacterial dilutions serially diluted from d0 to d7. On each plate, 100 µL of the seed wash macerate was plated. Positive and negative controls were included and 100 µL of each bacterial dilution was plated on mFS for visual quantification of CFU numbers after 4 days incubation. For each test, the number of CFUs counted on each control plate was used to estimate the number of CFUs present in each artificially inoculated seed wash subsamples.

All subsamples were tested using the DLH120-125/Zup2309-2310 duplex PCR method. The extractions from the untreated radish seeds and the disinfected cabbage seeds were also tested using the qPCR method. The number of CFUs detected by PCR and real-time PCR were identical for the untreated radish seed extractions. Results showed that the real-time PCR method was more sensitive on one of the subsample from cabbage disinfected seeds (Table 31).

A total of 12 subsamples were inoculated with either Xcc or Xcr: The mean CFU mL concentrations detected were 37.8 and 34.8 for Xcc and Xcr, respectively (Table 23). By adding 3 standard deviations as proposed in the *Dutch guideline for plant pathogens and pests version 2.* The detection limits were estimated to be 201 and 197 CFU mL.

Table 9. Gel-based PCR detection limits CFU/mL (mean+ 3 standard deviations) on radish and cabbage seeds

Subsample	Detectable concentrations (CFU/mL)	
	Xcc	Xcr
J46465.1	4.6	16
J46465.2	21	16
J46465.3	21	16
J73553.1	23	20
J73553.2	192	2
J73553.3	23	20
J63887.1	24	15
J63887.2	24	150
J63887.3	24	150
J73552.1	13	4
J73552.2	13	4
J73552.3	1.3	4
Mean	**37.8**	**34.8**
SD	54.3	54.2
Detection Limit	201	197

3.2.1. Radish untreated seed lots

Table 10. Number of CFUs observed on control plates 4 days after plating (CFU/100 µL)

Dilution	Number of colonies observed	
	Xcc	Xcr
D6	46	16
D6 (1:2 dilution)	21	6
D7	0	2

Table 11. PCR detection limits CFU/mL (mean+ 3 standard deviations) on untreated radish seeds

Subsample	Detectable concentrations (CFU/mL)	
	Xcc	Xcr
J46465.1	4.6	16
J46465.2	21	16
J46465.3	21	16
Mean	15.5	16
Standard Deviation	9.5	0
Detection Limit	**44**	**16**

3.2.2. Radish disinfected seed lots

Table 12. Number of CFUs observed on control plates 4 days after plating (CFU/100 µL)

Dilution	Number of colonies observed	
	Xcc	Xcr
D5	192	148
D6	23	20
D7	7	2

Table 13. PCR detection limits CFU/mL (mean+ 3 standard deviations) on disinfected radish seeds

Subsample	Detectable concentrations (CFU/mL)	
	Xcc	Xcr
J73553.1	23	20
J73553.2	192	2
J73553.3	23	20
Mean	79.3	14
Standard Deviation	97.6	10.4

Detection Limit	**372**	**45**

3.2.3. Cabbage untreated seed lots

Table 14. Number of CFUs observed on control plates 4 days after plating (CFU/100 µL)

Dilution	**Number of colonies observed**	
	Xcc	**Xcr**
D5	24	ND
D6	0	15
D7	0	0

Table 15. PCR detection limits CFU/mL (mean+ 3 standard deviations) on untreated cabbage seeds

Subsample	**Detectable concentrations (CFU/mL)**	
	Xcc	**Xcr**
J63887.1	24	15
J63887.2	24	150
J63887.3	24	150
Mean	24	105
Standard Deviation	0	78
Detection Limit	**24**	**339**

3.2.4. Cabbage disinfected seed lots

Table 16. Number of CFUs observed on control plates 4 days after plating (CFU/100 µL)

Dilution	**Number of colonies observed**	
	Xcc	**Xcr**
D5	ND	104
D6	13	4
D7	0	0

Table 17. PCR detection limits CFU/mL (mean+ 3 standard deviations) on disinfected cabbage seeds

Subsample	**Detectable concentrations (CFU/mL)**	
	Xcc	**Xcr**
J73552.1	13	4
J73552.2	13	4
J73552.3	1.3	4 (*0.4 qPCR*)
Mean	9.1	4
Standard Deviation	6.8	0

Detection Limit	29	4

3.3. REPEATABILITY AND REPRODUCIBILITY

Eight different untreated radish seed lots were selected for repeatability and reproducibility tests. A sample from each seed lot was divided into three sub-samples which were tested as independent samples using the Bio- PCR method. A test of repeatability was done by the same user testing the samples in duplicate. A second user tested the method at a different time point (Table 14).

Table 18. Overview of the validation process for *Xanthomonas* bio-PCR on untreated Radish seeds

Seedlot	N° lot	Dilution Plating result	Lecture	Test 1A User 1		Test 1B User 1		Test 2 User 2	
				PCR	qPCR	PCR	qPCR	PCR	qPCR
1	J53215	Infected (3/3)	D1	+	+	+	+	+	+
2	J65365	Infected (2/3)	D1	+	+	-	-	+	+
3	J65367	Infected (2/3)	D2	+	+	+	+	+	+
4	J64993	Infected (1/3)	D2	+	+	-	-	+	+
5	J64990	Infected (1/3)	D1	+	+	+	+	+	+
6	J64958	Infected (1/3)	D2	+	+	+	+	+	+
7	J65346	Non-Infected	D2	+	+	+	+	+	+
8	J60522	Non-Infected	D0	-	-	-	-	-	-

Infected= (number of sub-samples positive/total sub-samples)

For all tests for measuring repeatability and reproducibility, similar results were obtained from both the PCR and real-time PCR when used to test the same sub-samples. The consistency of the results obtained with the two methods, as well as the similar detection limits, allows the two amplification methods to be validated for use in this test. The repeatability between sub-samples is 14/16, thus reaching 87.5%. The reproducibility is 22/24, or 91.6%.

Two differences were observed in the repeatability tests: For both J65365 and J64993, only two of the three subsamples were detected as positive. This represents the medium to low contamination of these seed lots observed in the dilution-plating experiments. Using the dilution-plating method and due to the high saprophyte contamination, it was not possible to detect Xcc in the J65346 seed lot, however all three subsamples were detected as positive using the bio-PCR method.

3.4. ROBUSTNESS

The real-time PCR method was used in routine testing on several seed lots in parallel with the dilution- plating method (appendix 10).

Table 19. Comparison between real-time bio-PCR and dilution plating

Number of seed lots	Real-time bio-PCR positive	Real-time bio-PCR negative
Dilution-plating positive	4	0
Dilution-plating negative	3	9

No false-negative results were observed with the bio-PCR method (Table 19). However three seed lots were detected as positive with the bio-PCR method, while they were negative with the dilution-plating method. This depicts an increased sensitivity in the bio-PCR method as compared to the dilution plating method.

During these robustness tests inhibition in certain positive control real-time PCR reactions was observed. The volume of extract (5 µL) in each reaction was found to cause inhibition of the amplification. Different volumes of extract were tested for presence/absence of inhibition of the reaction. No PCR inhibition was observed when using 1 µl or 2 µL (data not shown). The volume of 2µl was selected for the DNA extract volume, in addition the real-time PCR reactions were divided into two simplex reactions to simplify melt curve interpretation.

3.5. CONCLUSION

In conclusion, the real-time PCR reaction conditions below applied on bio-PCR extracts were selected for validation in the ISHI-Veg inter-laboratory comparative test. It was also decided to restrict the scope of real-time bio-PCR method to untreated seeds for ISHI-Veg method.

4. Comparative test for pre-screening untreated cabbage seed by bio-PCR and seed-extract PCR

4.1. COMPARATIVE TEST ORGANIZATION

An interlaboratory comparative test for the detection of *Xanthomonas campestris* pv. *campestris* on Brassica seeds by dilution plating, bio-PCR and seed extract PCR was organized by Naktuinbouw.

4.1.1. Participants and protocol

Ten laboratories participated in this test and were randomly allocated to a number, so that the results remain anonymous. All ten performed the dilution plating according to ISTA rule 7-019a, six laboratories performed the bio-PCR and eight laboratories performed the seed extract PCR according to the PCR protocols as described in the new proposed version of ISTA rule 7-019a. Four laboratories performed both the bio-PCR and the seed extract PCR.

4.1.2. Seed samples

Each laboratory received 15 sub-samples of 10,000 untreated cabbage seeds each (see Table 20 and Appendix 2):

- three subsamples from a healthy lot.
- three of each of the three subsamples with a medium level of Xcc infection obtained by blending a healthy (ZZB-549) or slightly infected lot (ZZB-694) with a naturally Xcc infected lot (ZZB-695).
- three subsamples from a highly and naturally infected lot.

Based on the pre-tests, all healthy subsamples were expected to be negative, all positive samples to be positive with 10^3 – 10^6 CFU/mL for the medium and over 10^6 CFU/mL for the high infection level samples.

DNA of Xcc and *Acidovorax cattleyae* was sent to the laboratories as the Positive Amplification Control and the Positive Extraction Control, respectively, during the seed extract PCR method.

Table 20. Composition of samples for ISTA CT Xcc 2016.

Infection level	Category	no. of sub-samples	Composition of subsamples		
Healthy	1	3	10,000 seeds ZZB-700		
Medium	2.1	3	3,000 seeds ZZB-695	+	7,000 seeds ZZB-694
Medium	2.2	3	1,000 seeds ZZB-695	+	9,000 seeds ZZB-694
Medium	2.3	3	3,000 seeds ZZB-695	+	7,000 seeds ZZB-549
High level	3	3	10,000 seeds ZZB-689		

4.1.3. Notation of results

Participants reported a qualitative (positive/negative) result for each subsample.

4.2. STATISTICAL ANALYSIS

4.2.1. Homogeneity test

The homogeneity test results were analysed using tools provided by ISTA: Seedcalc8 and probpossample-V1. These tools calculate the expected number of positive subsamples in the comparative test and stability test, respectively. Using the results of the homogeneity test, the infection rate in the sample was calculated for each non-homogenous sample. Using the infection rate and a probability of 5%, the expected number of positive subsamples was calculated.

4.2.2. Stability test

The number of positive subsamples per category were compared to the expected number of positive subsamples as calculated based on the homogeneity test.

4.2.3. Comparative test: Analysis of qualitative data

Samples with no expected variation in the qualitative test result (i.e. positive or negative) were analysed according to the Standard NF EN ISO 16140 (AFNOR, 2003). Sensitivity, specificity, accuracy and reproducibility were calculated using the following formulas:

Sensitivity = ΣPA / (ΣPA+ΣND) x 100

Specificity = ΣNA / (ΣNA+ΣPD) x 100

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

Where:

PA = positive agreement

ND = negative deviation

NA = negative agreement

PD = positive deviation

Although there is no fixed rule, values over 80% for sensitivity, specificity and accuracy were deemed acceptable (ISTA, 2013). This minimum value is used here to determine whether a method is acceptable or not.

For the infection level categories where the expected outcome is variable and depends on the percentage of infection, results of the homogeneity test were used as a reference (ISTA, 2013). For each infection level category, accordance (repeatability of qualitative data) and concordance (reproducibility of qualitative data) were calculated using the method and tools developed by Langton *et al.* (2002).

4.3. ANALYSIS OF XCC CT: HOMOGENEITY AND STABILITY TESTS

4.3.1. Dilution plating

4.3.1.1. Homogeneity test

Three sets of 3 subsamples were tested per infection level before the comparative test and after packaging. This included nine healthy subsamples, 27 medium level infected subsamples (nine of each sub category 2.1, 2.2 and 2.3) and nine high infection level subsamples. The homogeneity test for dilution plating showed that all healthy subsamples were negative, 26 of medium infection level subsamples were positive and all high infection level subsamples were positive (see Table 21 and Appendix 3).

Table 21. Results of the homogeneity and stability test for dilution plating per infection level.

Infection level	Homogeneity results	Stability results
1 (healthy)	0/9	0/3
2.1 (medium)	9/9	3/3
2.2 (medium)	8/9	3/3
2.3 (medium)	9/9	3/3
3 (high)	9/9	3/3

Results present the number of positive subsamples over the total number of subsamples

Using the results of the homogeneity test, the sample infection rate was calculated to be 0.02% for category 2.2. At a probability of 5%, the expected number of positive subsamples is thus two to three for category 2.2 (Table 22). In the other infected categories (2.1, 2.3 and 3), all subsamples are expected to be positive, and in the healthy category (1), none of the subsamples are expected to be positive.

Table 22. Probability of number of positive subsamples (k) out of total number of subsamples (n) in the stability and comparative test for category 2.2 calculated using Seedcalc8 and probpossample-V1.

k	**Rate of *k* positive out of *n***
0	0.25%
1	4.75%
2	30.35%
3	64.65%

4.3.1.2. Stability test

The aim of the stability test is to control the stability of the infection status of samples. The test was performed when all participating laboratories had started their tests. All healthy subsamples were negative (no false-positives) and all medium and high infection subsamples were positive (no false-negatives; Table 3 and Appendix 4). Therefore, it was concluded that the infection status of samples was stable.

4.3.2. Bio-PCR

4.3.2.1. Homogeneity test

The seed extracts of the subsamples that were prepared for dilution plating were also used for the homogeneity test of the bio-PCR. The homogeneity test revealed that all healthy subsamples were negative, 26 medium infection level subsamples were positive and all high infection level subsamples were positive (Table 4 and Appendix 3).

Based on these results the expected number of positive subsamples was calculated using Seedcalc8 and probpossample-V1 to be two to three positive subsamples for category 2.2 (Table 3). In the other

infected categories (2.1, 2.3 and 3), all subsamples are expected to be positive, and in the healthy category (1), none of the subsamples are expected to be positive.

4.3.2.2. Stability test

The stability test for the bio-PCR was performed simultaneously with stability test for dilution plating on the same samples. All negative subsamples were negative (no false-positives) and all medium and high infection level subsamples were positive (no false-negatives; Table 23 and Appendix 4). Therefore, it was concluded that the stability of the samples was also suitable for bio-PCR testing.

Table 23. Results of the homogeneity and stability test for bio-PCR per infection level.

Infection level	Homogeneity results	Stability results
1 (healthy)	0/9	0/3
2.1 (medium)	9/9	3/3
2.2 (medium)	8/9	3/3
2.3 (medium)	9/9	3/3
3 (high)	9/9	3/3

Results present the number of positive subsamples over the total number of subsamples

4.3.3. Seed extract PCR

4.3.3.1. Homogeneity test

Together with the dilution plating and bio-PCR, the homogeneity was also tested for the seed extract PCR on the same subsamples. Results showed that all healthy subsamples were detected as negative, all medium infection level subsamples were found positive and all high infection level subsamples were found positive (Table 5 and Appendix 3).

This indicates that the expected values for each test set in the comparative test and stability test are three negatives for the healthy subsamples, and three positives for each of the medium and high infection level categories.

4.3.3.2. Stability test

The stability test was performed together with the dilution plating and bio-PCR stability test. All samples showed the expected results (Table 24 and Appendix 4), therefore the stability was good.

Table 24. Results of the homogeneity and stability test for the seed extract PCR per contamination level.

Infection level	Homogeneity results	Stability results
1 (healthy)	0/9	0/3
2.1 (medium)	9/9	3/3
2.2 (medium)	9/9	3/3
2.3 (medium)	9/9	3/3
3 (high)	9/9	3/3

Results present the number of positive subsamples over the total number of subsamples

4.4. COMPARATIVE TEST RESULTS

The raw data generated by the ten laboratories is presented in Appendix 5A, 5B5B and 5C5C. Laboratory 9 retracted their results from this comparative test and are therefore not included in the analysis. Laboratory 3 obtained dilution plating results that were not in line with the expectations based on the homogeneity and stability test. Laboratory 3 detected one false-positive. This laboratory was experienced in dilution plating and their hypothesis is that cross-contamination or exchanges between samples occurred during the CT. Since the dilution plating method is validated and the results of bio-PCR and seed extract PCR were compared to this validated method, results of laboratories that did not correspond to expected values with dilution plating were excluded from the analysis. Thus, the results of laboratory 3 were excluded from both the bio-PCR and seed extract PCR analysis.

4.4.1. Dilution plating

Analysis of qualitative results for each laboratory for healthy and high infection level subsamples has been carried out according to the norm ISO 16140. Results are presented in Tables 25 and 26. All laboratories obtained 100% sensitivity indicating no false-negatives were detected. All laboratories also obtained 100% specificity and accuracy.

The sensitivity, specificity and accuracy for the dilution plating method for the healthy, medium 2.1 and medium 2.3 and high level lots were calculated according to the formulas described in ISO 16140 (Table 26). For category 2.2, some variation was expected based on the homogeneity test and therefore this category was analysed separately. The observed variation was within the allowed range (Table 27).

Table 25. Results of dilution plating per laboratory. Number of positive subsamples in three subsamples per infection level category.

Lab code	1 (healthy)	2.1 (medium)	2.2 (medium)	2.3 (medium)	3 (high)
1	0	3	2	3	3
2	0	3	3	3	3
4	0	3	3	3	3
5	0	3	3	3	3
6	0	3	3	3	3
7	0	3	3	3	3
8	0	3	2	3	3
10	0	3	3	3	3

Table 26. Performance criteria dilution plating per category (N.A.: Not Applicable).

Infection level	Sensitivity	Specificity	Accuracy	Accordance	Concordance
1 healthy	N.A.	100%	100%	100%	100%
2.1 medium	100%	N.A.	100%	100%	100%
2.2 medium	As expected*	N.A.	As expected*	N.A.	N.A.
2.3 medium	100%	N.A.	100%	100%	100%
3 high	100%	N.A.	100%	100%	100%
Total	**100%**	**100%**	**100%**		

* As expected: although not all subsamples were positive they all performed according to expectations based on the homogeneity test.

Table 27. Expected and obtained results for the infection level category medium 2.2 subsamples with dilution plating.

	Infection level 2.2 (medium)	
Lab code	**Expected**	**Obtained**
1	2 - 3	2
2	2 - 3	3
4	2 - 3	3
5	2 - 3	3
6	2 - 3	3
7	2 - 3	3
8	2 - 3	2
10	2 - 3	3

Total	16 - 24	22

4.4.1.1. Conclusion

Eight laboratories obtained expected results for all healthy subsamples as well as for the three medium infection level categories and the high infection level subsamples. Therefore, it is concluded that the data from this sample set is suitable (homogenous and stable). All laboratories performed according to expectations.

4.4.2. Bio-PCR

After removal of laboratory 3 and 9, four laboratories remain for the analysis. Results are shown in Table 28. Sensitivity, specificity and accuracy for healthy, medium infection levels 2.1 and 2.3, and high infection level lots were calculated for bio-PCR according to ISO 16140 (Table 29). Results for medium 2.2 were expected to be variable based on the dilution plating homogeneity test and are therefore analysed separately (Table 30). All laboratories obtained expected results for medium 2.2 subsamples.

Table 28. Results for bio-PCR per laboratory.

Lab code	1 (healthy)	2.1 (medium)	2.2 (medium)	2.3 (medium)	3 (high)
4	0	3	3	3	3
6	0*	3	3	3	3
7	2	3	3	3	3
10	0	3	3	3	3

Figures in each infection level categories represent the number of positive subsamples. The yellow highlight as results deviating from expected results based on dilution plating. The asterisk (*) shows inconclusive results that were reported for two samples and that were excluded from the analysis.

Table 29. Performance criteria bio-PCR per infection level category.

Infection level	Sensitivity	Specificity	Accuracy	Accordance	Concordance
1 (healthy)	N.A.	77.8%	77.8%	77.8%	55.6%
2.1 (medium)	100%	N.A.	100%	100%	100%
2.2 (medium)*	As expected	N.A.	As expected	100%	100%
2.3 (medium)	100%	N.A.	100%	100%	100%
3 (high)	100%	N.A.	100%	100%	100%
Total	**100%**	**77.8%**	**94.5%**		

* As expected: although not all subsamples were positive they all performed according to expectations based on the homogeneity test.

Table 30. Expected and obtained bio-PCR results per laboratory for infection level category 2.2 medium samples. Number of positive subsamples per category.

	Infection level 2.2 (medium)	
Lab code	**Expected**	**Obtained**
4	2 - 3	3
6	2 - 3	3
7	2 - 3	3
10	2 - 3	3
Total	**8 - 12**	**12**

4.4.2.1. Conclusion

All performance criteria, namely sensitivity, specificity, accuracy, accordance and concordance, were acceptable for positive samples. For healthy samples, one laboratory obtained probable false-positives, although these results might also represent a low contamination level, which was not detected during pre-tests. It should however be noted that since the bio-PCR method is intented to be used as a pre-screening method only, false-positives are acceptable. The laboratory performances with bio-PCR was acceptable for its use as a pre-screening method.

4.4.3. Seed extract PCR

Seven laboratories performed the seed extract PCR in this comparative test, and results are summarized in Table 31. Sensitivity, specificity and accuracy for the healthy, medium 2.1, medium 2.3 and high infection level lots were calculated for seed extract PCR according to ISO 16140 (Table 32). Results of infection level category medium 2.2 are expected to be variable based on the dilution plating homogeneity test and are therefore analysed separately (Table 33). All seven labs obtained expected results for positive subsamples. For healthy subsamples, three positive and three inconclusive results were obtained.

Table 31. Results for Seed extract PCR results per laboratory for healthy and high level samples.

Lab code	1 (healthy)	2.1 (medium)	2.2 (medium)	2.3 (medium)	3 (high)
1	0	3	3	3	3
2	inconclusive*	3	3	3	3
4	0	3	3	3	3
5	0	3	3	3	3
7	3	3	3	3	3
8	0	3	3	3	3
10	0	3	3	3	3

Total	3	24	24	24	24

Figures in each infection level categories represent the number of positive subsamples. The yellow highlight indicates results deviating from expected results based on dilution plating. The asterisk (*) shows inconclusive results that were reported for two samples and that were excluded from the analysis. In this case, the laboratory also analysed the subsamples in parallel with an *in house* method and found them to be negative.

Table 32. Performance criteria seed extract PCR per infection level category seed extract PCR.

Infection level	Sensitivity	Specificity	Accuracy	Accordance	Concordance
1 (Healthy)	N.A.	83.3%	83.3%	100%	66.7%
2.1 (Medium)	100%	N.A.	100%	100%	100%
2.2 (Medium)*	As expected	N.A.	As expected	N.A.	N.A.
2.3 (Medium)	100%	N.A.	100%	100%	100%
3 (High)	100%	N.A.	100%	100%	100%
Total	**100%**	**83.3%**	**95.8%**		

* As expected: although not all subsamples were positive they all performed according to expectations based on the homogeneity test.

Table 33. Expected and obtained results for each laboratory for the infection level category medium 2.2 samples with seed extract PCR.

	Infection level 2.2 (medium)	
Lab code	Expected	Obtained
1	2 - 3	3
2	2 - 3	3
4	2 - 3	3
5	2 - 3	3
7	2 - 3	3
8	2 - 3	3
10	2 - 3	3
Total	**16 - 24**	**24**

Figures depict the number of positive subsamples out of three subsamples.

4.4.3.1. Conclusion

Xcc was detected in all positive subsamples. Laboratory 2 obtained inconclusive results on negative subsamples due to inhibition of the internal control. The same extract was repeated with the *in house* method routinely used in this laboratory and these results, which were negative, were shared with the test organizer. Therefore, the seed extract PCR results for laboratory 2 were excluded from the

analysis, since in practice these would be repeated or tested by dilution plating. Laboratory 7 obtained three false-positive results of which, two false-positive results obtained low Ct-values (24-27). These are likely false-positives, as they deviate from all other results in homogeneity, stability and CT results of other participants. However, since seed extract PCR is proposed as a pre-screening method, false-positives are deemed acceptable.

5. Discussion and general conclusions

As with dilution plating, bio-PCR and seed extract PCR showed a 100% sensitivity in the CT. Both bio-PCR and seed extract PCR show acceptable specificity and accuracy (Table 34). Both methods show some possibility of false-positives, which is acceptable since these methods are suggested as a pre-screening methods only. For positive samples both methods show high level accordance and concordance as shown in Table 34.

Therefore, the results support the use of bio-PCR and seed extract PCR as pre-screening methods for ISTA 7-019a. It is therefore proposed to include both bio-PCR and seed extract PCR as pre-screening methods in ISTA 7-019a.

Table 34. Summary of performance criteria of three methods for Xcc detection, dilution plating, bio-PCR and seed extract PCR.

Method	Sensitivity	Specificity	Accuracy	Accordance	Concordance
Dilution plating	100%	100%	100%	100%	100%
Bio-PCR	100%	77.8%	94.5%	100%	100%
Seed extract PCR	100%	83.3%	95.8%	100%	100%

Accordance and concordance are calculated for positive samples only.

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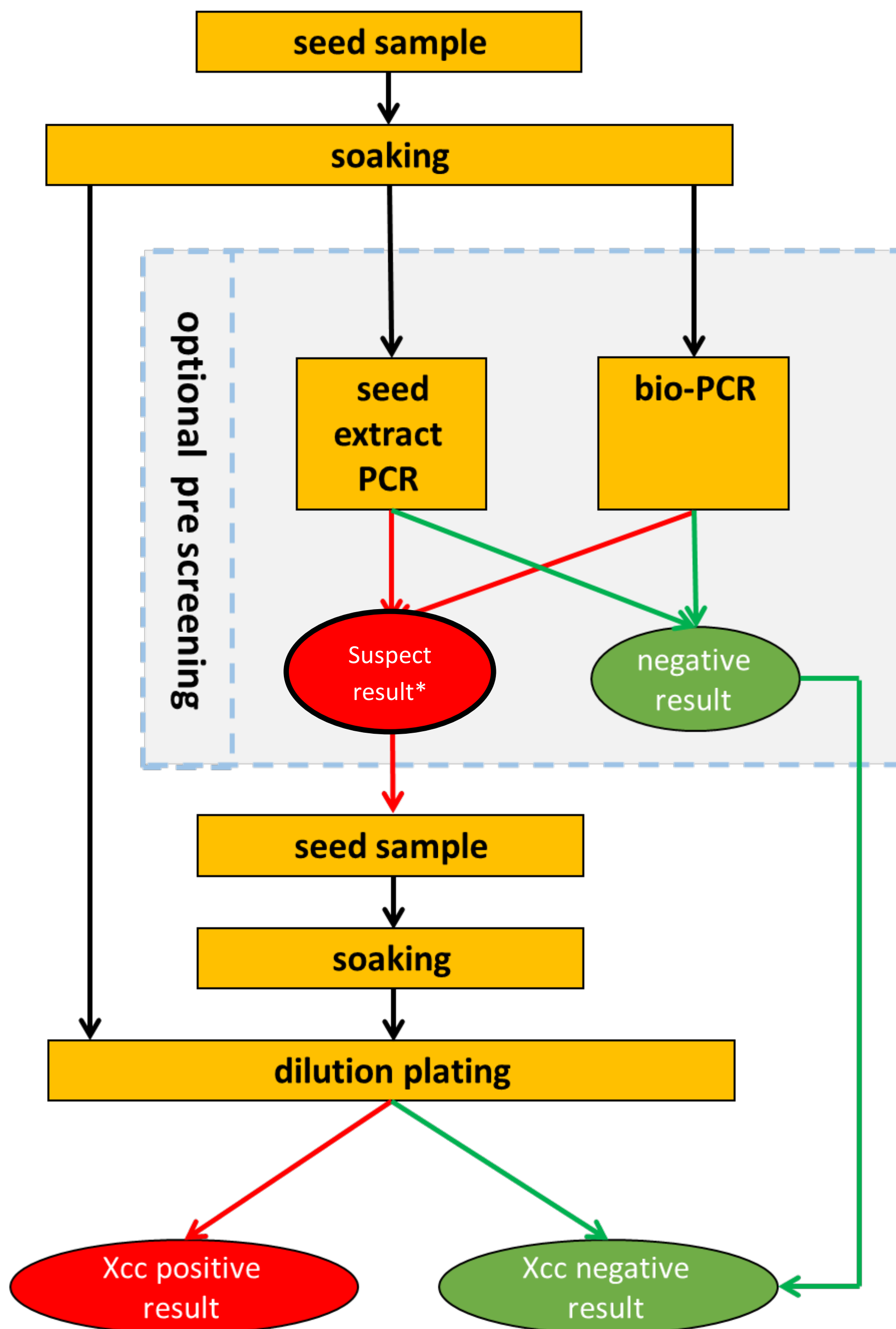
7. Appendices

Appendix 1: Flow chart ISTA 7-019a: detection of *Xanthomonas campestris* pv. *campestris* on untreated Brassica spp. seeds.



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*In case of inconclusive results for seed extract PCR or bio-PCR treat sample as suspect result.

Appendix 2: **Composition of the samples**

Infection level	Category	Sample number	Composition
healthy	1	3	10,000 seeds ZZB-700
healthy	1	6	
healthy	1	13	
medium	2.1	4	3,000 seeds ZZB-695 + 7,000 seeds ZZB-694
medium	2.1	11	
medium	2.1	14	
medium	2.2	1	1,000 seeds ZZB-695 + 9,000 seeds ZZB-694
medium	2.2	8	
medium	2.2	15	
medium	2.3	5	3,000 seeds ZZB-695 + 7,000 seeds ZZB-549
medium	2.3	9	
medium	2.3	12	
high	3	2	10,000 seeds ZZB-689
high	3	7	
high	3	10	

Appendix 3: Homogeneity test

Sample	Set	Infection level	Seed wash PCR			Bio-PCR		Dilution plating	
			Xcc (Fam)	Xc (Vic)	IAC (TR)	Zup	DLH	CS20ABN	FS
3	A	healthy	neg	neg	OK	neg	neg	neg	neg
6	A	healthy	neg	neg	OK	neg	neg	neg	neg
13	A	healthy	neg	neg	OK	neg	neg	neg	neg
3	B	healthy	neg	neg	OK	neg	neg	neg	neg
6	B	healthy	neg	neg	OK	neg	neg	neg	neg
13	B	healthy	neg	neg	OK	neg	neg	neg	neg
3	C	healthy	neg	neg	OK	neg	neg	neg	neg
6	C	healthy	neg	neg	OK	neg	neg	neg	neg
13	C	healthy	neg	neg	OK	neg	neg	neg	neg
4	A	medium 2.1	pos	pos	OK	pos	pos	pos	pos
11	A	medium 2.1	pos	pos	OK	pos	pos	pos	pos
14	A	medium 2.1	pos	pos	OK	pos	pos	pos	neg
4	B	medium 2.1	pos	pos	OK	pos	pos	pos	pos
11	B	medium 2.1	pos	pos	OK	pos	pos	pos	pos
14	B	medium 2.1	pos	pos	OK	pos	pos	pos	pos
4	C	medium 2.1	pos	pos	OK	pos	pos	pos	pos
11	C	medium 2.1	pos	pos	OK	pos	pos	pos	pos
14	C	medium 2.1	pos	pos	OK	pos	pos	pos	pos
1	A	medium 2.2	pos	pos	OK	pos	pos	pos	pos
8	A	medium 2.2	pos	pos	OK	pos	pos	pos	neg
15	A	medium 2.2	pos	pos	OK	pos	pos	pos	pos
1	B	medium 2.2	pos	pos	OK	pos	pos	pos	pos
8	B	medium 2.2	pos	pos	OK	pos	pos	pos	pos
15	B	medium 2.2	pos	pos	OK	pos	pos	pos	pos
1	C	medium 2.2	pos	pos	OK	pos	pos	pos	pos
8	C	medium 2.2	pos	pos	OK	pos	pos	pos	pos
15	C	medium 2.2	pos	pos	OK	neg	neg	neg	neg
5	A	medium 2.3	pos	pos	OK	pos	pos	pos	pos
9	A	medium 2.3	pos	pos	OK	pos	pos	pos	pos
12	A	medium 2.3	pos	pos	OK	pos	pos	pos	pos
5	B	medium 2.3	pos	pos	OK	pos	pos	pos	pos
9	B	medium 2.3	pos	pos	OK	pos	pos	pos	pos
12	B	medium 2.3	pos	pos	OK	pos	pos	pos	pos
5	C	medium 2.3	pos	pos	OK	pos	pos	pos	pos
9	C	medium 2.3	pos	pos	OK	pos	pos	pos	pos
12	C	medium 2.3	pos	pos	OK	pos	pos	pos	pos
2	A	high	pos	pos	OK	pos	pos	pos	neg
7	A	high	pos	pos	OK	pos	pos	pos	pos
10	A	high	pos	pos	OK	pos	pos	pos	pos
2	B	high	pos	pos	OK	pos	pos	pos	pos
7	B	high	pos	pos	OK	pos	pos	pos	pos
10	B	high	pos	pos	OK	pos	pos	pos	pos
2	C	high	pos	pos	OK	pos	pos	pos	pos
7	C	high	pos	pos	OK	pos	pos	pos	pos
10	C	high	pos	pos	OK	pos	pos	pos	pos
NC Lysis			neg	neg	N/A	neg	neg		
PC Xcc			pos	pos	36,72				

IAC	neg	neg	OK		

Appendix 4: Stability test

Sample	Infection level	Seed wash PCR			Bio-PCR		Dilution plating	
		Xcc (Fam)	Xc (Vic)	IAC (TR)	Zup	DLH	CS20ABN	FS
3	healthy	neg	neg	OK	neg	neg	neg	neg
6	healthy	neg	neg	OK	neg	neg	neg	neg
13	healthy	neg	neg	OK	neg	neg	neg	neg
4	medium 2.1	pos	pos	OK	pos	pos	pos	pos
11	medium 2.1	pos	pos	OK	pos	pos	pos	pos
14	medium 2.1	pos	pos	OK	pos	pos	pos	pos
1	medium 2.2	pos	pos	OK	neg	pos	neg	pos
8	medium 2.2	pos	pos	OK	pos	pos	pos	pos
15	medium 2.2	pos	pos	OK	pos	pos	pos	pos
5	medium 2.3	pos	pos	OK	pos	pos	pos	pos
9	medium 2.3	pos	pos	OK	pos	pos	pos	pos
12	medium 2.3	pos	pos	OK	pos	pos	pos	pos
2	high	pos	pos	OK	pos	pos	pos	pos
7	high	pos	pos	OK	pos	pos	pos	pos
10	high	pos	pos	OK	pos	pos	pos	pos

Appendix 5A: CT results: Dilution plating

Sample	Infection level	Laboratory									
		1	2	3*	4	5	6	7	8	9*	10
3	healthy	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
6	healthy	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg
13	healthy	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
4	medium 2.1	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos
11	medium 2.1	pos	pos	pos	pos	pos	pos	pos	pos	neg	pos
14	medium 2.1	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos
1	medium 2.2	pos	pos	pos	pos	pos	pos	pos	pos	neg	pos
8	medium 2.2	neg	pos	pos	pos	pos	pos	pos	pos	neg	pos
15	medium 2.2	pos	pos	neg	pos	pos	pos	pos	neg	neg	pos
5	medium 2.3	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos
9	medium 2.3	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos
12	medium 2.3	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos
2	high	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos
7	high	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos
10	high	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos

* Laboratory 3 and 9 were excluded from the analysis

Appendix 5B: CT results: Bio-PCR

Sample	Infection level	Replicate	Laboratory					
			3*	4	6	7	9*	10
3	healthy	A	pos	neg	neg	pos	pos	neg
		B	pos	neg	neg	neg	pos	neg
	spike		ok	pos	pos	pos	pos	pos
6	healthy	A	neg	neg	inc	neg	pos	neg
		B	neg	neg	inc	neg	pos	neg
	spike		ok	pos		pos	pos	pos
13	healthy	A	neg	neg	inc	pos	pos	neg
		B	neg	neg	inc	neg	pos	neg
	spike		not ok (1/10 ok)	pos		pos	pos	pos
4	medium 2.1	A	pos	pos	pos	pos	pos	pos
		B	pos	pos	pos	pos	pos	pos
	spike		ok	pos	pos	pos	pos	pos
11	medium 2.1	A	pos	pos	pos	pos	pos	pos
		B	pos	pos	pos	pos	pos	pos
	spike		not ok (1/10 ok)	pos	pos	pos	pos	pos
14	medium 2.1	A	neg	pos	pos	pos	pos	pos
		B	neg	pos	pos	pos	pos	pos
	spike		ok	pos	pos	pos	pos	pos
1	medium 2.2	A	pos	pos	pos	pos	pos	neg
		B	pos	pos	pos	pos	pos	pos
	spike		ok	pos	pos	pos	pos	pos
8	medium 2.2	A	pos	pos	pos	pos	pos	pos
		B	pos	pos	pos	pos	pos	pos
	spike		ok	pos	pos	pos	pos	pos
15	medium 2.2	A	neg	pos	pos	pos	pos	pos
		B	neg	pos	pos	pos	pos	pos
	spike		not ok (1/10 ok)	pos	pos	pos	pos	pos
5	medium 2.3	A	pos	pos	pos	pos	pos	pos
		B	pos	pos	pos	pos	pos	pos
	spike		ok	pos	pos	pos	pos	pos
9	medium 2.3	A	pos	pos	pos	pos	pos	pos
		B	pos	pos	pos	pos	pos	pos
	spike		ok	pos	pos	pos	pos	pos
12	medium 2.3	A	pos	pos	pos	pos	pos	pos
		B	pos	pos	pos	pos	pos	pos
	spike		ok	pos	pos	pos	pos	pos
2	high	A	pos	pos	pos	pos	pos	pos
		B	pos	pos	pos	pos	pos	pos

	spike		not ok (1/10 ok)	pos	pos	pos	pos	pos
7	high	A	pos	pos	pos	pos	pos	pos
		B	pos	pos	pos	pos	pos	pos
	spike		ok	pos	pos	pos	pos	pos
10	high	A	pos	pos	pos	pos	pos	pos
		B	pos	pos	pos	pos	pos	pos
	spike		not ok (1/10 ok)	pos	pos	pos	pos	pos
PC			ok	pos		pos		
NC			ok	neg		neg		
NTC			ok	neg		neg		

* Laboratory 3 and 9 were excluded from the analysis

Appendix 5C: CT results: Seed extract PCR

			1		2*		3**		4		5	7		8		10	
Sample	Category	Replicate	Conclusion target	Conclusion spike	Conclusion target	Conclusion spike	Conclusion target	Conclusion spike	Conclusion target	Conclusion spike	Conclusion target	Conclusion target	Conclusion spike	Conclusion target	Conclusion spike	Conclusion target	Conclusion spike
3	healthy	A	neg	good	inc	inhibition	neg	good	neg	good	neg	pos	good	Neg	good	Neg	good
		B	neg	good	inc	inhibition	neg	good	neg	good	neg	pos	good				
6	healthy	A	neg	good	inc	inhibition	neg	good	neg	good	neg	pos	good	Neg	good	Neg	good
		B	neg	good	inc	inhibition	neg	good	neg	good	neg	pos	good				
13	healthy	A	neg	good	inc	inhibition	neg	good	neg	good	neg	pos	good	Neg	good	neg	good
		B	neg	good	inc	inhibition	neg	good	neg	good	neg	pos	good				
4	medium 2.1	A	pos	good	pos	competition	pos	good	pos	good	pos	pos	good	Pos	good	Pos	good
		B	pos	good	pos	competition	pos	good	pos	good	pos	pos	good				
11	medium 2.1	A	pos	good	pos	competition	pos	good	pos	good	pos	pos	good	Pos	good	Pos	good
		B	pos	good	pos	competition	pos	good	pos	good	pos	pos	good				
14	medium 2.1	A	pos	good	pos	competition	pos	good	pos	good	pos	pos	good	Pos	good	Pos	good
		B	pos	good	pos	competition	pos	good	pos	good	pos	pos	good				
1	medium 2.2	A	pos	good	pos	competition	pos	good	pos	good	pos	pos	good	Pos	good	Pos	good
		B	pos	good	pos	competition	pos	good	pos	good	pos	pos	good				
8	medium 2.2	A	pos	good	inc	inhibition	pos	good	pos	good	pos	pos	good	Pos	good	Pos	good
		B	pos	good	pos	competition	pos	good	pos	good	pos	pos	good				
15	medium 2.2	A	pos	good	pos	competition	pos	good	pos	good	pos	pos	good	Pos	good	Pos	good
		B	pos	good	pos	competition	pos	good	pos	good	pos	pos	good				
5	medium 2.3	A	pos	good	pos	competition	pos	good	pos	good	pos	pos	good	Pos	good	Pos	good
		B	pos	good	pos	competition	pos	good	pos	good	pos	pos	good				
9	medium 2.3	A	pos	good	pos	competition	pos	good	pos	good	pos	pos	good	Pos	good	Pos	good
		B	pos	good	pos	competition	pos	good	pos	good	pos	pos	good				
12	medium 2.3	A	pos	good	pos	competition	pos	good	pos	good	pos	pos	good	Pos	good	Pos	good
		B	pos	good	pos	competition	pos	good	pos	good	pos	pos	good				
2	high	A	pos	good	pos	competition	pos	good	pos	good	pos	pos	good	Pos	good	Pos	good
		B	pos	good	pos	competition	pos	good	pos	good	pos	pos	good				
7	high	A	pos	good	pos	competition	pos	good	pos	good	pos	pos	good	Pos	good	Pos	good
		B	pos	good	pos	competition	pos	good	pos	good	pos	pos	good				
10	high	A	pos	good	pos	competition	pos	good	pos	good	pos	pos	good	Pos	good	Pos	good
		B	pos	good	pos	competition	pos	good	pos	good	pos	pos	good				
PC			pos	good	pos	competition	pos		pos	NA	pos			pos	good	pos	good
NC			neg	good	neg	inhibition	neg		neg	NA	pos			neg	good		
NTC			neg		no target	no spike	neg		neg	NA	neg			neg			

* inc: inconclusive

** Laboratory 3 was excluded from the analysis

Appendix 6: Published data on analytical specificity of Köhl and Berg Taqmans.
Table I. Analytical specificity of Berg Taqman from literature

Species/strain	Strain designation	PCR	Reference[a]
X. axonopodis pv. phaseoli	X18–DAR 58726	-	1
X. campestris pv. aberrans	X59–DAR 75944, ICMP 4805, LMG 9037, NCPPB 2986; race 5	+	1
X. campestris pv. armoraciae	X60–DAR 75942, ICMP 7, LMG 535, NCPPB 347	+	1
X. campestris pv. barbarae	X61–DAR 75945, ICMP 438, LMG 547, NCPPB 983	+	1
X. campestris pv. campestris	X50–DAR 75950, ATCC 33913, NCPPB 528, LMG568, ICMP 13; race 3	+	1
X. campestris pv. campestris	X110 PHDS 03/417	+	1
X. campestris pv. campestris	X219 PHDS 04/107	+	1
X. campestris pv. campestris	X227 3316-A	+	1
X. campestris pv. incanae	X62–DAR 75959, ICMP 574, LMG 7490, ATCC13462, NCPPB 937	+	1
X. campestris pv. raphani	X63–DAR 75960, ICMP 1404, LMG 860, NCPPB 1946; race 5	+	1
X. campestris from noncrucifers	X20 PHDS 02/564	-	1
Pseudomonas sp.	DAR 26838	-	1
X. sesame pv. sesame	X66 DAR 75547	-	1
X. c. pv. campestris	CFBP 6865[pt] NCPPB 2986	+	2
X. c. pv. campestris	CFBP 6863, NCPPB 875	+	2
X. campestris	CFBP 5824, LMG 7383, NCPPB 1930	–	2
X. c. pv. campestris	CFBP 3838[pt] , LMG 535, NCPPB 347	+	2
X. c. pv. raphani	756C[b]	–	2,3
X. campestris	CFBP 5825[pt] , LMG 547, NCPPB 983	–	2
X. campestris	CFBP 5826, LMG 7385	–	2
X. c. pv. campestris	CFBP 1119	+	2
X. c. pv. campestris	CFBP 1121	+	2
X. c. pv. campestris	CFBP 1869	+	2
X. c. pv. campestris	CFBP 4952	+	2
X. c. pv. campestris	CFBP 5127, SNES 3316	+	2

X. c. pv. campestris	CFBP 5128, SNES 3330	+	2
X. c. pv. campestris	CFBP 5129, SNES 3429	+	2
X. c. pv. campestris	CFBP 5815, Clause 628a1cs	+	2
X. c. pv. campestris	CFBP 5816, Clause 656C1FS1	+	2
X. c. pv. campestris	CFBP 5818, Clause 751a2FS	+	2
X. c. pv. campestris	HRI 3811[c]	+	2
X. c. pv. campestris	305[d]	+	2
X. c. pv. campestris	CFBP 5241[T] LMG 568, ATCC 33 913	+	2
X. c. pv. campestris	CFBP 5683	+	2
X. c. pv. campestris	277[d]	+	2
X. c. pv. campestris	CFBP 4956	+	2
X. c. pv. campestris	CFBP 5817, Clause 658pCS1	+	2
X. c. pv. campestris	Xcc 147[b]	+	2
X. c. pv. campestris	CFBP 1712	+	2
X. c. pv. campestris	CFBP 1713	+	2
X. c. pv. campestris	CFBP 4954	+	2
X. c. pv. campestris	CFBP 6943	+	2
X. c. pv. campestris	HRI 6181[c]	+	2
X. c. pv. campestris	CFBP 4953[e]	+	2
X. c. pv. campestris	CFBP 5130, SNES 3430	+	2
X. c. pv. campestris	CFBP 1124[e]	+	2
X. c. pv. campestris	CFBP 1710	+	2
X. c. pv. campestris	CFBP 1711	+	2
X. c. pv. campestris	CFBP 5820, Clause 2963	+	2
X. c. pv. campestris	CFBP 4955	+	2
X. c. pv. campestris	CFBP 5814, Clause 563apcs	+	2
X. c. pv. campestris	CFBP 6650[e] LMG 8004, NCPPB 1145	+	2
X. c. pv. incanae	CFBP 1371	+	2
X. c. pv. incanae	CFBP 1438, NCPPB 1934	+	2
X. c. pv. incanae	CFBP 1606	–	2
X. c. pv. incanae	LMG 7490, NCPPB 937	+	2
X. c. pv. incanae	CFBP 5686, Shmit J. 10 903	+	2
X. c. pv. raphani	CFBP 5827[pt] LMG 860, NCPPB 1946	–	2
X. c. pv. raphani	CFBP 5828, LMG 7505	–	2
X. c. pv. raphani	CFBP 5829, LMG 8134	–	2

[a] Reference 1: Berg *et al.* (2006), reference 2: Rijlaarsdam *et al.* (2004), reference 3: Kamdar *et al.* (1993)

[b] LIMP, Toulouse, France

[c] Vincente et al., (2001)

[d] Clause-Tezier Laboratory, Angers, France

[e] Additonal race type strains proposed for Xcc

Table II. Analytical specificity of the Taqman developed by Köhl et al (2011) from literature.

No.	Type of strains	PCR result
20	*X. c.* pv. *campestris* strains	Positive
1	*X. c.* pv. *armoraciae* strain IPO373	Positive
18	Related, plant pathogenic Xanthomonas species	Negative
19	Strains of plant pathogenic bacteria distantly related	Negative
7	Unidentified saprophytic bacteria from cabbage	Negative

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Appendix 7: Analytical specificity

Table III. Analytical specificity of Köhl and Berg Taqmans on Isolates from the Naktuinbouw collection.

Isolate no.	Year	Origin	UPBac	Kohl	Berg	Acat	AFLP tree	Pathogenicity assay
1	1987	Arizona	11,58	>40	>40	>40	Out	
2	2010	Unknown	13,32	>40	>40	>40	Out	
3	2006	Netherlands	12,04	35,27	34,63	>40	Out	Negative
4	1987	Arizona	10,67	>40	>40	>40	Out	
5	2006	Netherlands	10,23	44,1	>40	>40	Out	
6	2006	Netherlands	13,00	>40	>40	>40	Out	
7	2008	Unknown	10,80	24,32	>40	>40	Out	Negative
8	2006	Netherlands	11,11	35,12	>40	>40	Out	
9	2006	Netherlands	11,19	34,74	>40	>40	Out	Negative

10	2006	Netherlands	10,14	36,74	>40	>40	Out	
11	2010	France	13,18	>40	24,6	>40	In	Xca
12	2010	Unknown	11,67	>40	23,5	>40	In	
13	2010	France	12,23	>40	23,07	>40	In	Xca
14	2006	Netherlands	10,70	>40	21,83	>40	In	
15	2003	Unknown	11,73	>40	22,75	>40	In	
16	2003	Unknown	12,81	35,56	24,66	>40	In	
17	2010	Unknown	14,04	>40	26,12	>40	In	
18	2003	Unknown	11,97	36,25	23,95	>40	In	
19	2003	Unknown	12,02	>40	22,62	>40	In	
20	2010	Portugal	12,04	24,03	23,8	>40	In	
21	2003	Unknown	11,78	>40	23,82	>40	In	
22	2008	Unknown	10,09	36,43	>40	>40	In	Negative
23	2005	Unknown	11,01	23,08	>40	>40	In	Negative
24	2008	Unknown	10,66	21,09	>40	>40	In	Negative
25	2006	Unknown	11,93	>40	>40	>40	In	Negative
26	2010	Germany	12,20	23,9	23,64	>40	In	
27	1993	Unknown	10,11	22,31	22,04	>40	In	
28	2010	USA	13,13	>40	22,92	>40	In	Xca
29	2003	Unknown	11,01	22,31	22,12	>40	In	
30	2005	Unknown	11,88	23,92	23,61	>40	In	
31	1987	Arizona	12,74	23,35	23,3	>40	In	
32	2005	Netherlands	12,55	35,2	22,42	>40	In	
33	2010	Unknown	13,24	>40	>40	>40	In	Negative
34	2010	Russia	12,59	26,05	25,79	>40	In	
35	2005	Netherlands	10,34	23,21	22,98	>40	In	
36	2010	Netherlands	11,74	22	21,66	>40	In	
37	2010	Spain	13,15	25,03	24,9	>40	In	
38	2010	Italy	12,90	24,06	23,62	>40	In	
39	2010	Unknown	12,15	24,82	24,57	>40	In	
40	2010	Unknown	12,62	24,64	24,49	>40	In	
41	2010	Unknown	13,13	24,46	24,47	>40	In	
42	2004	Unknown	12,78	24,2	23,86	>40	In	
43	2006	Unknown	13,71	23,4	23,13	>40	In	
44	1992	California	11,69	22,89	22,59	>40	In	
45	2010	New Zealand	11,99	27,28	27,16	>40	In	
46	2011	Germany	11,14	21,56	21,59	>40	In	
47	2010	France	13,70	25,65	25,56	>40	In	
48	2009	Netherlands	9,86	23,83	23,64	>40	In	
49	2010	Netherlands	11,55	23,38	23,43	>40	In	
50	2010	Unknown	12,20	23,41	23,63	>40	In	
51	2010	Netherlands	13,93	24,82	24,54	>40	In	
52	2010	Netherlands	12,96	26,12	26,1	>40	In	

53	2010	Netherlands	11,96	22,03	21,58	>40	In	
54	2010	France	12,32	22,15	22,02	>40	In	
55	2010	Netherlands	12,00	22,85	22,65	>40	In	
56	2010	Germany	12,69	25,51	25,54	>40	In	
57	2010	Indonesia	12,08	24,09	23,69	>40	In	
58	2010	USA Florida	13,16	24,27	24,08	>40	In	
59	2010		13,04	23,04	22,89	>40	In	
60	2006	Unknown	10,08	21,09	20,53	>40	In	
61	2010	Australia	12,19	21,43	21,35	>40	In	
62	2011	Germany	12,02	23,24	23,07	>40	In	
63	2011	Germany	11,76	22,32	22,11	>40	In	
64	2005	Unknown	10,57	21,2	20,88	>40	In	
65	2007	Unknown	10,32	21,36	21,35	>40	In	
66	2004	Unknown	9,85	>40	35,57	>40	Out	Negative
67	2006	China	10,96	22,22	22,21	>40	Out	Xcc
68	2010	Netherlands	13,90	>40	>40	>40	Out	
69			12,56	35,63	36,41	>40	Out	
70			11,53	>40	>40	>40	Out	

Appendix 8: Repeatability

Table IV. Repeatability of three seed lots for the detection of *Xanthomonas campestris* pv. *campestris* (Xcc) on Brassica seeds (n = 8).

		Fam Xcc		Vic Xc		Texas red Acat	
Seed lot	Replicate	A	B	A	B	A	B
1	1	30.52	30.46	31.24	30.99	29.22	29.00
1	2	30.80	30.72	31.00	30.65	29.10	28.81
1	3	30.24	30.26	30.78	30.74	29.06	28.92
1	4	30.18	30.57	30.59	30.68	28.80	28.62
1	5	30.12	30.25	30.79	30.79	29.09	29.10
1	6	30.45	30.78	31.15	31.46	28.90	28.93
1	7	29.80	30.26	30.41	31.23	28.78	29.01
1	8	30.86	31.35	31.33	31.82	29.94	29.67
2	1	20.88	21.00	21.42	21.15	26.51	26.64
2	2	20.29	20.89	21.02	21.36	26.08	26.51
2	3	20.37	20.44	20.94	20.90	26.31	26.23
2	4	20.09	20.56	20.85	20.99	26.05	26.09
2	5	20.97	20.82	21.39	21.21	26.46	26.45
2	6	20.65	20.54	21.06	21.02	26.38	26.03
2	7	21.05	20.82	21.52	21.22	27.04	26.61
2	8	20.97	21.09	21.47	21.54	26.96	26.85
3	1	22.56	22.54	23.06	22.95	27.97	28.20
3	2	22.62	22.35	23.15	22.96	28.52	28.51
3	3	22.09	22.20	22.64	22.61	27.10	27.52
3	4	21.97	22.22	22.63	22.67	27.69	27.76
3	5	22.94	23.01	23.48	23.30	28.47	28.22
3	6	21.76	22.14	22.42	22.66	27.62	27.70
3	7	22.73	22.81	23.11	23.25	28.28	28.26
3	8	23.02	22.70	23.51	23.13	28.34	28.14
Controls	Spike	>45	>45	>45	>45	29.10	29.59
	PC DNA	18.06	18.01	18.37	18.40	>45	>45
	NTC	>45	36.56	>45	>45	>45	>45

Table IV. Reproducibility of three seed lots for the detection of *Xanthomonas campestris* pv. *campestris* (Xcc) on Brassica seeds (n = 8).

FAM Xcc	VIC Xc	Texas Red Acat

Seed lot	Replicate	A	B	A	B	A	B
A	1	31.26	31.21	30.97	31.30	29.51	29.36
A	2	30.80	31.03	30.77	31.35	29.25	29.46
A	3	31.31	31.15	31.27	31.40	29.10	28.74
A	4	31.40	31.37	32.09	31.52	29.95	30.00
A	5	31.01	30.86	31.22	31.29	29.29	29.16
A	6	31.47	32.40	31.57	31.62	29.97	29.94
A	7	30.84	31.48	31.49	31.58	29.01	28.93
A	8	31.26	31.09	32.57	31.28	29.34	28.73
B	1	21.40	21.42	22.02	22.00	26.67	26.61
B	2	21.87	21.62	22.13	22.14	27.07	26.99
B	3	21.80	21.90	22.04	22.17	27.17	27.11
B	4	21.90	21.81	22.15	22.19	27.05	27.02
B	5	21.22	21.60	21.76	22.10	26.53	27.05
B	6	21.23	21.16	21.38	21.76	26.39	26.24
B	7	21.14	21.41	21.68	22.00	26.02	26.30
B	8	21.37	21.34	21.97	21.94	26.55	26.46
C	1	23.37	23.94	24.04	24.05	28.32	28.46
C	2	23.28	23.76	23.89	24.01	28.67	28.56
C	3	23.23	23.33	23.75	23.74	27.38	27.38
C	4	23.05	23.08	23.22	23.22	28.49	28.56
C	5	22.98	23.01	23.21	23.18	27.23	27.32
C	6	23.07	23.06	23.69	23.58	29.20	28.71
C	7	23.24	23.16	23.74	23.75	28.97	28.55
C	8	22.91	22.90	23.11	23.12	27.91	28.07
Controls	NTC	>45		>45		>45	
	NTC	>45		>45		>45	
	PC DNA	15.94		16.39		>45	
	PC DNA	16.01		16.46		>45	

Appendix 9: Robustness

Table VI. Ct-values for three samples (n=8) for six treatments. A) freeze after soaking B) cool after soaking, C) freeze pellet, D) freeze after lysis, E) freeze DNA, F) no freezing or cooling.

		A			B			C			D			E			F		
Monster	Herhaling	Fam Xcc	Vic Xc	TR Acat	Fam Xcc	Vic Xc	TR Acat	Fam Xcc	Vic Xc	TR Acat	Fam Xcc	Vic Xc	TR Acat	Fam Xcc	Vic Xc	TR Acat	Fam Xcc	Vic Xc	TR Acat
1	1	22.07	22.48	29.65	21.36	21.86	27.45	21.19	21.73	27.27	21.09	21.47	29.89	20.88	21.37	28.86	21.27	21.73	28.23
	2	19.23	19.86	28.21	19.03	19.65	27.45	18.96	19.5	27.06	19.52	19.93	29.7	18.78	19.23	27.46	19.22	19.73	27.18
	3	17.34	18.09	28.96	17.23	17.52	27.24	17.13	17.62	26.97	17.45	17.83	30.2	16.67	17.03	26.65	16.75	17.39	27.02
	4	17.96	18.25	29.36	17.71	18.11	27.52	18.16	18.45	26.72	17.66	18.11	29.51	19.15	19.86	29.17	19.45	19.96	28.6
	5	19.16	19.94	28.29	19.35	19.81	27.59	19.32	19.9	27.08	19.43	20.05	29.68	17.78	18.14	28.66	17.64	18.36	28.5
	6	18.73	19.16	29.05	18.48	18.91	28.13	18.69	19.08	26.37	18.84	19.18	29.03	18.22	18.97	28.89	18.5	18.88	28.36
	7	19.05	19.4	29.04	18.58	19.02	27.21	18.72	19.13	27.19	18.62	19.08	28.66	18.69	19.1	28.75	18.51	19.18	28.15
	8	22.7	23.08	29.31	21.94	22.39	27.44	21.83	22.1	27.06	22.28	22.73	29.29	22.46	23.18	29.94	22.12	22.66	28.72
2	1	18.34	19.07	28.79	18.1	18.53	28.21	17.9	18.35	27.44	17.81	18.38	29.19	17.46	18.2	27.36	18.45	19.17	27.3
	2	21.08	21.44	29.15	20.63	21.04	28.38	20.43	21.05	28.1	21.04	21.32	29.94	20.95	21.35	29	21.37	21.92	28.91
	3	23.02	23.35	29.48	22.54	23.12	29	23.01	23.63	28.44	22.56	23.18	29.41	21.82	22.28	28.5	22.69	23.25	29.07
	4	28.85	29.41	30.76	28.18	28.97	27.01	28.5	28.96	28.76	28.43	28.86	30.59	27.72	28.2	29.36	28.4	29.18	29.4
	5	24.12	24.85	30.02	24.12	24.46	29.6	23.81	24.26	29.13	24.02	24.36	30.16	24.06	24.88	29.58	24.4	24.87	29.23
	6	22.6	22.93	29.21	21.83	22.22	28.34	21.91	22.35	27.83	22.02	22.27	29.3	22.65	23.1	29.36	22.26	22.64	28.64
	7	22.75	23.16	30.05	22.43	22.82	29.11	22.42	22.88	28.2	22.07	22.64	29.86	22.93	23.35	29.83	22.46	23.02	28.58
	8	20.13	20.82	29.73	19.49	20.01	28.23	19.06	19.81	27.4	19.4	19.75	29.76	20.22	21	29.51	20.11	20.45	28.13
3	1	45	45	30.39	41.05	35.5	28.58	35.2	45	29.27	34.12	36.7	29.79	45	45	29.86	41.45	36.89	29.52
	2	45	45	30.63	45	45	28.14	45	45	29.18	45	45	30.15	45	45	30.19	42.85	35.36	30.13
	3	45	45	30.79	35.45	36.84	28.89	45	45	29.03	35.28	45	29.68	35.78	45	30.27	36.29	45	30.53
	4	45	45	30.52	45	45	28.78	45	45	28.87	45	45	30.7	42.8	37.84	29.72	42.57	37.8	29.55
	5	45	45	30.3	34.78	45	29.67	45	45	29.19	35.37	45	30.85	33.86	34.34	28.56	41.56	37.06	29.04
	6	45	45	31.43	42.81	35.43	29.26	45	45	28.76	45	45	30.11	36.02	45	30.53	36.39	45	30.35
	7	45	45	30.46	36.17	45	30.02	45	45	29.2	34.92	45	29.25	35.13	45	29.6	34.84	45	29.38
	8	45	45	31.09	45	45	29.55	45	45	29.11	35.04	45	29.5	35.91	45	29.9	35.11	45	29.17
	Acat spike	45	45	29.88	45	45	29.73	45	45	28.03	45	45	29.1	45	45	30.14	45	45	29.02
	PC Xcc	16.44	17.24	45	16.59	17.1	45	16.08	16.49	45	16.51	16.87	45	15.09	15.47	45	16.59	17.34	45

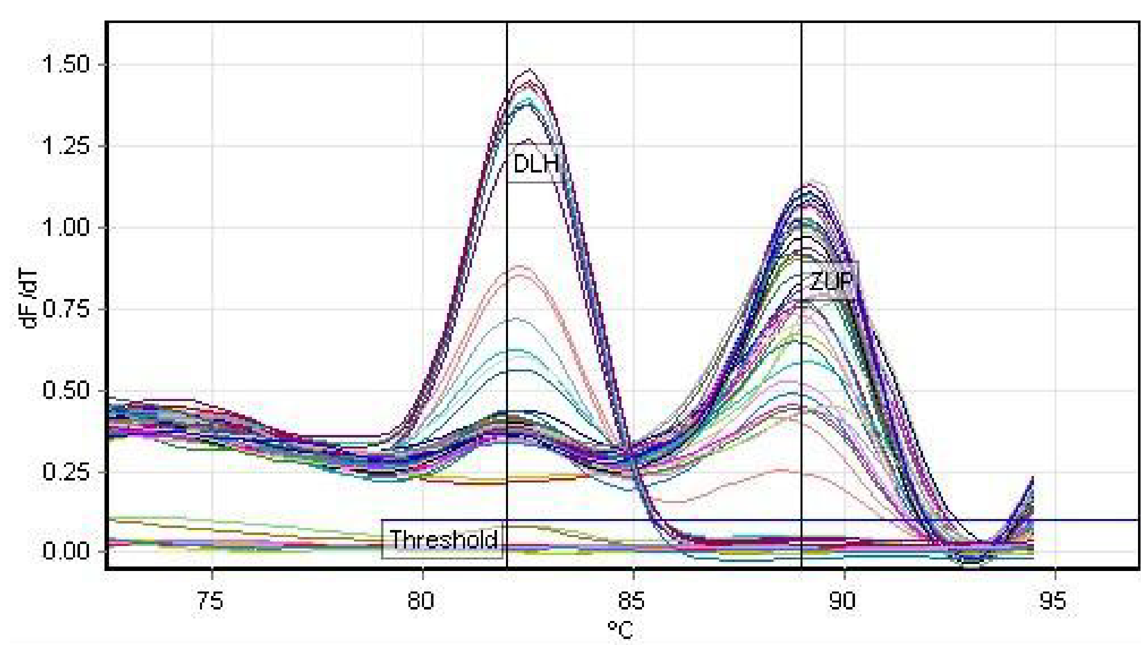
Appendix 10: Raw data results from the repeatability/reproducibility tests

User 1 (Repeatability tests 1A and 1B)

Real-time PCR Melt Report

Temp. Threshold	79°c
Threshold dF/dT	0.1

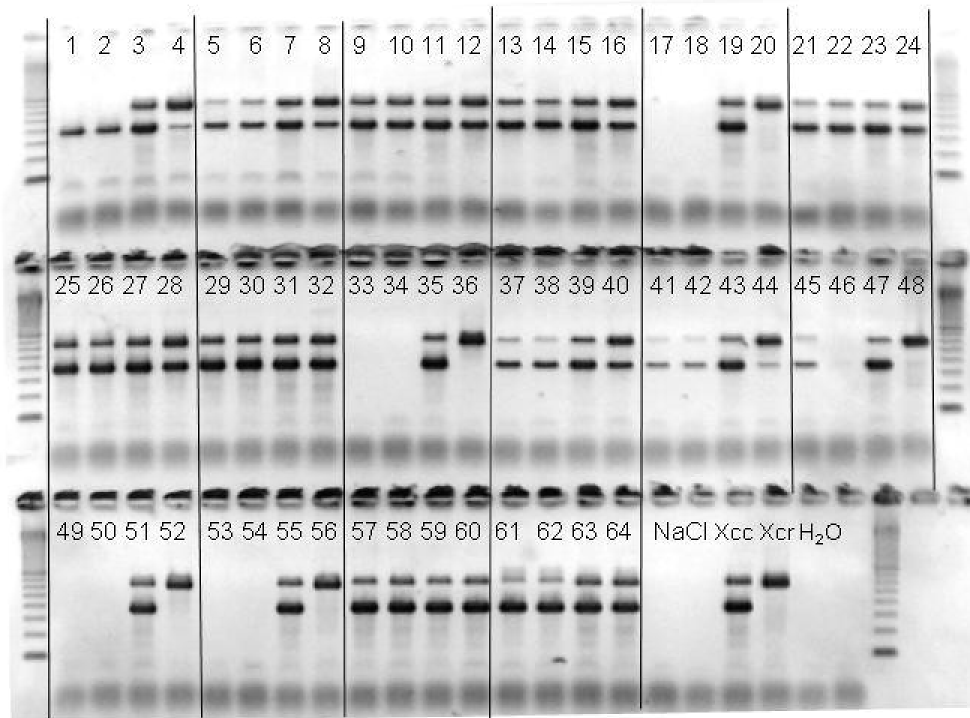
Melt data for Melt A.Green



No	Sample	Genotype	Peak 1	Peak 2
1	J65365-2	Xcc	89.0 (ZUP)	
2	J65365-2	Xcc	89.0 (ZUP)	
3	J65365-2-xcc	Xcc	82.2 (DLH)	89.0 (ZUP)
4	J65365-2-xcr	Xcr	82.5 (DLH)	
5	J65367-2	Xcc	81.8 (DLH)	88.7 (ZUP)
6	J65367-2	Xcc	82.0 (DLH)	88.8 (ZUP)
7	J65367-2-xcc	Xcc	82.0 (DLH)	89.0 (ZUP)
8	J65367-2-xcr	Xcc	82.3 (DLH)	88.5 (ZUP)
9	J64958-2	Xcc	82.3 (DLH)	89.5 (ZUP)
10	J64958-2	Xcc	82.0 (DLH)	89.5 (ZUP)
11	J64958-2-xcc	Xcc	82.0 (DLH)	89.0 (ZUP)
12	J64958-2-xcr	Xcc	82.2 (DLH)	89.2 (ZUP)
13	J64990-2	Xcc	82.2 (DLH)	89.8 (ZUP)
14	J64990-2	Xcc	81.8 (DLH)	89.7 (ZUP)

15	J64990-2-xcc	Xcc	82.0 (DLH)	89.0 (ZUP)
16	J64990-2-xcr	Xcc	82.2 (DLH)	89.3 (ZUP)
17	J60522-2	Non-Infected		
18	J60522-2	Non-Infected		
19	J60522-2-xcc	Xcc	81.7 (DLH)	89.0 (ZUP)
20	J60522-2-xcr	Xcr	82.5 (DLH)	
21	J64993-2	Xcc	82.0 (DLH)	89.0 (ZUP)
22	J64993-2	Xcc	82.0 (DLH)	89.0 (ZUP)
23	J64993-2-xcc	Xcc	82.0 (DLH)	89.0 (ZUP)
24	J64993-2-xcr	Xcc	82.3 (DLH)	88.8 (ZUP)
25	J53215-2	Xcc	82.3 (DLH)	89.5 (ZUP)
26	J53215-2	Xcc	82.2 (DLH)	89.5 (ZUP)
27	J53215-2-xcc	Xcc	82.0 (DLH)	89.0 (ZUP)
28	J53215-2-xcr	Xcc	82.3 (DLH)	89.2 (ZUP)
29	J65346-3	Xcc	82.0 (DLH)	89.2 (ZUP)
30	J65346-3	Xcc	82.0 (DLH)	89.2 (ZUP)
31	J65346-3-xcc	Xcc	82.0 (DLH)	89.0 (ZUP)
32	J65346-3-xcr	Xcc	82.0 (DLH)	89.0 (ZUP)
33	J65365-3	Non-Infected		
34	J65365-3	Non-Infected		
35	J65365-3xcc	Xcc	82.0 (DLH)	89.0 (ZUP)
36	J65365-3-xcr	Xcr	82.5 (DLH)	
37	J65367-3	Xcc	82.0 (DLH)	88.8 (ZUP)
38	J65367-3	Xcc	82.0 (DLH)	89.0 (ZUP)
39	J65367-3-xcc	Xcc	82.2 (DLH)	89.0 (ZUP)
40	J65367-3-xcr	Xcc	82.3 (DLH)	88.5 (ZUP)
41	J64958-3	Xcc	82.0 (DLH)	88.8 (ZUP)
42	J64958-3	Xcc	82.3 (DLH)	88.8 (ZUP)
43	J64958-3-xcc	Xcc	82.0 (DLH)	89.0 (ZUP)
44	J64958-3-xcr	Xcr	82.5 (DLH)	
45	J64990-3	Xcc	82.0 (DLH)	89.5 (ZUP)
46	J64990-3	Non-Infected		
47	J64990-3-xcc	Xcc	82.2 (DLH)	89.2 (ZUP)
48	J64990-3-xcr	Xcr	82.5 (DLH)	
49	J60522-3	Non-Infected		
50	J60522-3	Non-Infected		
51	J60522-3-xcc	Xcc	82.2 (DLH)	89.0 (ZUP)
52	J60522-3-xcr	Xcr	82.5 (DLH)	

53	J64993-3	Non-Infected		
54	J64993-3	Non-Infected		
55	J64993-3-xcc	Xcc	82.0 (DLH)	89.2 (ZUP)
56	J64993-3-xcr	Xcr	82.5 (DLH)	
57	J65346-3	Xcc	82.0 (DLH)	89.0 (ZUP)
58	J65346-3	Xcc	82.0 (DLH)	89.2 (ZUP)
59	J65346-3-xcc	Xcc	82.0 (DLH)	89.2 (ZUP)
60	J65346-3-xcr	Xcc	82.0 (DLH)	89.0 (ZUP)
61	J53215-3	Xcc	82.0 (DLH)	89.0 (ZUP)
62	J53215-3	Xcc	82.0 (DLH)	89.0 (ZUP)
63	J53215-3-xcc	Xcc	82.0 (DLH)	89.0 (ZUP)
64	J53215-3-xcr	Xcc	82.2 (DLH)	88.8 (ZUP)
65	NaCl	Non-Infected		
66	NaCl	Non-Infected		
67	T+xcc	Xcc	82.0 (DLH)	89.2 (ZUP)
68	T+xcr	Xcr	82.5 (DLH)	
69	H2O	Non-Infected		
70	H2O	Non-Infected		

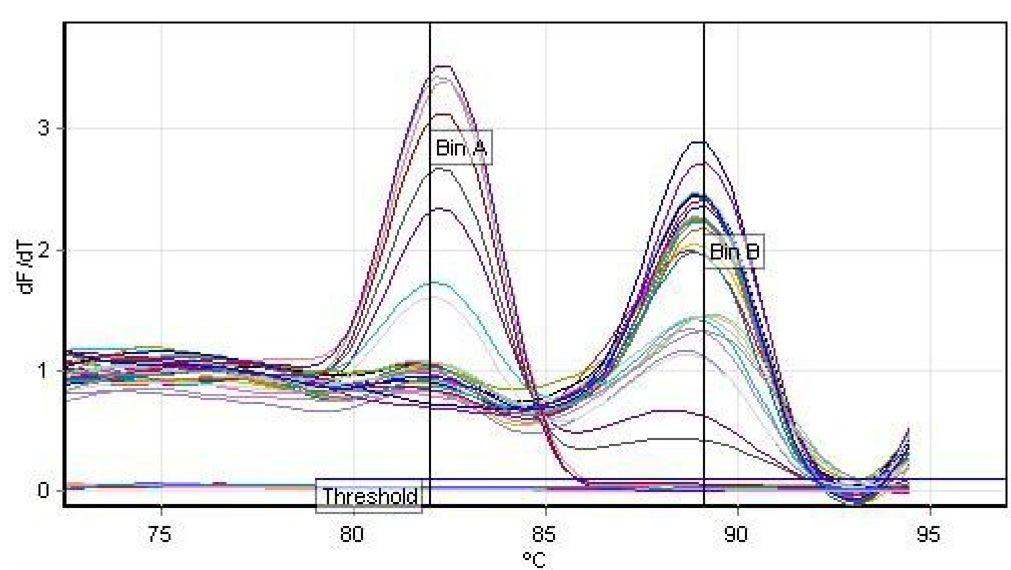


User 2 (Reproducibility test 2)

Real-time PCR Melt Report

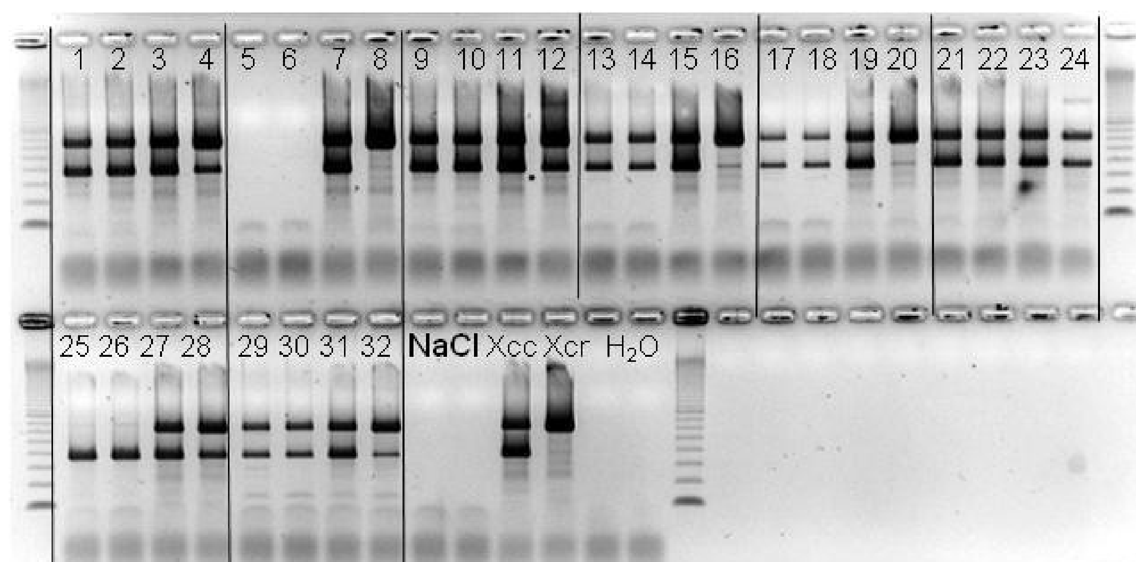
Temp. Threshold	79°c
Threshold	0.1

Melt data for Melt A.Green



No	Sample	Genotype	Peak 1	Peak 2
1	53215	Xcc	81.5 (DLH)	88.8 (ZUP)
2	53215	Xcc	81.7 (DLH)	89.0 (ZUP)
3	53215+xcc	Xcc	81.5 (DLH)	89.0 (ZUP)
4	53215+xcr	Xcc	82.3 (DLH)	88.3 (ZUP)
5	60522	Non-Infected		
6	60522	Non-Infected		
7	60522+xcc	Xcc	81.8 (DLH)	89.0 (ZUP)
8	60522+xcr	Xcr	82.5 (DLH)	
9	64958	Xcc	81.5 (DLH)	89.0 (ZUP)
10	64958	Xcc	81.5 (DLH)	89.0 (ZUP)
11	64958+xcc	Xcc	81.5 (DLH)	89.0 (ZUP)
12	64958+xcr	Xcc	82.0 (DLH)	88.8 (ZUP)
13	64990	Xcc	81.8 (DLH)	89.2 (ZUP)
14	64990	Xcc	81.7 (DLH)	89.3 (ZUP)
15	64990+xcc	Xcc	81.2 (DLH)	89.0 (ZUP)
16	64990+xcr	Xcr	82.3 (DLH)	

17	64993	Xcc	81.7 (DLH)	88.7 (ZUP)
18	64993	Xcc	81.8 (DLH)	88.8 (ZUP)
19	64993+xcc	Xcc	80.5	89.0 (ZUP)
20	64993+xcr	Xcr	82.3 (DLH)	
21	65346	Xcc	81.8 (DLH)	89.0 (ZUP)
22	65346	Xcc	81.7 (DLH)	89.0 (ZUP)
23	65346+xcc	Xcc	81.8 (DLH)	89.0 (ZUP)
24	65346+xcr	Xcc	81.8 (DLH)	89.0 (ZUP)
25	65365	Xcc	89.0 (ZUP)	
26	65365	Xcc	89.0 (ZUP)	
27	65365+xcc	Xcc	89.0 (ZUP)	
28	65365+xcr	Xcc	82.0 (DLH)	88.7 (ZUP)
29	65367	Xcc	82.0 (DLH)	89.0 (ZUP)
30	65367	Xcc	82.0 (DLH)	89.2 (ZUP)
31	65367+xcc	Xcc	82.0 (DLH)	89.0 (ZUP)
32	65367+xcr	Xcc	82.3 (DLH)	88.3 (ZUP)
33	nacl	Non-Infected		
34	nacl	Non-Infected		
35	xcc	Xcc	81.8 (DLH)	89.0 (ZUP)
36	xcr	Xcr	82.3 (DLH)	
37	H2O	Non-Infected		
38	H2O	Non-Infected		



ISHI-Veg validation report to add TaqMan PCR as an option to conventional Polymerase Chain Reaction (PCR) for the identification of *Xanthomonas campestris* pv. *campestris* and pv. *raphani* in *Brassica* seed to support C.7.1.

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1. INTRODUCTION

Xanthomonas campestris pv. *campestris* (Xcc) and pv. *raphani* (Xcr) are gram-negative, seed-borne bacteria that cause disease (Xcc - black rot, and Xcr - leaf spot disease) on Brassica crops. An *Xcc/Xcr* outbreak in commercial farming may have significant environmental, economic, and legal repercussions for the parties involved.

The seed industry developed, validated and proposed a method to be adopted as an ISTA Rule. The ISTA Rule 7-019a is a bacterial plating method for detection of Xcc/Xcr on untreated Brassica seed and includes confirmation of suspect isolates by medium-specific morphological evaluation, PCR assays, or host-pathogenicity testing.

It employs DHL/Zup conventional PCR assays (Rijlaarsdam et al., 2004, Berg et al., 2005) to confirm the identity of suspect isolates. The method also offers two additional options - the Berg and Köhl TaqMan PCR assays - for PCR confirmation. The universal domain-*Bacteria* Wu TaqMan PCR assay, (Wu et al., 2008) functions as an internal process control (IPC) for the Berg (Berg et al., 2006) and Köhl (Köhl et al., 2011) TaqMan PCR assays.

2. PROTOCOL VALIDATION

This report assesses the elements critical to the development, validation, and routine implementation of Berg-Wu and Köhl-Wu duplex TaqMan PCR assays for confirmation of suspect Xcc/Xcr isolates. Performance characteristics were evaluated independently for the TaqMan PCR and included analytical sensitivity and specificity, selectivity, accuracy, repeatability, reproducibility and robustness. This was followed by a comparative test (CT) where the performance criteria accuracy, repeatability, and reproducibility were evaluated. To finalize the validation, the CT examined the TaqMan based PCR method in relation to the conventional PCR reference methods (ISTA Rule 7-019a).

3. ASSAY VALIDATION- PERFORMANCE CRITERIA

Assay validation was performed in two sequential parts. First, all performance parameters of the TaqMan PCR assay were validated. All performance parameters of the total assay (bacterial DNA preparation and TaqMan PCR) were validated thereafter. The presentation of findings in this section is organized by performance parameter, and does not represent the validation process flow. All validation experiments were conducted in accordance to the assay protocol unless stated otherwise.

3.1. Sensitivity

Sensitivity of an assay is determined by two parameters, the limit of detection and limit of quantification of that assay.

Requirement Show that both selective assays are always able to identify the suspect (Xcc or Xcr) by being far more sensitive than the practice sample load in routine testing.

In a TaqMan PCR assay, the limit of detection (LOD) is the lowest concentration, or concentration range, at which the analyte, a target DNA sequence, can be detected in 95% of replicates tested. The limit of quantification (LOQ) is the lowest concentration, or concentration range, at which the analyte, a target DNA sequence, can be detected within limits of replicate variation and amplification efficiency determined adequate by the project owner.

Requirement

The new assay should be as sensitive as dilution plating. Dilution plating is not in this test but refers to data presented in the validation report for SE-PCR as a pre-screen for detecting Xcc in Brassica seed where plating LOD/LOQ is around the 100 CFU/ml on semi-selective media.

3.1.1. TaqMan PCR Assay Sensitivity

Evaluate the LOD and LOQ of the TaqMan PCR component of the assay with respect to purified Xcc and/or Xcr DNA.

Experimental Method

Ninety replicates of the positive amplification controls (PAC=10 pg) by both TaqMan PCR Berg-Wu and Köhl-Wu duplex TaqMan PCR assays were assayed. A ten-fold dilution serie of purified genomic Xcc DNA ranging from 10 ng to 10 fg were tested by PCR in triplicate per dilution with the both Berg-Wu and Köhl-Wu duplex TaqMan PCR assays. After 10 days, the experiment was repeated using different TaqMan PCR reagents, DNA templates, TaqMan PCR thermocyclers, micropipettes, and labware. At this time 12 replicates of each dilution were tested. All data from each experiment is combined to generate TaqMan PCR assay sensitivity data.

Results[1]

Table 1 - TaqMan PCR Assay sensitivity data to determine the LOD of the assay (100% detection)

Xcc DNA	Berg/Wu Duplex		Köhl/Wu Duplex	
	Berg	Wu	Köhl	Wu
10 ng	15 of 15	15 of 15	15 of 15	15 of 15
1 ng	15 of 15	15 of 15	15 of 15	15 of 15
100 pg	15 of 15	15 of 15	15 of 15	15 of 15
10 pg	15 of 15	15 of 15	15 of 15	15 of 15
1 pg	15 of 15	15 of 15	15 of 15	15 of 15
100 fg	15 of 15	15 of 15	15 of 15	15 of 15
10 fg	15 of 15	15of 15	3 of 15	10 of 15
LOD	10 fg	10 fg	100 fg	100 fg

[1] Reference Appendix 1.v-genomic DNA standard curve data (p.26), Appendix 1.i.-TaqMan PCR optimization data (p.21), Appendix 1.iii-Positive amplification control range data (p.25)

The Berg component of the Berg-Wu assay shows 100% Xcc DNA detection at 10 fg; the limit of detection exists at 10 fg. The Köhl component of the Köhl-Wu detects all replicates of Xcc DNA at 100 fg, so the limit of detection exists at 100 fg or less.

Table 2 - TaqMan PCR Assay sensitivity data to determine LOQ of the assay. (Reliability of 100% of the replicates within a standard deviation of 0.67 Ct (Applied Biosystems standard))

		Berg/Wu Duplex		Köhl/Wu Duplex	
Xcc DNA		Berg	Wu	Köhl	Wu
10 ng	Ct Mean	12.37	12.40	13.58	14.04
	Ct σ	0.459	0.461	0.212	0.266
1 ng	Ct Mean	16.03	16.05	16.85	17.15
	Ct σ	0.228	0.241	0.194	0.269
100 pg	Ct Mean	19.64	19.70	20.37	20.66
	Ct σ	0.224	0.218	0.229	0.168
10 pg	Ct Mean	23.22	23.07	23.90	23.88
	Ct σ	0.210	0.215	0.283	0.259
1 pg	Ct Mean	26.76	26.63	27.38	27.51
	Ct σ	0.302	0.383	0.327	0.254
100 fg	Ct Mean	29.83	29.78	30.83	31.00
	Ct σ	0.256	0.256	0.411	0.388
10 fg	Ct Mean	30.62	30.55	34.67	34.82
	Ct σ	0.316	0.319	1.073	1.170
	LOQ	100 fg	100 fg	100 fg	100 fg

Reliable quantification for the TaqMan PCR component of an assay is determined by ΔCT, a measure of amplification efficiency between two DNA concentrations (an international recommendation, see Bustin et al. (2009), is 90% efficiency, an Applied Biosystems standard corresponds to 3.0-3.67 Ct between ten-fold dilutions of cells and Ct -standard deviation - internal recommendation is 0.67 Ct or less) at each given concentration in the test range.

For both duplex TaqMan PCR assays, these recommendations are observed through 100 fg of Xcc DNA. If only Ct values were used, the LOQ level determination 10 fg would be considered as the LOQ. However, due to the standard deviation values of the Köhl-Wu Duplex and the deviation of the Ct values between 100 fg and 10 fg with the Berg-Wu duplex, the limit of quantification for the TaqMan PCR assay was determined to be approximately 100 fg.

3.1.2. Total Assay Sensitivity

Evaluate the LOD and LOQ of the total assay with respect to Xcc/Xcr cell suspensions.

Experimental Method

A positive process control (PPC) suspension (OD_{600}=0.1*10^{-2}) was prepared, boiled and assayed in 40 replicates by both Berg-Wu and Köhl-Wu duplex TaqMan PCR assays. In addition to the 40 replicates,

six ten-fold serial dilutions on each suspension from OD_{600}=0.100 - 0.100×10^{-6} were prepared, boiled and tested in triplicate per dilution by both Berg-Wu and Köhl-Wu duplex TaqMan PCR assays. As with the experiment described in 3.1.1 the test was repeated with 12 replicates after 10 days using different TaqMan PCR reagents, DNA templates, TaqMan PCR thermocyclers, micropipettes, and labware. All data from from both experiments were combined to generate total assay sensitivity data.

Results[2]

Table 3 - TaqMan PCR Assay sensitivity data to determine the LOD of the assay (100% detection)

Dilution	Berg/Wu Duplex		Köhl/Wu Duplex	
	Berg	**Wu**	**Köhl**	**Wu**
OD_{600}=0.100	15 of 15	15 of 15	15 of 15	15 of 15
10^{-1}	15 of 15	15 of 15	15 of 15	15 of 15
10^{-2}	15 of 15	15 of 15	15 of 15	15 of 15
10^{-3}	15 of 15	15 of 15	15 of 15	15 of 15
10^{-4}	15 of 15	15 of 15	15 of 15	15 of 15
10^{-5}	15 of 15	15 of 15	15 of 15	15 of 15
10^{-6}	13 of 15	13 of 15	1 of 15	4 of 15
LOD	10^{-5}	10^{-5}	10^{-5}	10^{-5}

The Berg component of the Berg-Wu assay falls below 95% Xcc/Xcr detection at OD_{600}= 0.100×10^{-6}; therefore, its limit of detection is approximated to be OD_{600}= 0.100×10^{-5}. Like for the Berg component the Köhl component of the Köhl-Wu assay falls below 95% Xcc/Xcr detection at OD_{600}= 0.100×10^{-6}; therefore, its limit of detection as well exists at OD_{600}= 0.100×10^{-5}.

The limit of detection can be approximated to be 10 CFU/ml for the Berg-Wu assay, and 100 CFU/ml for the Köhl-Wu assay using a Ct threshold of <32 for both the Berg and Köhl components.

Table 4 - TaqMan PCR Assay sensitivity data to determine the LOQ of the assay. Reliability of 100% of the replicates within a standardeviation of 0.67 Ct (Applied Biosystems standard))

		Berg/Wu Duplex		Köhl /Wu Duplex	
Dilution		**Berg**	**Wu**	**Köhl**	**Wu**
OD_{600}=0.100	Ct Mean	14.23	13.81	15.94	16.55
	Ct σ	0.127	0.213	0.320	0.336
10^{-1}	Ct Mean	17.85	17.37	19.33	20.19
	Ct σ	0.137	0.344	0.278	0.299
10^{-2}	Ct Mean	21.38	21.09	22.96	23.87
	Ct σ	0.074	0.206	0.311	0.269

[2] Reference Appendix 1.ii-Total assay optimization data (p.24), Appendix 1. iv.-Positive process control range data (p.25)

10^{-3}	Ct Mean	24.62	25.11	26.20	27.39
	Ct σ	0.154	0.534	0.330	0.326
10^{-4}	Ct Mean	27.75	28.28	29.81	30.98
	Ct σ	0.357	0.372	0.226	0.395
10^{-5}	Ct Mean	31.03	31.68	33.20	34.52
	Ct σ	0.664	0.410	0.661	0.510
10^{-6}	Ct Mean	33.23	33.30	36.95	38.15
	Ct σ	0.892	1.362	UD	0.858
	LOQ	10^{-5}	10^{-5}	10^{-5}	10^{-5}

Reliable quantification for the total assay is determined by ΔCT, a measure of amplification efficiency between two DNA concentrations (an international recommendation, see Bustin et al. (2009), is 90% efficiency, an Applied Biosystems standard corresponds to 3.0-3.67 Ct between ten-fold dilutions of cells and Ct standard deviation - internal recommendation is 0.67 Ct or less) at each given concentration in the test range.

Based on the above it can be observed that the Wu component of Köhl/Wu duplex assays does not meet the recommended ΔCT standards from OD_{600}= 10^{-1} to 10^{-2} dilution. The computation of the ΔCT values between both dilutions resulted in a 3.68 Ct difference for the Wu component. For the Berg-Wu complex the same result was obtained at the 10^{-1} and 10^{-2} dilution with an even higher Ct difference of 3.72.

The OD_{600}= 0.100*10^{-1} dilution can be used to satisfy the intended purpose of the assay due to the binary nature of results scoring.

Conclusion

For both duplex TaqMan PCR assays, these standards are observed consistently through OD_{600}= 0.100×10^{-5} of Xcc/Xcr cell suspension which is equal to 1000 Xcc cells per ml. Suspect screening is done by transferring a loop of bacteria and boil this in 1ml H_2O which is contains 0.5 ng of pure isolate genomic DNA. The LOD of a 1000 cells is representing a genomic DNA concentration of 5.48 fg which is almost a million times more sensitive. With the above mentioned number we can declare that the assays are in all routine suspect screening test (following the protocol in ISTA 07-19) able to detect if the isolate tested is Xcc/Xcr or not and with this meets the requirement.

3.2. Specificity

Specificity of an assay is determined by three parameters: the inclusivity, exclusivity, and selectivity of the assay.

In a TaqMan PCR assay, inclusivity is determined by the fraction of its intended targets that the assay can detect. Exclusivity is determined by the ability of the assay to exclude non-targets from detection. Selectivity refers to the ability of the assay to detect its target(s) in the presence of interference from the sample matrix.

Requirement

For this assay, the requirement is that the Wu component in both the duplex assays does not miss any isolates.

3.2.1. Inclusivity

i. TaqMan PCR Assay Inclusivity

Evaluate the inclusivity of the Wu component of the TaqMan PCR duplex assays (Berg-Wu/ Köhl-Wu) with respect to bacterial DNA from Brassica bacterial isolates. Note: Inclusivity of the Berg and Köhl assay already proven in validation report supporting version 6.

Experimental Method

Genomic DNA from a variety of bacterial organisms isolated from Brassica seeds were tested with both Berg-Wu and Köhl-Wu duplex TaqMan PCR assays. A total of 148 DNA samples from Brassica saprophytes and Xcc/Xcr look-alike strains were screened with both duplex Taqman PCR assays. The used DNA samples were pre-existing, and not normalized prior to use.

Results[3]

Of 148 genomic DNA isolates tested, 148 were detected by the Wu component of both Berg-Wu and Köhl-Wu assays. The purpose of these results is to validate the inclusivity of Wu primers against bacteria that may be present on Brassica seed. This data set supplements the validation of total assay inclusivity, which is more representative of Wu performance as an internal process control.

ii. Total Assay Inclusivity

Evaluate the inclusivity of the Wu component of the total assay with respect to bacterial DNA from Brassica bacterial strains.

Experimental Method

Thirty bacterial strains recovered from routine application of the Xcc/Xcr ISTA 7-19a protocol were tested for inclusivity with the use of both Taqman duplex assays.

Results[4]

Of 30 Xcc/Xcr suspects assayed, 30 were detected by the Wu component of both Berg-Wu and Köhl-Wu duplex TaqMan PCR assays. Berg and Köhl did not detect any of the isolates tested. These results correlated 100% to the pathogenicity assay, confirming none of the suspects were Xcc/Xcr. All controls performed within their designated ranges. Due to the low frequency of Xcc/Xcr suspects in the bacterial plating assay, Wu assay inclusivity should be monitored closely after implementation. Accumulation of supporting data is recommended.

3.2.2. Exclusivity

Exclusivity of the Wu assay was not examined, as the assay targets all bacteria, and test samples used in this assay will all be bacterial cultures.

Conclusion

[3] Reference Appendix 1.vi-TaqMan PCR inclusivity data (p.27)

[4] Reference Appendix 1.vii-Total assay inclusivity data (p.28)

100% detection of the bacterial isolates with the WU assay in both duplexes was accomplished for Xcc/Xcr isolates as well as for non-Xcc/Xcr isolates. Therefore, the requirement for Specificity are met.

3.2.3. Selectivity

The ability of a method to distinguish the target organism (Xcc/Xcr) from other components in the sample.

Requirement: The requirement for the selectivity criteria for the Wu-assay was determined by ΔCt a measure of 90% amplification efficiency.

i. TaqMan PCR Assay Selectivity

The TaqMan PCR assay component cannot be evaluated alone due to the absence of sample matrix. Selectivity was only evaluated with respect to the total assay.

ii. Total Assay Selectivity

Evaluate the effect of the TaqMan PCR efficiency and precision.

Experimental Method

A bacteria growth solution containing OD_{600}= 2.00 of Xcc-BHK92 (prepared at a separate time than other dilutions tested) was prepared and boiled for 10 minutes in ddH_2O. All boiled-extracts (n=20 replicates) were tested by both Berg-Wu and Köhl-Wu duplex TaqMan PCR assays. To determine total assay precision, assay reproducibility data was compared (reference section 3.4.2. p.12).

Results[5]

Table 5 - Total Assay Selectivity

		Berg/Wu Duplex		Köhl /Wu Duplex	
Dilution		Berg	Wu	Berg	Wu
OD_{600}=0.100	Ct Mean	14.24	14.38	14.77	14.92
	Ct σ	0.17	0.20	0.11	0.55
10^{-1}	Ct Mean	18.14	18.20	18.23	18.30
	Ct σ	0.01	0.04	0.32	0.50
10^{-2}	Ct Mean	21.82	21.90	21.85	21.96
	Ct σ	0.22	0.16	0.31	0.21
10^{-3}	Ct Mean	25.26	25.06	25.58	25.64

[5] Reference Appendix 1. ii-Total assay optimization data (p.24), Appendix 1.iv-Positive process control range data (p.25), Appendix 1.ix-Total assay robustness data (p.30)

	Ct σ	0.12	0.06	0.20	0.27
10^{-4}	Ct Mean	28.80	28.80	28.06	28.08
	Ct σ	0.19	0.14	0.21	0.28
10^{-5}	Ct Mean	33.17	33.05	33.55	33.54
	Ct σ	0.50	0.49	0.07	0.11
10^{-6}	Ct Mean	38.09	38.92	37.33	38.30
	Ct σ	1.45	1.52	1.49	1.55

Selectivity for the total assay was determined by ΔCt a measure of amplification efficiency (internal recommendation is 90% efficiency, an Applied Biosystems standard corresponds to 3.0-3.67 Ct between ten-fold dilutions of cells, 3.9-4.77 Ct between twenty-fold dilutions and Ct standard deviation - internal recommendation is 0.67 CT or less) at each given concentration in the test range.

When a 20x stock of positive process control is tested, the Berg component of the Berg-Wu duplex exhibits a ΔCT around 4.2, and a Ct standard deviation of 1.20. The Wu component of the Berg-Wu duplex exhibits a ΔCT around 4.2, and a Ct standard deviation of 1.21. Although guidelines of ΔCTs are met, the standard deviation detected for the Berg-Wu duplex is the cause that the Berg-Wu duplex is not recommended for use at a concentration of 20 × positive process control.

The Köhl component of the Köhl -Wu assay exhibits a ΔCt around 4.2 and a Ct standard deviation of 1.35. The Wu component of the Köhl -Wu assay exhibits a ΔCt around 3.5 and a CT standard deviation of 0.33. The Köhl -Wu duplex is also not recommended for use at a concentration of 20 × positive process control.

Conclusion

The Wu component of both duplex assays does not meet the recommended ΔCT standards from OD_{600}= 0.100 to 10^{-1} dilution but it is detecting the OD_{600}= 0.100 concentration precisely, indicating that internal process controls will produce valid results when bacterial suspensions are used at this concentration. The OD_{600}= 0.100 dilution can be used to satisfy the intended purpose of the assay due to the binary nature of results scoring. It would also be acceptable to use a 10^{-1} dilution of the suspension for routine purposes and therefore thus meets the set requirement.

3.3. Accuracy

Accuracy of an assay consists of the trueness (or bias) and precision of that assay

3.3.1. Trueness (Bias)

i. TaqMan PCR Assay Bias

Bias ~~were~~ is evaluated on a total assay level rather than for the TaqMan PCR assay alone. Bias ~~are~~ is determined by comparing test samples to a reference sample, which would yield minimal bias at this

level of the test because the TaqMan PCR assay was validated using the reference sample (Xcc/Xcr-GRS1 purified genomic DNA) in the absence of matrix.

ii. Total Assay Bias

Due to a lack of CT data normalization methods, test samples could not be compared to a purified genomic DNA reference. Instead, a less resolving survey of bias was performed, which compared CT means from test strains to a reference strain (Xcc/Xcr-GRS1).

Requirement

The representation of total assay bias to demonstrate that various strains of Xcc/Xcr at the standard concentration (OD_{600}=0 .100) will consistently be detected by the proposed assay and produce a positive result.

Experimental Method

Bacteria cell suspensions containing OD_{600}=0 .100 of Xcc 355, Xcc 466, and Xcc 474 in sterile, nuclease-free water were prepared to demonstrate bias. All four different were boiled and tested (n=16 per suspension) by both Berg-Wu and Köhl-Wu duplex TaqMan PCR assays. Results were compared to the Ct values found for the positive process control (section 3.4.2. p.12).

Results[6]

Table 6A - Total Assay Bias, Berg-Wu

Xcc Isolate#	MVS252		MVS253		MVS254		BX-92	
	Berg	Wu	Berg	Wu	Berg	Wu	Berg	Wu
Ct Mean	14.66	14.54	14.85	14.74	14.44	14.36	14.56	14.43
Ct σ	0.196	0.152	0.156	0.185	0.238	0.230	0.78	0.52

Table 6B - Total Assay Bias, Köhl -Wu

Xcc Isolate#	MVS252		MVS253		MVS254		BX-92	
	Köhl	Wu	Köhl	Wu	Köhl	Wu	Köhl	Wu
Ct Mean	16.37	16.36	16.41	16.40	16.16	16.14	16.05	16.38
Ct σ	0.167	0.155	0.152	0.121	0.138	0.127	0.10	0.06

Xcc/Xcr MVS 252, 253 and 254 yielded CT means and standard deviations consistent with those of the reference strain, but do not necessarily fall within the positive process control ranges determined under reproducibility conditions. A tolerable range for Xcc/Xcr strains other than the positive process control is not required to satisfy the intended purpose of the assay, as it reports TaqMan PCR results in a binary fashion.

Strain bias are a limited assessment of assay bias. Other components of assay bias were not assessed due to the lack of reference methods for comparison against the proposed assay.

Conclusion

[6] Reference Appendix 1.viii- Total assay bias data (p.29)

The representation of total assay bias given here demonstrates that various strains of Xcc/Xcr at the standard concentration (OD_{600}=0 .100) were consistently detected by the proposed assay and produce a positive result for the different seed lods tested and therefore the results are in line with the set requirement.

3.3.2. Precision

Precision of an assay must be assessed under both repeatability and reproducibility conditions to assess different ranges of variables.

Repeatability conditions represent the most fundamental level of short-term inherent variability of a process. Under repeatability conditions, a single technician at a single location is processing replicates of a sample, using the same reagents and equipment, over a short duration of time.

Reproducibility conditions represent the long-term inherent variability of a process. Under reproducibility conditions, multiple technicians at multiple locations may process different samples, using different reagents and equipment, over separate intervals of time. Reproducibility data is produced by calculating the mean and standard deviation of collective data between all repeatability experiments.

Requirement

Demonstrate adequate precision, $\sigma < 0.67$ Ct under repeatability conditions and $\sigma < 1.00$ Ct under reproducibility conditions (applied biosystems) throughout the operational range of the assay (10 ng to 100 fg of Xcc DNA).

i. TaqMan PCR Assay Precision

Evaluate the precision of the Berg-Wu and Köhl-Wu duplex TaqMan PCR assays against Xcc/Xcr DNA under repeatability and reproducibility conditions.

Experimental Method

90 replicates of the positive amplification control were assayed by both TaqMan PCR Berg-Wu and Köhl-Wu duplex TaqMan PCR assays. This is TaqMan PCR assay repeatability experiment 1. In addition to this, seven ten-fold dilutions of Xcc purified genomic DNA ranging from 10 ng to 10 fg were prepared and tested in triplicate by both Berg-Wu and Köhl-Wu duplex TaqMan PCR assays. This is TaqMan PCR assay repeatability experiment 2. After 10 days experiment 2 was repeated using different TaqMan PCR reagents, DNA templates, TaqMan PCR thermocyclers, micropipettes, and labware by 12 replicates of each dilution (rather than triplicates). This is TaqMan PCR assay repeatability experiment 3. All data from each of the three experiments were combined to evaluate TaqMan PCR assay reproducibility.

Results [7]

[7] Reference Appendix 1.iii-Positive amplification control range data (p.25), Appendix 1.i-TaqMan PCR optimization data (p.21), Appendix 1.v- Genomic DNA standard curve data (p.26)

Table 7 - TaqMan PCR Assay Precision

TaqMan PCR Assay Repeatability Experiment 1 (t0)

		Berg/Wu Duplex		Köhl /Wu Duplex	
Dilution		Berg	Wu	Köhl	Wu
10 pg	Ct Mean	23.23	22.87	23.89	23.78
	Ct σ	0.371	0.309	0.151	0.125

TaqMan PCR Assay Repeatability Experiment 2 (t1)

		Berg/Wu Duplex		Köhl /Wu Duplex	
Dilution		Berg	Wu	Köhl	Wu
10 ng	Ct Mean	12.67	12.83	13.60	13.88
	Ct σ	0.156	0.081	0.236	0.165
1 ng	Ct Mean	16.31	16.39	17.09	17.32
	Ct σ	0.232	0.093	0.176	0.129
100 pg	Ct Mean	19.75	19.93	20.55	20.77
	Ct σ	0.194	0.115	0.176	0.08
10 pg	Ct Mean	23.31	23.47	24.15	24.20
	Ct σ	0.293	0.095	0.057	0.047
1 pg	Ct Mean	26.94	27.07	27.81	27.84
	Ct σ	0.264	0.363	0.252	0.253
100 fg	Ct Mean	30.06	29.56	30.94	31.07
	Ct σ	0.336	0.362	0.144	0.284
10 fg	Ct Mean	30.80	30.62	32.98	32.90
	Ct σ	0.451	0.427	0.804	0.813

TaqMan PCR Assay Repeatability Experiment 3 (t2)

Dilution		Berg	Wu	Köhl	Wu
10 ng	Ct Mean	12.30	12.30	13.60	13.88
	Ct σ	0.483	0.457	0.236	0.165
1 ng	Ct Mean	15.96	15.96	17.09	17.32
	Ct σ	0.170	0.183	0.176	0.129
100 pg	Ct Mean	19.61	19.64	20.55	20.77
	Ct σ	0.228	0.200	0.176	0.080
10 pg	Ct	23.13	22.96	24.15	24.20

TaqMan PCR Assay Reproducibility (xbar, [t0, t1, t2])

Dilution		Berg	Wu	Köhl	Wu
10 ng	Ct Mean	12.37	12.40	13.58	14.04
	Ct σ	0.459	0.461	0.212	0.266
1 ng	Ct Mean	16.03	16.05	16.85	17.15
	Ct σ	0.228	0.241	0.194	0.269
100 pg	Ct Mean	19.64	19.70	20.37	20.66
	Ct σ	0.224	0.218	0.229	0.168
10 pg	Ct Mean	23.22	23.07	23.90	23.88

	Mean				
	Ct σ	0.079	0.113	0.057	0.047
1 pg	Ct Mean	26.71	26.53	27.81	27.84
	Ct σ	0.302	0.311	0.252	0.253
100 fg	Ct Mean	29.78	29.70	30.94	31.07
	Ct σ	0.212	0.175	0.144	0.284
10 fg	Ct Mean	30.58	30.54	32.98	32.90
	Ct σ	0.283	0.307	0.804	0.813

	Ct σ	0.210	0.215	0.283	0.259
1 pg	Ct Mean	26.76	26.63	27.38	27.51
	Ct σ	0.302	0.383	0.327	0.254
100 fg	Ct Mean	29.83	29.78	30.83	31.00
	Ct σ	0.256	0.256	0.411	0.388
10 fg	Ct Mean	30.62	30.55	34.67	34.82
	Ct σ	0.316	0.319	1.073	1.170

Both Berg-Wu and Köhl-Wu duplex qPCR assays demonstrate adequate precision (international recommendations are σ < 0.67 Ct under repeatability conditions, σ < 1.00 Ct under reproducibility conditions (Applied biosystems) throughout the operational range of the assay (10 ng to 100 fg of *Xcc* DNA). The Wu component of each duplex assay does not appear to adversely affect the precision of the Berg or Köhl components under repeatability and reproducibility conditions of precision. Assay variability under reproducibility conditions increases considerably, but not beyond the adopted standard.

ii. Total Assay Precision

Evaluate the precision of the Berg-Wu and Köhl-Wu duplex TaqMan PCR assays against Xcc/Xcr cell suspensions under repeatability and reproducibility conditions.

Experimental Method

A new positive process control suspension (OD_{600}=0.1) was prepared 40 times. All were boiled and assayed by both Berg-Wu and Köhl-Wu duplex TaqMan PCR assays to determine assay repeatability experiment 1. Prepare a positive process control suspension. Perform six times ten-fold serial dilutions on each suspension from OD_{600}=0.100 - 0.100×10-6. Boil extract all seven dilutions. Test extracts in triplicate by both Berg-Wu and Köhl-Wu duplex TaqMan PCR assays. This is assay repeatability experiment 2. Repeat the experiment after 10 days using different TaqMan PCR reagents, DNA templates, TaqMan PCR thermocyclers, micropipettes, and labware. This is assay repeatability experiment 3. Combine all data from each experiment to generate assay reproducibility data.

Results [8]

Table 8 - Total Assay Precision

qPCR Assay Repeatability Experiment 1 (t0)

	Berg/Wu Duplex	Köhl /Wu Duplex

qPCR Assay Repeatability Experiment 2 (t1)

	Berg/Wu Duplex	Köhl /Wu Duplex

[8] Reference Appendix 1. iv-Positive process control data (p.25), Appendix 1.ii-Total assay optimization data (p.24)

Dilution		Berg	Wu	Köhl	Wu
OD_{600}=0 .100	Ct Mean	13.67	13.87	16.47	16.89
	Ct σ	0.365	0.403	0.254	0.158

Dilution		Berg	Wu	Köhl	Wu
OD_{600}=0 .100	Ct Mean	14.21	13.99	16.05	16.38
	Ct σ	0.140	0.107	0.104	0.055
10^{-1}	Ct Mean	17.82	17.65	19.60	19.40
	Ct σ	0.083	0.036	0.078	0.062
10^{-2}	Ct Mean	21.41	21.08	23.18	22.89
	Ct σ	0.085	0.053	0.070	0.095
$10^{-.3}$	Ct Mean	24.51	24.23	26.44	26.33
	Ct σ	0.085	0.149	0.125	0.131
10^{-4}	Ct Mean	28.16	27.65	29.90	29.74
	Ct σ	0.061	0.089	0.165	0.090
10^{-5}	Ct Mean	31.61	31.29	32.98	33.10
	Ct σ	0.276	0.171	0.825	0.813
10^{-6}	Ct Mean	33.82	33.35	36.95	37.06
	Ct σ	0.368	0.375	-	-

qPCR Assay Repeatability Experiment 3 (t2)

Dilution		Berg	Wu	Köhl	Wu
OD_{600}=0 . 0100	Ct Mean	14.24	13.76	15.91	16.60
	Ct σ	0.130	0.210	0.352	0.364
10^{-1}	Ct Mean	17.85	17.30	19.26	20.13
	Ct σ	0.150	0.351	0.267	0.313
10^{-2}	Ct Mean	21.37	21.10	22.91	23.86
	Ct σ	0.073	0.232	0.325	0.301
$10^{-.3}$	Ct Mean	24.65	25.32	26.30	27.27
	Ct σ	0.157	0.313	0.288	0.123
10^{-4}	Ct Mean	27.65	28.43	29.78	30.90
	Ct σ	0.323	0.201	0.239	0.407
10^{-5}	Ct Mean	30.86	31.78	32.63	34.38

qPCR Assay Reproducibility (xbar,[t0,t1,t2])

Dilution		Berg	Wu	Köhl	Wu
OD_{600}=0 .100	Ct Mean	14.23	13.81	15.94	16.55
	Ct σ	0.127	0.213	0.320	0.336
10^{-1}	Ct Mean	17.85	17.37	19.33	20.19
	Ct σ	0.137	0.344	0.278	0.299
10^{-2}	Ct Mean	21.38	21.09	22.96	23.87
	Ct σ	0.074	0.206	0.311	0.269
$10^{-.3}$	Ct Mean	24.62	25.11	26.20	27.39
	Ct σ	0.154	0.534	0.330	0.326
10^{-4}	Ct Mean	27.75	28.28	29.81	30.98
	Ct σ	0.357	0.372	0.226	0.395
10^{-5}	Ct Mean	31.03	31.68	33.20	34.52

	Ct σ	0.652	0.394	0.636	0.308
10^{-6}	Ct Mean	33.13	33.29	UD*	38.52
	Ct σ	0.924	1.476	UD	0.556

	Ct σ	0.664	0.410	0.661	0.510
10^{-6}	Ct Mean	33.23	33.30	36.95	38.15
	Ct σ	0.892	1.362	UD	0.858

Both Berg-Wu and Köhl-Wu duplex TaqMan PCR assays demonstrate adequate precision international recommendations are σ < 0.67 Ct under repeatability conditions, σ < 1.00 Ct under reproducibility conditions (applied biosystems) throughout the operational range of the assay (OD_{600}=0.100- 0.100×10^{-5} for Xcc cell suspensions). The Wu component of each duplex assay does not appear to adversely affect the precision of the Berg or Köhl components under repeatability and reproducibility conditions of precision. Assay variability under reproducibility conditions increases considerably, but not beyond the adopted standard. All CT values from the repeatability experiments fall within the positive process control ranges (reference section 3.4.2. p.12).

Reproducibility should be evaluated over a broader range of time, technicians, instruments, and laboratories. A more accurate measure of reproducibility will be assessed using data generated from routine assay. Periodic evaluation of reproducibility is critical to monitoring of the assay.

Conclusion

The requirement set for both Taqman PCR duplex assays is met over a multitude of different experiments performed to demonstrate assay repeatability and reproducibility.

3.4. Control Range Determination:

Determine quantitative ranges for positive amplification and process controls. Negative amplification and process controls are included in all the experiments performed within the scope of this project, but are evaluated on a binary scale rather than a continuous range. Negative controls must not produce CT data.

Requirement

TaqMan PCR positive control results within three standard deviations above or below mean CT values for the assays.

3.4.1. Positive amplification control

Experimental Method

TaqMan PCR assay precision - TaqMan PCR assay reproducibility data was referenced to determine the positive process control range (reference section 3.3.2.i. p.9).

Results[9]

Table 9 - Positive Amplification Control Range

	Berg/Wu duplex	
	Berg	**Wu**

	Köhl /Wu duplex	
	Köhl	**Wu**

[9] Reference Appendix 1.v-Genomic DNA standard curve data (p.26), Appendix 1.i-TaqMan PCR assay optimization data (p.21), Appendix 1.iii-Positive amplification control data (p.25)

Ct mean	24.07	23.73
Ct σ	0.48	0.39
Range min	23.01	22.78
Range max	24.96	24.43

Ct mean	26.55	26.49
Ct σ	0.85	0.73
Range min	24.10	24.16
Range max	27.51	27.30

Average CT values and standard deviations for both assays were calculated. Positive amplification control results are considered acceptable within three standard deviations above or below the mean CT values. Under the experimental conditions occurring during this study, 99.7% of replicates are expected to yield results within the acceptable range. The control limits determined above are provisional, and result from limited process variability. Following implementation, control limits must be continuously re-assessed for new ranges to be set under conditions representative of routine processing.

3.4.2. Positive process control

Experimental Method

To determine total assay precision, the assay reproducibility data was refferenced for the range of the positive process control (section 3.3.2.ii p.10).

Results[10]

Table 10 - *Positive* Process Control Range

	Berg/Wu duplex	
	Berg	Wu
Ct mean	13.82	13.86
Ct σ	0.405	0.361
Range max	14.35	14.78
Range min	12.85	13.05

	Köhl /Wu duplex	
	Köhl	Wu
Ct mean	16.32	16.80
Ct σ	0.360	0.266
Range max	16.90	17.19
Range min	15.36	16.04

Average CT values and standard deviations for both assays were calculated.

Conclusion

TaqMan PCR positive control results are considered acceptable within three standard deviations above or below mean CT values.

3.5. Robustness

[10] Reference Appendix 1. ii-Total assay optimization (p.24), Appendix 1.iv for and positive process control data (p.25)

Evaluate the effects of systematic (introduced) process variability on assay performance. Robustness was evaluated with respect to the total assay, but not the TaqMan PCR component of the assay, as systematic error is much more likely to occur during culture preparation and processing that during TaqMan PCR setup.

3.5.1. Total Assay Robustness

Evaluate the effects of un-boiled and over-concentrated positive process control suspensions, and positive process control suspensions prepared from overgrown culture, on the performance of the assay.

Requirement

The effect of boiling or direct use of the extract in the protocol on the detection of Xcc was investigated as well as concentration differences and the difference of growth time of the isolates prior to testing.

Experimental Method

Twenty-four positive process control suspensions (OD_{600}=0.1) were prepared and directly tested with both Berg-Wu and Köhl-Wu duplex TaqMan PCR assays. Another 20 × stock solution of *Xcc-BHK92* [OD_{600}= 2.00] was prepared, boiled and tested (n=24 replicates) by both Berg-Wu and Köhl-Wu duplex TaqMan PCR assays. And as third discrimination a stock solution positive of a process control culture which was grown for 96 h on YDC agar was prepared, boiled and tested (n=24 replicates) by both Berg-Wu and Köhl-Wu duplex TaqMan PCR assays. All data from the three different preperations were compared to positive process control data (section 3.4.2, p.12) to determine if the assays deliver robust results.

Results[11]

Table 11A - Total Assay Robustness: Berg-Wu Duplex

	96 h isolate		**Unboiled (OD=0.100)**		**Overloaded (OD=2.00)**		**PPC**	
	Berg	**Wu**	**Berg**	**Wu**	**Berg**	**Wu**	**Berg**	**Wu**
Ct mean	16.05	16.06	13.71	13.71	8.01	8.15	13.67	13.87
Ct σ	0.29	0.30	0.16	0.17	1.20	1.21	0.36	0.40

Table 11B - Total Assay Robustness: Köhl-Wu Duplex

	96 h isolate		**Unboiled (OD=0.100)**		**Overloaded (OD=2.00)**		**PPC**	
	Köhl	**Wu**	**Köhl**	**Wu**	**Köhl**	**Wu**	**Köhl**	**Wu**

[11] Reference Appendix 1.ix-Total assay robustness data (p.30)

Ct mean	16.89	17.07	14.19	14.42	8.57	9.46	14.47	14.89
Ct σ	0.37	0.31	0.28	0.11	1.35	0.33	0.25	0.16

The 96 h positive process control is detected approximately two cycles later than the standard control used for this assay. This is likely due to excessive growth and metabolic waste on the agar from which bacterial culture is retrieved, as well as a lower DNA concentration to sample matrix ratio in the cell suspension prepared. Although precision does not appear to be significantly affected by the older culture, the shift in trueness makes this control variant unsuitable for use in routine assay.

The un-boiled positive process control demonstrates equivalence of both trueness and precision to the standard control. Note that TaqMan PCR cycling parameters used include a 10 min, 95°C interval to heat-activate polymerase enzymes in the TaqMan PCR mastermix.

The over-concentrated positive process control performs appropriately with respect to the Berg component of the assay, but the $\Delta CT_{(standard\text{-}overloaded)}$ shows a significant reduction of reaction efficiency with respect to the Wu component of the assay.

Conclusion

These robustness experiments confirm the importance of assay standardization. Operators must ensure that samples are processed with close attention to time and extent of culture growth, as well as optical density of the cell suspension prior to boiling. These factors aside, the assay demonstrates considerable tolerance to an inefficacious boiling process.

4. COMPARATIVE TEST

4.1. Organization

An interlaboratory comparative test was organized by Monsanto Holland B.V. for confirmation by Taqman PCR and conventional PCR of *Xanthomonas campestris* pv. *campestris* (Xcc) and *raphani* (Xcr) on suspect colonies found in dilution plating of Brassica seeds.

4.1.1. Aim

The aim of this comparative test was to validate the use of a quantitative PCR method for the confirmation of Xcc/ Xcr suspect colonies found in dilution plating in comparison to the conventional PCR option currently described in ISTA Rule 7-019a. The Taqman PCR and conventional PCR were compared to each other. The objective is that the Taqman PCR should perform at least as well as the conventional PCR before it can be added as an additional PCR option.

4.1.2. Participants

Seven laboratories participated in this test and were randomly allocated to a number, so that the results remain anonymous. All seven laboratories performed the TaqMan PCR according to the protocol shared in the CT test plan. The conventional PCR was performed according to ISTA rule 7-019a by one laboratory only.

4.1.3. DNA samples

All laboratories received one set of samples in May 2017. Each set consisted of 60 genomic DNA samples (10 pg generated via a Qiagen DNeasy Genomic DNA isolation and concentration measurement on a Nanodrop) of different bacterial isolates (Appendix 2) and were divided among the following four categories:

- 20 *Xanthomonas campestris* pv. *campestris*
- 10 *Xanthomonas campestris* pv. *raphani*
- 18 *Xanthomonas* look-alike bacterial isolates
- 12 *Xanthomonads* species commonly found on various vegetable crops

In addition to the samples, a positive Xcc control (1 ng, PPC1), positive Xcr control (1 ng, PPC2), a negative process control (NPC= 1 ng *Acidovorax cattleyae*) and a non-template control (NTC= ddH_2O were included in the package.

Each participating lab is also asked to include at least one Xcc positive boiled bacterial extract (PPC3) from their own collection to demonstrate the effectiveness of the Taqman-PCR assay on bacterial isolates extracted as described in ISTA methods 7-019a and 7-019b. The boiled samples are prepared by transferring a small quantity of Xcc bacteria, grown for 2-4 days on YDC-media, using a tooth pick to a tube containing 1ml dH_2O and vortexing to obtain a homogenised suspension, which is boiled for 5 min at 100°C.

4.1.4. Notation of results

The participants reported quantitative (Ct-Values) as well as qualitative (positive/negative) results for each subsample. Statistical analysis was performed only on the qualitative data since Ct-values are difficult to compare due a multitude of deviants such as equipment, chemicals used, thresholds, primer probe supplier, etc.

4.2. Statistical analysis

4.2.1. Homogeneity test

Since genomic DNA was used in this comparative test no homogeneity test was performed.

4.2.2. Stability test

To determine stability of the samples over time and during transport an extra set of samples was shipped intercontinentally to a participant and immediately returned to the test organiser for testing. Stability was determined by performing Taqman PCR only.

4.2.3. Analysis CT: Statistical analysis for qualitative methods

Samples with no expected variation in the qualitative test result were analysed according to the Standard NF EN ISO 16140 (AFNOR, 2003). Calculation of sensitivity, specificity, accuracy and reproducibility was performed according to the following mathematical formulas:

Sensitivity = ΣPA/(ΣPA+ΣND) x 100

Specificity = ΣNA/(ΣNA+ΣPD) x 100

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

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

Although no fixed rule is determined, as guidance, it is mentioned that values >80% are acceptable (ISTA, 2013). This number is used here to determine whether a method is acceptable or not.

For each of the four categories of DNA samples, accordance (repeatability of qualitative data) and concordance (reproducibility of qualitative data) were evaluated using the method and tool developed by Langton et al. (2002). Accordance was evaluated only on stability data. Accordance is expressed as the probability that two sample give the same result, then the probability is averaged over all laboratories. Accordance = number accords/number of possible accords in one laboratory. Concordance is number of accords / number of possible accords between laboratories (see ISTA, 2013).

4.3. Analysis of XCC CT: Stability tests

The stability test was performed once all participating laboratories had started their tests (June 2017). The Internal control (IC) assay Wu were not always in accordance with the scoring acceptance criteria described in the test plan: "*To determine if a sample is properly prepared the Ct-value of the Wu in Xcc/Xcr negative samples should be at least 3.3 Ct's lower than the NTC. In other words, the sample needs to contain 10 × more microbial DNA than present in the NTC*". However, aside from the IC data, neither the sample set stored on site nor the transcontinentally shipped sample set yielded any false positive or false negative results, in all four sample categories (Appendix 3).

4.4. Analysis of Xcc CT: Comparative test results for reproducibility

Raw data of all seven laboratories are given in a separate file, and the summary of results is presented in Appendix 4. Laboratories did set their own thresholds internally.

4.4.1. Taqman PCR

Analysis of qualitative results from Taqman PCR for each laboratory for all four sub categories was carried out according to the ISO 16140 standard. The results are presented in Tables 12 and 13. All laboratories together obtained 98.94% for sensitivity, meaning no false-negatives were obtained. All laboratories combined also obtained 99.61% for specificity and 99.27% on accuracy.

Table 12 - Results of TaqMan PCR per (total amount of preselection isolates tested/positive reaction in accordance to preselection). *Missing value.

Lab code	Xcc isolates	Xcr isolates	Look-alike isolates	Other xanthomonads
1	20/19	10/11	18/17*	12/12
2	20/20	10/9	18/19	12/12
3	20/20	10/10	18/18	12/12
4	20/20	10/10	18/18	12/12
5	20/20	10/10	18/18	12/12
6	20/20	10/11	18/17	12/12
7	20/20	10/10	18/18	12/12

The sensitivity, specificity and accuracy of the Taqman assay for the Xcc, Xcr, look-alike and other Xanthomonad bacteria isolates were calculated according to the formulas described in ISO 16140. The observed variation was within the allowed range (Table 13).

Table 13 - Performance criteria Taqman PCR per category (N.A.: Not Applicable).

Category	sensitivity	specificity	accuracy
Xcc isolates	99.29%	N.A.	99.29%
Xcr isolates	98.59%	N.A.	98.59%
Look-alike isolates	N.A.	99.21%	99.21%
Other xanthomonads	N.A.	100.00%	100.00%
Total	**98.94%**	**99.61%**	**99.27%**

Accordance and concordance was calculated by target and non-target separately and combined are showing all above 98% for both accordance and concordance which means repeatability and reproducibility are way higher than the 95%.

Confidence limits: 95
Bootstrap samples: 5000
Bootstrap method: representative

Laboratory	Number of samples	positives
1	30	30
2	30	29
3	30	30
4	30	30
5	30	30
6	30	30
7	30	30
8		
9		
10		
11		
12		

	Estimate	Bootstrap s.e.	Bootstrap 95% limits lower	upper
Within lab pairs	3045			
Within lab matched pairs	3016			
Accordance (propn)	0,990	0,0090	0,9714286	1
Accordance (percentage)	99,0%	0,90%	97,14%	100,00%
Total pairs	21945			
Total matched pairs	21736			
Between pairings	18900			
same between	18720			
Concordance (propn)	0,990	0,0089	0,971746	1
Concordance (percentage)	99,0%	0,89%	97,17%	100,00%
COR	1,00		0,9885658	1

Above results are based on 5000 bootstrap samples of 7 labs using representative method

Figure 1a- Target calculation existing of *Xanthomonas campestris campestris* and *raphani* isolates

Confidence limits: 95
Bootstrap samples: 5000
Bootstrap method: representative

Laboratory	Number of samples	positives
1	30	29
2	30	30
3	30	30
4	30	30
5	30	30
6	30	29
7	30	30
8		
9		
10		
11		
12		

	Estimate	Bootstrap s.e.	Bootstrap 95% limits lower	upper
Within lab pairs	3045			
Within lab matched pairs	2987			
Accordance (propn)	0,981	0,0114	0,9619048	1
Accordance (percentage)	98,1%	1,14%	96,19%	100,00%
Total pairs	21945			
Total matched pairs	21529			
Between pairings	18900			
same between	18542			
Concordance (propn)	0,981	0,0112	0,9625397	1
Concordance (percentage)	98,1%	1,12%	96,25%	100,00%
COR	0,99		0,9826847	1

Above results are based on 5000 bootstrap samples of 7 labs using representative method

Figure 1b - Non target calculation for look a likes and *Xanthomonas* isolates

Confidence limits: 95
Bootstrap samples: 5000
Bootstrap method: representative

Laboratory	Number of samples	positives
1	60	59
2	60	59
3	60	60
4	60	60
5	60	60
6	60	59
7	60	60
8		
9		
10		
11		
12		
13		

	Estimate	Bootstrap s.e.	Bootstrap 95% limits lower	upper
Within lab pairs	12390			
Within lab matched pairs	12213			
Accordance (propn)	0,986	0,0063	0,9714286	0,9952381
Accordance (percentage)	98,6%	0,63%	97,14%	99,52%
Total pairs	87990			
Total matched pairs	86739			
Between pairings	75600			
same between	74526			
Concordance (propn)	0,986	0,0062	0,9718254	0,9952381
Concordance (percentage)	98,6%	0,62%	97,18%	99,52%
COR	0,99		0,9857085	1

Above results are based on 5000 bootstrap samples of 7 labs using representative method

Figure 1c - Accordance and concordance calculation of all target and non-target isolates tested in the CT combined.

Discussion and Conclusion

Five out of the seven labs obtained results for the Internal control (IC) assay Wu that were not in accordance with the scoring acceptance criteria described in the test plan . *To determine if a sample is properly prepared the Ct-value of the Wu in Xcc/Xcr negative samples should be at least 3.3 Ct values lower than the NTC. In other words, the sample needs to contain 10 × more microbial DNA than present in the NTC*. However, aside from the IC data, all seven labs performed according to expectations. Lab 1 had three deviants, and lab 2 and 5 showed to have one deviation from the original shared samples.

An explanation as to the discrepancy in the obtained IC results regarding the described rule can be proposed. All genomic DNA samples shared with the participants were of a concentration of 10 pg/µl, which is a concentration nearly equal to the concentration of residual eukaryotic DNA (originating from the Taq polymerase) present in some PCR master mixes. The Wu assay reacts to all 16S DNA present (independently of its origin), thus to ensure representative results, a 10 × concentration difference to the NTC rule was included in the test plan.

Under standard laboratory conditions, suspect bacterial colonies found with dilution plating are boiled directly prior to performing the suspect DNA identification PCR. Boiled colonies give an estimated concentration of 0.5 ng/µL of DNA. Due to import restrictions on live bacteria in some countries, it was decided to use genomic DNA for this comparative test. Although the results for the IC in this test on the genomic samples are not as expected, there is no doubt in the success of the IC-assay as laboratories the labs showed when they performed the Taqman PCR assays on participants in house boiled Xcc/Xcr colony material and all showed to be able to detect Xcc and Xcr properly (Appendix 4).

4.4.2. Taqman-PCR vs Conventional PCR

A pre-trial for this comparative test was organized in February 2017 to demonstrate that each participating laboratory could perform the protocols included in the test plan. Difficulties were experienced in running one of the conventional (gel-based) protocols as described in ISTA method 7-019a. After evaluation of the results, it was decided that the only the test organizers would perform the conventional PCR in addition to the Taqman PCR, in order to compare results of both tests. PCR option 1 from ISTA method 7-019a was used on the same sample set used for the Taqman PCR of the test organizer.

The results for the Taqman PCR and the conventional PCR are shown in Table 14 (data in Appendix 5).

Table 14 - Qualitative results Taqman and conventional PCR per category (N.A.: Not Applicable) (total amount of preselection isolates tested/positive reaction in accordance to preselection).

			Sensitivity		Specificity		Accuracy	
Category	**Taqman PCR**	**Conventional PCR**	**TaqMan**	**Conv. PCR**	**TaqMan**	**Conv. PCR**	**TaqMan**	**Conv. PCR**
Xcc isolates	20/20	20/20	100%	100%	N.A.	N.A.	100%	100%
Xcr isolates	10/10	9/10	100%	91%	N.A.	N.A.	100%	91%
Look-alike isolates	18/18	17/18	N.A.	N.A.	100%	91%	100%	91%
Other Xanthomonads	12/12	12/12	N.A.	N.A.	100%	100%	100%	100%
Inconclusive	0	2						
		Total	100%	95.5%	100%	95.5%	100%	95.5%

Conclusion

Comparison of the results from the Taqman PCR and the conventional PCR showed that the Taqman PCR gave the expected results, whereas the conventional PCR had one sample that scored as a false negative. This sample should have been retested with conventional PCR as no bands were observed on the gel.

5. CONCLUSIONS

The TaqMan PCR assay for confirmation of suspect Xcc/Xcr colonies demonstrated the necessary sensitivity, specificity, accuracy, and robustness to satisfy its intended purpose. When evaluating the sensitivity, specificity and accuracy of the TaqMan PCR for the 60 samples tested in this comparative test, all criteria scored well above the general acceptance rule of >80% (Grimault et al*.,* 2012).

With the explanation of the concentration difference used for this comparative test compared to the practice of using boiled suspect bacteria DNA as input for the PCR instead of genomic DNA, there is no doubt that the internal (amplification) control will function as expected. This was proven by the results of all laboratories showing expected results for Xcc/ Xcr (PPCs) when testing a boiled bacterial isolate from participants in-house collection directly picked from a semi-selective media plate.

Comparison of the results of conventional PCR option 1 (ISTA method 7-019a) with Taqman PCR on the same sample set showed that the Taqman PCR has comparable performance to the conventional PCR option (ISTA method 7-019a V5.0). Results of the CT assessment indicate that the Taqman PCR method can be included as a third PCR option next to the other two conventional PCR options already present in the ISTA protocol 7-019a.

The proposed assay is suitable for implementation under the provisions of monitoring during routine use and accumulation of supporting validation data.

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Appendix 1

Raw Data

i. TaqMan PCR Optimization Data

Berg-Wu Duplex (Xcc)

	Berg 500mM/ WU 100mM		Berg 500mM/ WU 200mM		Berg 500mM/ WU 500mM	
[DNA]	**Berg**	**WU**	**Berg**	**WU**	**Berg**	**WU**
10 ng	12.52	12.84	17.17	14.89	UD	16.7
10 ng	12.65	12.74	16.93	14.77	UD	16.75
10 ng	12.83	12.90	17.21	14.88	UD	16.76
Mean Ct	12.67	12.83	17.10	14.85	UD	16.74
Ct σ	0.156	0.081	0.151	0.067	UD	0.032
1 ng	16.25	16.36	21.01	18.41	UD	20.01
1 ng	16.57	16.49	20.83	18.39	UD	20.1
1 ng	16.12	16.31	20.77	18.32	UD	20.17
Mean Ct	16.31	16.39	20.87	18.373	UD	20.093
Ct σ	0.232	0.093	0.125	0.047	UD	0.08
100 pg	19.80	19.80	24.64	21.96	UD	23.48
100 pg	19.92	19.97	24.36	21.69	UD	23.86
100 pg	19.54	20.02	24.75	21.79	UD	23.63
Mean Ct	19.75	19.93	24.583	21.813	UD	23.657
Ct σ	0.194	0.115	0.201	0.137	UD	0.191
10 pg	23.04	23.40	28.23	25.17	UD	26.76
10 pg	23.26	23.44	UD	25.27	UD	27.09
10 pg	23.62	23.58	UD	25.12	UD	26.8
Mean Ct	23.31	23.47	28.23	25.187	UD	26.883
Ct σ	0.293	0.095	UD	0.076	UD	0.18
1 pg	26.71	26.90	31.62	28.64	UD	30.17
1 pg	26.89	26.83	UD	28.49	UD	30.27
1 pg	27.23	27.49	UD	28.3	UD	30.19
Mean Ct	26.94	27.07	31.62	28.477	UD	30.21
Ct σ	0.264	0.363	UD	0.17	UD	0.053

100 fg	29.71	29.93	UD	30.99	UD	32.77
100 fg	30.09	29.90	UD	31.02	UD	33.2
100 fg	30.38	30.47	UD	31.07	UD	33.03
Mean Ct	30.06	29.56	UD	31.027	UD	33
Ct σ	0.336	0.362	UD	0.04	UD	0.217
10 fg	30.91	30.74	UD	32.55	UD	33.96
10 fg	31.18	30.98	UD	32.35	UD	33.99
10 fg	30.30	30.15	UD	31.82	UD	34.04
Mean Ct	30.80	30.62	UD	32.24	UD	33.997
Ct σ	0.451	0.427	UD	0.377	UD	0.04

Köhl-Wu Duplex (Xcc)

	Köhl 500mM/ WU 100mM		Köhl 500mM/ WU 200mM		Köhl 500mM/ WU 500mM	
[DNA]	**Köhl**	**WU**	**Köhl**	**WU**	**Köhl**	**WU**
10 ng	13.86	14.06	16.23	15.55	UD	16.55
10 ng	13.54	13.83	16.22	15.45	UD	16.57
10 ng	13.4	13.74	16.33	15.49	UD	16.64
Mean Ct	13.60	13.88	16.26	15.50	UD	16.59
Ct σ	0.236	0.165	0.061	0.05	UD	0.047
1 ng	17.11	17.36	19.88	18.99	UD	19.96
1 ng	16.9	17.18	19.9	18.98	UD	19.99
1 ng	17.25	17.43	19.88	18.85	UD	20.03
Mean Ct	17.09	17.32	19.89	18.94	UD	19.99
Ct σ	0.176	0.129	0.012	0.078	UD	0.035
100 pg	20.38	20.69	23.52	22.34	UD	23.19
100 pg	20.53	20.76	23.36	22.15	UD	23.35
100 pg	20.73	20.85	23.47	22.37	UD	23.29
Mean Ct	20.55	20.77	23.45	22.29	UD	23.28
Ct σ	0.176	0.08	0.082	0.119	UD	0.081
10 pg	24.1	24.16	26.99	25.54	UD	26.52
10 pg	24.21	24.25	27	25.69	UD	26.86
10 pg	24.13	24.18	27.13	25.65	UD	26.54
Mean Ct	24.15	24.20	27.04	25.63	UD	26.64
Ct σ	0.057	0.047	0.078	0.078	UD	0.191
1 pg	27.53	27.57	30.29	29.45	UD	30.15
1 pg	27.88	27.89	30.2	29.23	UD	29.97
1 pg	28.02	28.07	30.43	29.24	UD	30.21
Mean Ct	27.81	27.84	30.31	29.31	UD	30.11
Ct σ	0.252	0.253	0.116	0.124	UD	0.125
100 fg	30.98	30.84	32.97	32.76	UD	33.01
100 fg	30.78	31.39	33.74	32.29	UD	33.92
100 fg	31.06	30.99	33.36	32.46	UD	33.12

Mean Ct	30.94	31.07	33.36	32.50	UD	33.35
Ct σ	0.144	0.284	0.385	0.238	UD	0.497
10 fg	32.89	32.68	36.21	35.9	UD	38.22
10 fg	33.22	33.22	35.08	35.41	UD	35.71
10 fg	33.82	33.8	36.7	35.49	UD	35.7
Mean Ct	32.98	32.90	36.00	35.60	UD	36.54
Ct σ	0.804	0.813	0.831	0.263	UD	1.452

Berg-Wu Duplex (*Xcr)*

	Berg 500mM/ WU 100mM	
[DNA]	**Berg**	**WU**
10 ng	12.900	12.870
10 ng	12.370	12.190
10 ng	12.570	12.480
Mean Ct	12.613	12.513
Ct σ	0.268	0.341
1 ng	16.120	15.670
1 ng	16.570	16.250
1 ng	16.380	16.200
Mean Ct	16.357	16.040
Ct σ	0.226	0.321
100 pg	20.260	20.100
100 pg	20.320	19.990
100 pg	19.960	19.620
Mean Ct	20.180	19.903
Ct σ	0.193	0.251
10 pg	23.950	23.360
10 pg	23.780	23.420
10 pg	23.840	23.530
Mean Ct	23.857	23.437
Ct σ	0.086	0.086
1 pg	27.420	27.010
1 pg	27.490	27.180
1 pg	27.280	26.820
Mean Ct	27.397	27.003
Ct σ	0.107	0.180
100 fg	29.880	29.520
100 fg	30.310	29.840

100 fg	29.980	29.470
Mean Ct	30.057	29.610
Ct σ	0.225	0.201
10 fg	30.260	29.920
10 fg	30.980	30.690
10 fg	31.000	30.580
Mean Ct	30.747	30.397
Ct σ	0.422	0.416

ii. **Total Assay Optimization Data**

	Berg/WU Duplex (*Xcc*)				Köhl/WU Duplex(*Xcc*)				Berg/WU Duplex (*Xcr*)			
	Run 1		Run 2		Run 1		Run 2		Run 1		Run 2	
Dilution	**Berg**	**WU**	**Berg**	**WU**	**Köhl**	**WU**	**Köhl**	**WU**	**Berg**	**WU**	**Berg**	**WU**
	14.05	13.9	13.98	13.5	16.11	16.43	16.24	15.89	14.49	14.52	15.04	14.86
OD600=0.100	14.25	13.97	14.18	13.83	15.93	16.32	16.15	15.91	13.81	13.88	14.75	14.4
	14.32	14.11	14.41	14.1	16.11	16.38	16.04	15.79	14.37	14.9	14.93	14.68
Mean Ct	14.21	13.99	14.19	13.81	16.05	16.38	16.14	15.86	14.557	14.433	14.907	14.647
Ct σ	0.14	0.107	0.215	0.3	0.104	0.055	0.100	0.064	0.782	0.515	0.146	0.232
	17.89	17.62	17.46	17.42	19.69	19.35	19.35	19.54	17.83	18.63	18.16	17.78
10^{-1}	17.85	17.69	17.64	17.44	19.54	19.47	19.14	19.44	17.84	17.72	18.37	18.11
	17.73	17.64	17.81	17.24	19.58	19.38	19.89	19.31	18.47	18.75	18.5	18.09
Mean Ct	17.82	17.65	17.64	17.37	19.60	19.40	19.46	19.43	18.05	18.37	18.34	17.99
Ct σ	0.083	0.036	0.175	0.110	0.078	0.062	0.387	0.115	0.367	0.563	0.172	0.185
	21.33	21.04	21.24	20.84	23.11	22.82	23.01	23.06	22.91	21.98	22.32	22
10^{-2}	21.5	21.14	21.28	21.14	23.19	22.86	22.92	22.94	22.04	21.76	22.16	21.87
	21.41	21.06	21.12	20.96	23.25	23	22.74	22.91	23.41	22.54	21.99	21.61

Mean Ct	21.41	21.08	21.21	20.98	23.18	22.89	22.89	22.97	22.79	22.09	22.16	21.83
Ct σ	0.085	0.053	0.083	0.151	0.070	0.095	0.137	0.079	0.693	0.402	0.165	0.199
10^{-3}	24.43	24.12	24.21	24.22	26.54	26.47	26.41	26.45	26.12	24.81	25.55	25.08
	24.51	24.4	24.52	23.85	26.48	26.32	26.64	26.23	25.5	25.16	25.62	25.21
	24.6	24.17	23.96	24.35	26.3	26.21	26.32	26.05	26.76	25.83	26.1	25.65
Mean Ct	24.51	24.23	24.23	24.14	26.44	26.33	26.46	26.24	26.13	25.27	25.76	25.31
Ct σ	0.085	0.149	0.281	0.259	0.125	0.131	0.165	0.200	0.630	0.518	0.299	0.299
10^{-4}	28.12	27.62	27.51	27.61	30.07	29.83	29.42	30	29.93	28.37	28.7	28.29
	28.13	27.58	28.07	27.01	29.89	29.75	29.51	29.19	29.8	29.21	28.92	28.46
	28.23	27.75	27.61	26.84	29.74	29.65	29.53	28.99	29.83	29.2	29.3	28.91
Mean Ct	28.16	27.65	27.73	27.15	29.90	29.74	29.49	29.39	29.85	28.93	28.97	28.55
Ct σ	0.061	0.089	0.299	0.405	0.165	0.090	0.059	0.535	0.068	0.482	0.304	0.320
10^{-5}	31.37	31.13	30.64	31.14	33.65	33.75	33.01	32.99	31.96	30.73	30.84	30.34
	31.54	31.27	32.05	30.5	32.06	32.19	33.36	32.75	31.68	31.05	31.55	30.97
	31.91	31.47	31.36	29.85	33.24	33.37	32.71	32.2	32.41	31.38	31.39	30.82
Mean Ct	31.61	31.29	31.35	30.50	32.98	33.10	33.03	32.65	32.02	31.05	31.26	30.71
Ct σ	0.276	0.171	0.705	0.645	0.825	0.813	0.325	0.405	0.368	0.325	0.372	0.329
10^{-6}	UD	UD	32.56	32	36.95	37.06	34.43	34.05	32.1	31.91	29.48	29.14
	33.56	33.08	32.58	32.04	UD	UD	35.96	35.62	33.5	32.63	31.35	30.93
	34.08	33.61	31.82	31.24	UD	UD	36.99	36.69	33.47	32.37	31.47	30.99

Mean Ct	33.82	33.345	32.32	31.76	36.95	37.06	35.793	35.453	33.023	32.303	30.767	30.353
Ct σ	0.368	0.375	0.433	0.451	-	-	1.288	1.328	0.8	0.365	1.116	1.051

iii. **A. Positive Amplification Control Range, OD_{600}=0.100**

Berg positive process control			
Berg CT		Wu CT	
14.03	13.23	14.43	13.87
13.84	13.51	14.07	13.05
13.28	13.95	13.68	13.14
12.85	14.32	13.36	13.49
13.70	13.34	13.88	13.92
14.20	13.81	14.24	14.56
13.44	14.22	13.76	13.24
13.82	13.39	13.83	14.48
13.14	13.42	13.38	14.11
13.47	13.45	13.64	13.47
13.73	13.53	13.98	13.48
13.59	13.86	14.14	14.12
13.68	13.84	14.78	13.63
13.31	13.68	13.91	14.09
13.95	13.51	13.63	14.07
12.91	14.14	13.94	14.05
13.30	14.14	13.53	13.97
13.75	14.00	13.46	14.29
13.46	14.35	13.56	14.41
13.85	13.9	13.95	14.32

Köhl positive process control			
Köhl CT		Wu CT	
16.19	16.14	16.83	16.85
16.21	16.44	16.77	17.08
16.15	16.64	16.70	16.76
16.49	16.83	16.87	16.94
16.07	16.20	16.62	16.68
16.81	16.57	16.72	17.11
16.03	16.49	16.94	17.15
16.40	16.71	16.88	16.71
16.47	16.90	17.02	16.95
16.71	16.20	17.16	17.05
16.68	16.64	17.03	16.98
16.18	16.75	16.61	16.80
16.73	16.45	16.95	16.91
15.99	16.40	16.82	17.04
16.43	16.64	17.08	16.88
16.49	16.58	16.96	16.61
16.89	16.44	16.98	16.64
16.56	16.21	17.09	16.67
16.58	16.19	16.92	16.99
16.81	16.33	17.02	16.91

iv. Positive Process Control Range

Berg positive process control			
Berg CT		Wu CT	
24.68	24.30	25.45	25.26
24.26	24.24	25.16	25.16
24.30	24.34	25.37	25.40
24.26	24.04	25.20	25.08
24.44	24.15	25.30	25.20
24.15	24.39	25.07	25.36
24.37	24.28	25.21	25.25
24.43	24.37	25.45	25.30
24.37	24.23	25.31	25.25
24.01	24.22	25.03	25.20
23.82	24.43	24.86	25.33
24.32	24.26	25.27	25.27
24.54	24.45	25.41	25.35
24.19	24.15	25.15	25.15
24.16	24.32	25.14	25.21
24.31	24.28	25.32	25.44
24.30	24.56	25.20	25.51
24.26	24.43	25.32	25.47
24.12	24.24	25.16	25.22
23.78	24.12	24.87	25.18

Köhl positive process control			
Köhl CT		Wu CT	
26.03	26.14	27.17	28.06
25.99	26.02	27.09	27.18
25.81	26.19	27.84	27.27
25.85	26.06	26.97	27.20
26.46	25.94	27.38	27.12
26.79	25.82	27.52	27.69
26.70	26.70	27.58	27.37
25.87	25.70	27.73	27.58
26.05	25.86	27.41	27.26
25.94	26.17	27.66	27.30
26.34	25.82	27.02	27.55
26.01	25.44	27.05	27.39
25.96	25.94	27.22	27.34
26.07	26.52	27.96	27.41
26.65	26.57	27.01	27.58
26.72	25.84	26.87	26.86
26.02	25.98	27.27	28.07
26.03	26.75	28.06	28.09
26.05	26.74	27.63	26.86
26.43	26.05	26.83	27.71

v. Genomic DNA Standard Curve

Berg-Wu DUPLEX

	Berg CT	WU CT		Berg CT	WU CT
10 ng	12.39	12.34	**1 pg**	26.55	26.35
	12.75	12.8		26.32	26.12
	12.32	12.58		26.74	26.68
	12.37	12.39		27.49	27.34
	12.52	12.34		26.95	26.74
	12.23	12.26		26.51	26.39
	12.62	12.5		26.62	26.41
	10.96	11.05		26.53	26.34
	11.82	11.85		26.62	26.43
	12.45	12.4		26.54	26.3
	12.46	12.42		26.91	26.62
	12.65	12.65		26.74	26.58
Ct Mean	12.30	12.30	**Ct Mean**	26.71	26.53
Ct σ	0.483	0.457	**Ct σ**	0.302	0.311
1 ng	16.03	16.06	**100fg**	29.81	29.93
	15.89	15.8		29.77	29.59
	16	16.09		29.82	29.78
	16.09	16.06		29.51	29.53
	16.1	16.03		29.86	29.74
	15.99	15.79		29.75	29.85
	15.91	15.82		29.99	29.72
	16.1	16.13		29.82	29.75
	15.48	15.57		29.63	29.28
	15.9	15.92		29.93	29.8
	15.94	16.13		30.1	29.84
	16.08	16.16		29.31	29.64
Ct	15.96	15.96	**Ct**	29.78	29.70

Köhl -Wu DUPLEX

	Köhl CT	WU CT		Köhl CT	WU CT
10 ng	13.73	14.55	**1 pg**	26.93	27.12
	13.69	14.22		27.09	27.34
	13.66	14.04		27.25	27.44
	13.4	14.13		27.47	27.51
	13.61	14.07		27.52	27.57
	13.74	14.12		27.67	27.78
	13.61	14.11		27.35	27.43
	13.81	14.33		27.46	27.57
	13.85	14.27		27.34	27.37
	13.22	13.78		27.17	27.54
	13.2	13.47		27.29	27.35
	13.45	13.92		26.8	27.16
Ct Mean	13.58	14.08	**Ct Mean**	27.28	27.43
Ct σ	0.217	0.275	**Ct σ**	0.250	0.183
1 ng	16.75	16.89	**100 fg**	30.68	30.94
	16.66	17.28		30.78	31.03
	16.78	16.98		31	31.22
	16.83	17.38		30.6	30.69
	17	17.42		30.88	30.99
	16.73	17.32		31.91	31.96
	16.95	16.93		30.6	30.83
	16.89	16.92		30.25	30.41
	16.57	16.82		30.66	30.96
	16.58	16.58		30.33	30.5
	16.72	17.26		31.36	31.46
	17.02	17.43		30.58	30.85
Ct	16.79	17.10	**Ct**	30.80	30.99

Mean			Mean		
Ct σ	0.170	0.183	Ct σ	0.212	0.175
100 pg	19.93	19.95	10 fg	30.96	30.88
	19.36	19.3		30.25	30.07
	19.57	19.6		30.39	30.24
	19.68	19.61		30.63	30.32
	19.38	19.5		30.66	30.41
	20.09	20.02		30.23	30.99
	19.36	19.6		30.15	30.94
	19.54	19.6		30.77	30.48
	19.77	19.82		30.43	30.22
	19.56	19.49		30.9	30.69
	19.47	19.57		30.72	30.43
	19.56	19.65		30.89	30.75
Ct Mean	19.61	19.64	Ct Mean	30.58	30.54
Ct σ	0.228	0.200	Ct σ	0.283	0.307
10 pg	23.01	22.83			
	23.03	22.78			
	23.05	22.91			
	23.28	23.16			
	23.14	22.94			
	23.11	22.93			
	23.2	22.97			
	23.18	23.14			
	23.17	22.99			
	23.18	22.97			
	23.07	22.89			
	23.14	23.05			
Ct Mean	23.13	22.96			
Ct σ	0.079	0.113			

Mean			Mean		
Ct σ	0.152	0.280	Ct σ	0.455	0.419
100 pg	20.03	20.55	10 fg	34.92	35.26
	20.2	20.62		34.34	34.64
	20.44	20.6		34.53	34.75
	20.4	20.62		34.16	34.18
	20.49	20.79		34.32	34.54
	20.38	20.62		36.75	36.98
	20.63	20.81		35.45	35.79
	20.65	21.05		36.3	36.42
	20.07	20.59		34.36	34.52
	20.28	20.54		34.53	34.62
	20.38	20.46		36.02	36.21
	19.96	20.38		34.48	34.65
Ct Mean	20.33	20.64	Ct Mean	35.01	35.21
Ct σ	0.224	0.177	Ct σ	0.891	0.911
10 pg	23.68	24			
	23.79	24.1			
	23.96	24.22			
	23.77	24.03			
	23.83	24.22			
	24.17	24.26			
	23.87	24.07			
	24.2	24.51			
	23.84	24.06			
	23.71	24.13			
	24	24.26			
	23.51	23.88			
Ct Mean	23.86	24.15			
Ct σ	0.198	0.163			

vi. qPCR Inclusivity

Wu Coverage of Bacterial DNA isolates other than *Xcc*/*Xcr* (n=85)

	Berg	Köhl		Berg	Köhl		Berg	Köhl
Sample Name	**Wu Ct**	**Wu Ct**	**Sample Name**	**Wu Ct**	**Wu Ct**	**Sample Name**	**Wu Ct**	**Wu Ct**
BX02	16.26	17.34	BX37	15.82	16.48	BX76	17.55	18.67
BX03	12.89	14.02	BX38	18.47	18.55	BX77	29.34	31.71
BX04	23.56	25.09	BX39	13.70	14.82	BX78	32.72	34.08
BX05	21.22	22.31	BX40	12.48	13.85	BX79	26.63	27.50
BX06	29.49	30.57	BX41	17.89	18.26	BX80	25.37	25.29
BX07	23.59	25.01	BX42	19.54	21.28	BX81	34.11	34.59
BX08	21.62	21.99	BX43	17.56	18.71	BX82	17.61	18.52
BX09	19.81	20.73	BX44	11.49	12.36	BX83	11.18	12.09
BX13	14.66	15.97	BX46	17.71	17.63	BX86	15.67	17.22
BX14	16.21	17.35	BX47	21.49	22.57	BX87	24.32	25.97
BX17	23.89	25.24	BX48	22.23	23.47	BX88	22.07	23.68
BX18	20.35	21.75	BX49	19.87	21.06	BX89	16.49	16.66
BX19	32.90	34.72	BX50	14.58	15.39	BX90	18.08	17.52
BX20	29.18	30.85	BX51	17.45	18.66	Sap1	27.98	30.14
BX21	27.31	28.94	BX52	20.45	20.67	Sap2	23.17	26.01
BX22	26.72	27.64	BX53	21.33	21.46	Sap3	21.79	21.79
BX23	25.11	27.03	BX54	32.68	34.01	Sap4	23.88	25.41
BX24	18.11	20.10	BX58	27.37	28.60	Sap5	32.80	33.97
BX25	15.67	16.81	BX59	18.32	18.99	Sap6	30.74	29.97
BX26	12.48	13.67	BX64	14.52	15.64	Sap7	18.89	19.71
BX27	31.58	32.97	BX65	13.27	12.58	Sap8	17.43	17.96
BX28	22.34	24.05	BX66	20.05	21.16	Sap9	20.17	21.05
BX29	26.71	26.44	BX67	22.44	22.57	Sap10	29.08	31.13
BX30	29.08	31.07	BX68	18.93	20.22	Sap11	24.22	25.61
BX31	22.47	24.16	BX69	19.54	19.80	Sap12	23.52	23.98
BX32	20.11	22.19	BX70	28.17	29.39			
BX33	17.79	18.63	BX71	25.71	26.37			
BX34	16.79	18.12	BX72	19.77	21.82			
BX35	14.79	15.60	BX73	12.71	13.86			
BX36	10.80	13.71	BX74	19.87	18.11			

vii. Total Assay Inclusivity

The results below represent pathogenicity verified non-*Xcc/Xcr* isolates.

The total assay inclusivity experiments were performed before assay optimization, as a critical component of the feasibility study. Due to this context, bacterial cultures assayed were not standardized to a specific optical density; therefore, the CT values below represent the inclusivity of the assay only, but not the accuracy of the assay. Furthermore, *Xcc/Xcr* suspects rarely arise in routine testing. The isolates below comprise the complete collection of *Xcc/Xcr* suspects available for inclusivity testing.

	Berg-Wu		Köhl-Wu		Pathogenicity
Isolate#	Berg Ct	Wu Ct	Köhl Ct	Wu Ct	+/-
1	UD	21.44	UD	20.36	-
2	UD	20.82	UD	19.83	-
3	UD	18.62	UD	17.69	-
4	UD	19.77	UD	18.84	-
5	UD	21.13	UD	20.15	-
6	UD	20.52	UD	19.40	-
7	UD	21.20	UD	20.11	-
8	UD	20.63	UD	19.52	-
9	UD	21.06	UD	20.00	-
10	UD	21.16	UD	20.15	-
11	UD	20.36	UD	19.25	-
12	UD	19.92	UD	18.73	-
13	UD	17.58	UD	16.73	-
14	UD	19.94	UD	18.79	-
15	UD	33.95	UD	35.42	-
16	UD	33.13	UD	34.39	-
17	UD	31.74	UD	31.59	-
18	UD	19.94	UD	18.79	-
19	UD	33.95	UD	35.42	-
20	UD	33.13	UD	34.39	-
21	UD	31.74	UD	31.59	-
22	UD	15.05	UD	13.96	-
23	UD	21.35	UD	19.99	-
24	UD	20.44	UD	19.25	-
25	UD	21.30	UD	20.98	-
26	UD	21.76	UD	21.44	-
27	UD	21.83	UD	21.64	-
28	UD	22.78	UD	22.40	-
29	UD	19.28	UD	20.37	-
30	UD	22.88	UD	23.19	-

viii. Total Assay Bias

Berg-WU Various Xcc Isolate Evaluation

Xcc Isolate#	**MVS252**		**MVS253**		**MVS254**	
	Berg	**WU**	**Berg**	**Wu**	**Berg**	**Wu**
	14.27	14.33	14.55	14.52	14.18	14.17
	14.49	14.41	15.03	14.92	14.60	14.55
	14.58	14.41	14.95	14.89	14.77	14.76
	14.72	14.50	15.00	14.88	14.81	14.63
	14.80	14.63	14.82	14.67	14.35	14.23
	14.71	14.60	14.71	14.52	14.11	14.01
	14.89	14.82	14.63	14.59	14.29	14.24
	14.37	14.38	15.01	14.93	14.31	14.24
	14.84	14.68	14.98	14.89	14.70	14.57
	14.67	14.43	14.87	14.89	14.23	14.15
	14.86	14.70	14.84	14.77	14.57	14.46
	14.66	14.59	14.83	14.43	14.41	14.25
CT mean	14.66	14.54	14.85	14.74	14.44	14.36
CTσ	0.20	0.15	0.16	0.19	0.24	0.23

Köhl -WU Various Xcc Isolate Evaluation

Xcc Isolate#	**MVS252**		**MVS253**		**MVS254**	
	Berg	**WU**	**Berg**	**Wu**	**Berg**	**Wu**
	16.18	16.17	16.26	16.26	16.16	16.12
	16.41	16.35	16.40	16.43	16.31	16.30
	16.03	16.06	16.44	16.40	16.29	16.26
	16.49	16.49	16.36	16.33	16.15	16.12
	16.36	16.33	16.41	16.36	16.01	15.97

	16.38	16.35	16.21	16.27	15.83	15.86
	16.33	16.28	16.51	16.49	16.07	16.05
	16.38	16.45	16.73	16.64	16.31	16.24
	16.35	16.30	16.54	16.50	16.18	16.20
	16.52	16.51	16.26	16.36	16.13	16.15
	16.70	16.64	16.51	16.54	16.23	16.14
	16.29	16.35	16.25	16.26	16.21	16.22
CT mean	16.37	16.36	16.41	16.40	16.16	16.14
CTơ	0.17	0.15	0.15	0.12	0.14	0.13

ix. Assay Robustness

Assay Robustness Berg-Wu Duplex

	96 h Isolate		Unboiled (OD=0.100)		Overloaded (OD=2.0)	
	Berg Ct	Wu Ct	Berg Ct	Wu Ct	Berg Ct	Wu Ct
	16.18	16.17	16.27	16.36	8.87	8.98
	16.41	16.35	15.80	15.75	4.57	5.04
	16.03	16.06	15.83	16.08	8.07	8.30
	16.49	16.49	15.61	15.86	8.09	8.42
	16.36	16.33	16.38	16.29	8.28	8.56
	16.38	16.35	16.33	16.38	8.34	8.59
	16.33	16.28	16.18	16.17	7.96	8.40
	16.38	16.45	16.41	16.35	8.02	8.28
	16.35	16.30	16.03	16.06	8.54	8.92
	16.52	16.51	16.49	16.49	7.41	7.74
	16.70	16.64	16.36	16.33	8.84	9.01
	16.29	16.35	16.38	16.35	8.97	9.00
	16.26	16.26	16.33	16.28	7.67	4.67
	16.40	16.43	16.38	16.45	4.25	8.15
	16.44	16.40	16.35	16.30	8.43	8.91
	16.36	16.33	16.52	16.51	8.03	8.35
	16.41	16.36	16.70	16.64	7.85	7.95
	16.21	16.27	16.29	16.35	8.97	9.03
	16.51	16.49	15.86	16.36	8.27	8.54
	16.73	16.64	16.05	15.97	7.79	8.25
	16.54	16.50	15.77	16.02	8.78	5.87
	16.26	16.36	15.94	16.17	8.46	8.80
	16.51	16.54	15.31	15.50	8.79	8.90
	16.25	16.26	15.67	15.86	8.99	9.00
Ct Mean	16.39	16.38	16.14	16.20	8.01	8.15
Ct σ	0.157	0.138	0.343	0.270	1.196	1.211

Assay Robustness Köhl -Wu Duplex

	96 hour Isolate		Unboiled (OD=0.100)		Overloaded (OD=2.0)	
	Köhl Ct	Wu Ct	Köhl Ct	Wu Ct	Köhl Ct	Wu Ct
	18.39	18.68	18.05	18.43	9.50	9.73
	18.06	18.27	18.01	18.38	8.95	9.52
	18.26	18.56	18.26	18.69	8.91	9.27
	18.36	18.58	18.25	18.52	9.45	9.78
	18.28	18.48	18.40	18.74	8.68	9.19
	18.27	18.59	18.86	19.25	9.24	9.73
	18.11	18.32	17.86	17.76	8.54	9.24
	18.38	18.51	18.03	17.91	9.70	9.90
	17.89	18.18	18.00	17.89	4.91	8.94
	18.18	18.45	17.93	17.75	9.24	9.58
	18.50	18.66	17.96	17.61	8.05	8.77
	18.33	18.65	17.87	17.43	8.43	9.36
	17.87	17.96	18.09	17.67	8.67	9.32
	18.40	18.35	18.24	17.91	8.17	8.81
	18.43	18.68	18.1	17.78	9.53	9.85
	17.21	18.46	17.96	17.73	9.62	9.85
	18.98	17.89	18.14	17.83	9.51	9.69
	17.93	17.98	17.86	17.81	9.33	9.59
	18.35	18.72	18.00	17.76	9.30	9.64
	17.99	18.48	18.01	17.74	8.93	9.41
	18.60	18.91	18.31	18.75	5.58	9.65
	18.58	18.84	17.93	18.14	8.81	9.38
	17.87	18.14	18.47	19.24	9.30	9.73
	18.19	18.48	17.39	18.14	5.34	9.14
Ct Mean	18.23	18.45	18.08	18.12	8.57	9.46
Ct σ	0.341	0.271	0.274	0.512	1.352	0.326

Appendix 2 Composition of the sample set used in CT

Sample	Isolate	Bacteria species	Isolate	Berg	Köhl	Wu	Gel	Pathogenicity*
1	MVS384	-	lal^{+}	-	-	+	-	
2	MVS381		Xcr	+	-	+	+	+
3	MVS268	-	lal	-	-	+	-	
4	MVS283	-	lal	-	-	+	-	
5	MVS240		Xcc	+	+	+	+	+
6	MVS50	X. melonis	neg	-	-	+	-	
7	wk44-1		Xcc	+	+	+	+	+
8	MVS343		Xcr	+	-	+	+	+
9	MVS220		Xcr	+	-	+	+	+
10	MVS335	-	lal	-	-	+	-	
11	MVS264		Xcc	+	+	+	+	+
12	MVS352		Xcc	+	+	+	+	+
13	MVS199	X. fuscans	neg	-	-	+	-	
14	MVS338		Xcc	+	+	+	+	+
15	MVS248		Xcc	+	+	+	+	+
16	MVS255		Xcc	+	+	+	+	+
17	MVS82	X. perforans	neg	-	-	+	-	
18	MVS382		Xcr	+	-	+	+	+
19	MVS363		Xcr	+	-	+	+	+
20	MVS278		Xcc	+	+	+	+	+
21	MVS344		Xcc	+	+	+	+	+
22	MVS49	X. cucurbitae	neg	-	-	+	-	
23	MVS262		Xcc	+	+	+	+	+
24	MVS362		Xcr	+	-	+	+	+
25	MVS334		Xcc	+	+	+	+	+
26	MVS33		Xcr	+		+	+	+
27	MVS415	-	lal	-	-	+	-	
28	MVS270		Xcc	+	+	+	+	+
29	MVS361		Xcr	+	-	+	+	+
30	MVS333	-	lal	-	-	+	-	
31	MVS258		Xcr	+	-	+	+	+
32	MVS52	X. vesicatoria	neg	-	-	+	-	
33	MVS189	X. pelargonii	neg	-	-	+	-	
34	MVS391	-	lal	-	-	+	-	
35	MVS56	X. euvesicatoria	neg	-	-	+	-	
36	MVS274		Xcc	+	+	+	+	+

37	MVS260		Xcc	+	+	+	+	+
38	MVS272		Xcc	+	+	+	+	+
39	MVS252		Xcc	+	+	+	+	+
40	MVS386	-	lal	-	-	+	-	
41	wk41-2	-	lal	-	-	+	-	
42	MVS387	-	lal	-	-	+	-	
43	MVS385	-	lal	-	-	+	-	
Sample	Isolate	Bacteria species	Isolate	Berg	Köhl	Wu	Gel	Pathogenicity*
44	MVS51	X. cucurbitae	neg	-	-	+	-	
45	MVS169	X. gardneri	neg	-	-	+	-	
46	wk12		lal	-	-	+	-	
47	MVS198	X. phaseoli	neg	-	-	+	-	
48	MVS247		Xcc	+	+	+	+	+
49	MVS394	-	lal	-	-	+	-	
50	MVS390	-	lal	-	-	+	-	
51	MVS350	X. vitians	neg	-	-	+	-	
52	MVS282	-	lal	-	-	+	-	
53	MVS392	-	lal	-	-	+	-	
54	MVS250		Xcc	+	+	+	+	+
55	MVS383	-	lal	-	-	+	-	
56	MVS364		Xcr	+	-	+	-	
57	wk2-1	-	lal	-	-	+	-	
58	MVS266		Xcc	+	+	+	+	+
59	MVS245		Xcc	+	+	+	+	+
60	MVS223	X. hortorum	neg	-	-	+	-	

*Pathogenicity performed in multiple runs to construct organizers inhouse isolate database, not repeated within this test. PCR negative were not tested by a pathogenicity assay.

+ The LAL (Look A Like) resembles the target organism on the basis of morphology on (semi-selective) growth media and genetic features. An example of a LAL is the use of a different subspecies or pathovar of the target organism.

Appendix 3 Stability test

Sample nr.	Expected results	9/5/2017 (date prior to shipment)				16/6/2017 (date equal to participant perfroming the CT)			
		TaqMan PCR				TaqMan PCR			
		Berg	Köhl	Wu	results	Berg	Köhl	Wu	results
1	neg	ND	36,36	31,22	*neg*	36,23	35,33	29,19	*neg*
2	Xcr pos	26,91	ND	29,59	*Xcr pos*	28,67	35,44	29,13	*Xcr pos*
3	neg	ND	ND	33,41	*neg*	35,83	35,55	34,12	*neg*
4	Xcc pos	27,60	26,75	31,71	*Xcc pos*	29,73	29,71	32,46	*Xcc pos*
5	Xcr pos	28,11	37,23	28,92	*Xcr pos*	29,80	32,80	28,88	*Xcr pos*
6	Xcr pos	26,80	ND	28,72	*Xcr pos*	27,89	34,76	28,88	*Xcr pos*
7	Xcr pos	23,29	36,60	28,53	*Xcr pos*	28,03	35,34	28,72	*Xcr pos*
8	neg	ND	36,32	30,23	*neg*	34,79	35,52	30,59	*neg*
9	Xcc pos	27,00	25,89	27,84	*Xcc pos*	27,07	27,09	28,49	*Xcc pos*
10	Xcc pos	28,12	27,15	29,97	*Xcc pos*	28,96	28,83	29,87	*Xcc pos*
11	neg	ND	ND	27,06	neg	36,00	36,66	26,86	neg
12	Xcc pos	27,51	26,36	29,03	Xcc pos	28,92	28,89	29,16	Xcc pos
13	Xcc pos	27,50	26,90	28,54	Xcc pos	28,48	28,42	29,01	Xcc pos
14	Xcc pos	28,09	27,27	30,20	Xcc pos	29,49	29,45	29,97	Xcc pos
15	neg	ND	35,25	28,68	neg	35,57	36,23	27,99	neg
16	Xcr pos	27,33	33,76	28,15	Xcr pos	27,97	33,19	29,10	Xcr pos
17	Xcr pos	26,80	ND	28,77	Xcr pos	27,83	35,38	29,33	Xcr pos
18	Xcc pos	27,45	26,43	32,39	Xcc pos	31,41	30,98	31,34	Xcc pos
19	Xcc pos	28,21	27,13	29,28	Xcc pos	28,91	28,87	29,59	Xcc pos
20	neg	ND	ND	28,99	neg	35,95	35,77	28,98	neg
21	Xcc pos	26,26	25,45	28,88	Xcc pos	28,58	28,50	28,63	Xcc pos
22	Xcc pos	28,23	27,29	28,64	Xcc pos	28,18	28,06	28,38	Xcc pos
23	Xcr pos	26,76	35,89	30,21	Xcr pos	29,62	35,35	31,52	Xcr pos
24	neg	ND	36,76	30,11	neg	35,29	35,17	31,49	neg
25	Xcr pos	28,96	ND	28,89	Xcr pos	28,16	34,30	29,11	Xcr pos
26	neg	ND	ND	32,85	neg	35,04	34,62	32,75	neg
27	Xcr pos	27,62	37,51	28,59	Xcr pos	27,82	34,65	29,53	Xcr pos
28	neg	ND	37,49	27,91	neg	34,17	34,11	28,96	neg
29	neg	ND	36,49	29,57	neg	36,19	35,38	29,84	neg
30	neg	ND	35,97	28,37	neg	34,86	35,32	29,12	neg
31	neg	ND	36,18	30,17	neg	36,36	35,55	30,69	neg
32	Xcc pos	28,81	27,64	28,94	Xcc pos	28,76	28,58	29,08	Xcc pos
33	Xcc pos	28,48	27,45	28,55	Xcc pos	27,85	27,66	28,46	Xcc pos
34	neg	ND	ND	29,71	neg	35,22	34,22	29,12	neg
35	neg	ND	35,66	29,20	neg	35,59	34,96	29,59	neg
36	neg	ND	37,95	25,25	neg	35,63	34,40	25,47	neg
37	neg	ND	36,36	28,66	neg	36,12	34,33	28,46	neg
38	Xcc pos	28,52	27,61	28,73	Xcc pos	28,56	28,73	29,89	Xcc pos
39	Xcr pos	28,28	36,52	30,46	Xcr pos	30,67	33,58	31,13	Xcr pos
40	neg	ND	37,80	29,89	neg	35,98	35,03	31,41	neg
41	neg	39,06	36,18	27,06	neg	35,61	33,62	27,60	neg
42	Xcc pos	29,16	28,17	28,48	Xcc pos	28,38	28,40	29,08	Xcc pos
43	neg	ND	36,68	30,43	neg	35,45	30,60	30,56	neg
44	Xcc pos	26,91	25,79	26,90	Xcc pos	26,93	26,74	26,62	Xcc pos
45	Xcc pos	27,66	29,15	31,59	Xcc pos	30,13	31,62	31,34	Xcc pos
46	Xcc pos	29,16	28,29	28,78	Xcc pos	28,54	28,28	29,07	Xcc pos
47	Xcc pos	27,18	26,14	32,95	Xcc pos	35,00	33,00	33,59	Xcc pos
48	neg	ND	38,12	29,03	neg	36,39	29,96	28,69	neg
49	neg	39,23	38,64	32,22	neg	35,39	30,26	30,56	neg
50	neg	ND	36,70	28,82	neg	32,02	28,92	27,68	neg
51	Xcc pos	28,41	27,94	27,88	Xcc pos	27,45	27,03	27,45	Xcc pos
52	neg	ND	ND	22,02	neg	34,93	30,94	27,12	neg
53	Xcc pos	27,94	27,02	27,92	Xcc pos	27,86	27,53	27,68	Xcc pos
54	Xcc pos	28,52	27,33	28,18	Xcc pos	27,96	27,51	27,85	Xcc pos
55	neg	ND	35,39	28,32	neg	35,57	30,36	29,00	neg
56	neg	38,30	33,61	30,66	neg	35,35	32,53	30,98	neg
57	neg	ND	35,37	30,70	neg	36,78	30,16	30,63	neg
58	Xcc pos	ND	38,41	28,16	Xcc pos	+	+	+	Xcc pos
59	Xcc pos	ND	ND	28,61	Xcc pos	+	+	+	Xcc pos
60	neg	36,03	34,95	29,34	neg	-	-	+	neg

Appendix 4 CT results: Taqman PCR

Summary	Lab1	Lab2	Lab3	Lab4	Lab5	Lab6	Lab7
	Conclusion	Conclusion	Conclusion	Conclusion	Conclusion	Conclusion	Conclusion
1	Neg	neg	neg	neg	neg	neg	neg
2	Xcr	xcr	xcr	xcr	xcr	xcr	xcr
3	Neg	neg	neg	neg	neg	neg	neg
4	Neg	neg	neg	neg	neg	neg	neg
5	Xcc	xcc	xcc	xcc	xcc	xcc	xcc
6	neg	neg	neg	neg	neg	neg	neg
7	Xcc	xcc	xcc	xcc	xcc	xcc	xcc
8	Xcr	neg	xcr	xcr	xcr	xcr	xcr
9	Xcr	xcr	xcr	xcr	xcr	xcr	xcr
10	Neg	neg	neg	neg	neg	neg	neg
11	Xcc	xcc	xcc	xcc	xcc	xcc	xcc
12	Xcc	xcc	xcc	xcc	xcc	xcc	xcc
13	Neg	neg	neg	neg	neg	neg	neg
14	Xcc	xcc	xcc	xcc	xcc	xcc	xcc
15	Xcc	xcc	xcc	xcc	xcc	xcc	xcc
16	Xcc	xcc	xcc	xcc	xcc	xcc	xcc
17	Neg	neg	neg	neg	neg	neg	neg
18	Xcr	xcr	xcr	xcr	xcr	xcr	xcr
19	Xcr	xcr	xcr	xcr	xcr	xcr	xcr
20	Xcc	xcc	xcc	xcc	xcc	xcc	xcc
21	Xcc	xcc	xcc	xcc	xcc	xcc	xcc
22	Neg	neg	neg	neg	neg	neg	neg
23	Xcc	xcc	xcc	xcc	xcc	xcc	xcc
24	Xcr	xcr	xcr	xcr	xcr	xcr	xcr
25	Xcc	xcc	xcc	xcc	xcc	xcc	xcc
26	Xcr	xcr	xcr	xcr	xcr	xcr	xcr
27	Neg	neg	neg	neg	neg	neg	neg
28	Xcc	xcc	xcc	xcc	xcc	xcc	xcc
29	Xcr	xcr	xcr	xcr	xcr	xcr	xcr
30	Neg	neg	neg	neg	neg	neg	neg
31	Xcr	xcr	xcr	xcr	xcr	xcr	xcr
32	Xcr	neg	neg	neg	neg	neg	neg
33	Neg	neg	neg	neg	neg	neg	neg
34	neg	neg	neg	neg	neg	neg	neg
35	Neg	neg	neg	neg	neg	neg	neg
36	Xcc	xcc	xcc	xcc	xcc	xcc	xcc
37	neg	xcc	xcc	xcc	xcc	xcc	xcc
38	Xcc	xcc	xcc	xcc	xcc	xcc	xcc
39	Xcc	xcc	xcc	xcc	xcc	xcc	xcc
40	Neg	neg	neg	neg	neg	neg	neg
41	Neg	neg	neg	neg	xcr	neg	neg
42	Neg	neg	neg	neg	neg	neg	neg
43	Neg	neg	neg	neg	neg	neg	neg
44	Neg	neg	neg	neg	neg	neg	neg
45	Neg	neg	neg	neg	neg	neg	neg
46	Neg	neg	neg	neg	neg	neg	neg
47	Neg	neg	neg	neg	neg	neg	neg
48	Xcc	xcc	xcc	xcc	xcc	xcc	xcc
49	neg	neg	neg	neg	neg	neg	neg
50	missing value	neg	neg	neg	neg	neg	neg
51	Neg	neg	neg	neg	neg	neg	neg
52	Neg	neg	neg	neg	neg	neg	neg
53	Neg	neg	neg	neg	neg	neg	neg

54	Xcc	xcc	xcc	xcc	xcc	xcc	xcc
55	Neg	neg	neg	neg	neg	neg	neg

	Lab1	Lab2	Lab3	Lab4	Lab5	Lab6	Lab7
	Conclusion	Conclusion	Conclusion	Conclusion	Conclusion	Conclusion	Conclusion
56	Xcr	xcr	xcr	xcr	xcr	xcr	xcr
57	Neg	neg	neg	neg	neg	neg	neg
58	Xcc	xcc	xcc	xcc	xcc	xcc	xcc
59	Xcc	xcc	xcc	xcc	xcc	xcc	xcc
60	Neg	neg	neg	neg	neg	neg	neg

Appendix 5 CT results: Taqman vs Conventional PCR

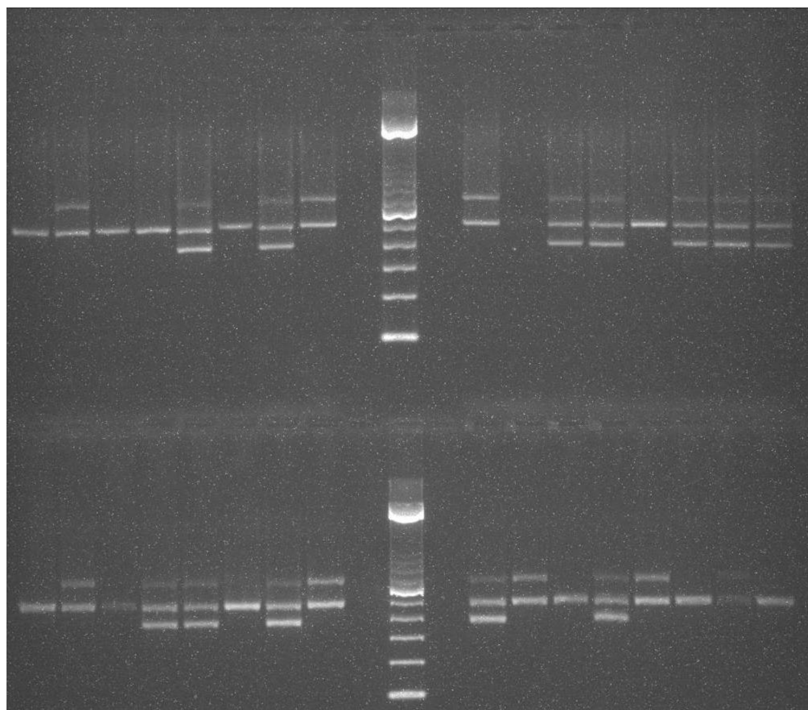
	sample	Conv. PCR	Taqman PCR
row 1	1	neg	neg
	2	xcr	xcr
	3	neg	neg
	4	neg	neg
	5	xcc	xcc
	6	neg	neg
	7	xcc	xcc
	8	xcr	xcr
	x		
	100bp ladder		
	x		
	9	xcr	xcr
	10	no band	neg
	11	xcc	xcc
	12	xcc	xcc
	13	neg	neg
	14	xcc	xcc
	15	xcc	xcc
	16	xcc	xcc

	sample	Conv. PCR	Taqman PCR
row 2	17	neg	neg
	18	xcr	xcr
	19	neg	xcr
	20	xcc	xcc
	21	xcc	xcc
	22	neg	neg
	23	xcc	xcc
	24	xcr	xcr
	x		
	100bp ladder		
	x		
	25	xcc	xcc
	26	xcr	xcr
	27	neg	neg
	28	xcc	xcc
	29	xcr	xcr
	30	neg	neg
	31	xcr	xcr
	32	neg	neg

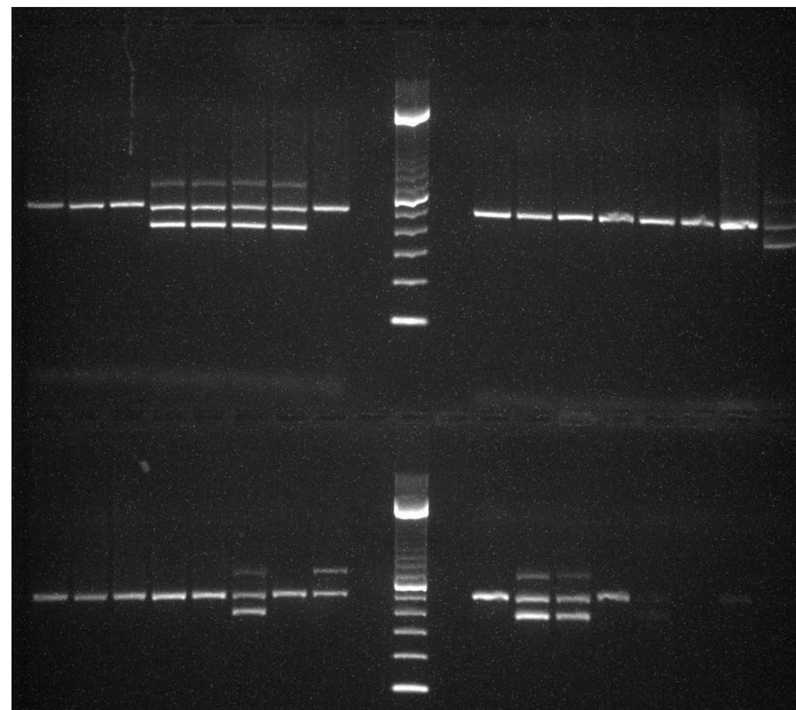
	sample	Conv. PCR	Taqman PCR
row 3	33	neg	neg
	34	neg	neg
	35	neg	neg
	36	xcc	xcc
	37	xcc	xcc
	38	xcc	xcc
	39	xcc	xcc
	40	neg	neg
	x		
	100bp ladder		
	x		
	41	neg	neg
	42	neg	neg
	43	neg	neg
	44	neg	neg
	45	neg	neg
	46	neg	neg
	47	neg	neg
	48	xcc	xcc

	sample	Conv. PCR	Taqman PCR
row 4	49	neg	neg
	50	neg	neg
	51	neg	neg
	52	neg	neg
	53	neg	neg
	54	xcc	xcc
	55	neg	neg
	56	xcr	xcr
	x		
	100bp ladder		
	x		
	57	neg	neg
	58	xcc	xcc
	59	xcc	xcc
	60	neg	neg

Conventional PCR gel pictures representing the results found in the table of appendix 5 above.



Gel 1: from left to right row 1 (1 to 8, 100bp ladder, 9 to 16) and 2 (17 to 32)



Gel 2: from left to right row 3 (33 to 48) and 4 (49 to 60)

Proposal for the addition of *Cicer arietinum* (Desi type) as a species to which the conductivity test for seed vigour can be applied to support C.15.1.

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Summary

The conductivity test identified differences in field emergence of 11 seed lots of the Desi type of chickpea (*Cicer arietinum*). Six of these seed lots, all having a laboratory germination of >85%, were tested by four laboratories using the electrical conductivity test, as described in the ISTA Rules (ISTA 2015). All laboratories consistently identified the same significant differences in the seed lot conductivity and the results were repeatable within laboratories and reproducible between laboratories. This provides evidence in support of the addition of *Cicer arietinum* (Desi type), to the ISTA Rules as a species for which the conductivity test can be applied.

Introduction

The conductivity test is validated in the ISTA Rules as a test that can be applied to species of *Pisum sativum*, *Phaseolus vulgaris*, *Glycine max, Cicer arietinum* (Kabuli type) and *Raphanus sativus* (ISTA, 2017). This test is based on the leakage of solutes that occurs from all seeds that are soaked in water. These solutes include sugars, amino acids and most importantly for the test, electrolytes. Thus the incidence of leakage can be detected by measurement of the electrical conductivity (EC) of the seed soak-water. The test was developed following the observation of the correlation between solute leakage and field emergence in wrinkled-seeded vining peas (*Pisum sativum*). Low leakage and therefore low conductivity was associated with seeds that emerged well, that is seeds with high vigour; whereas low vigour seeds with poor emergence had high levels of leakage and conductivity (Matthews and Whitbread, 1968). The conductivity test has also been used as an indicator of field emergence in field beans (*Vicia faba*, Hegarty, 1977), *Phaseolus beans* (Powell *et al*., 1986), soybean (Oliveria *et al*., 1984; Yaklich *et al*., 1984) and long bean (*Vigna sesquipedalis*; Abdullah *et al*., 1991). Leakage has also been related to emergence in the light-coloured, larger seeded Kabuli type chickpea (*Cicer arietinum*) (Khajeh-Hosseini *et al.,* 2007; Khajeh-Hosseini and Rezazadeh, 2011) leading to the validated method and inclusion of the Kabuli type chickpea as a species to which the EC test can be applied (ISTA,2014). There is however another, distinctly different and well recognised type of chickpea, the Desi type which has smaller, coloured seeds (Smartt and Simmonds, 1995). The objective of this study was to demonstrate that the conductivity test can also predict the field emergence of the smaller-seeded coloured Desi type chickpea (*Cicer arietinum)* and that the test is both repeatable within laboratories and reproducible between laboratories.

Materials and Methods

Field emergence: Samples of eleven seed lots of the Desi type of chickpea (*Cicer arietinum*) were obtained from Plant Research Institute, Ferdowsi University of Mashhad. Nine seed lots originated from Iran, the other two lots from Ethiopia and Tanzania. All lots had standard germinations above 89%. Field emergence was carried out in a completely randomized block design with four replications of 25 seeds from each seed lot in a clay loam soil where seeds were sown by hand at a depth of 4 cm. The average air and soil temperatures at sowing depth during the experiment were 15 and 13.5 ^{o} C respectively. Emergence was counted daily for 35 days until no further increase was observed.

Comparative test: Six of the seed lots used for field emergence were used in the comparative test, five from Iran and one (lot C) from Ethiopia. The lots were selected from the original 11 so that they included two high, two medium and two low vigour lots. All lots had standard germinations above 89%. Coded samples of the seed lots were sent from Mashhad, Iran to the participating laboratories, namely Department of Crop Science, Ferdowsi University of Mashhad, Mashhad, Iran; Department of Horticulture, Ege, University, Izmir, Turkey; National Institute of Agricultural Research, Oliveros Experimental Station, Oliveros, Argentina; GEVES, Station Nationale d'Essais de Semences (SNES), Angers, France. Each laboratory completed the conductivity test using the same method as that described for chickpea (Kabuli type) in the ISTA Rules (ISTA, 2015) i.e. four weighed replicates of 50 seeds, each soaked in 250 ml deionised/ distilled water for 24 hours at 20° C. The conductivity was measured after 24 hours on the same sample. The conductivity was expressed as µS cm^{-1} g^{-1} of seed.

The data from conductivity was analysed using (a) two-way Analysis of Variance, (b) calculation of z-scores and (c) the statistical tool developed by S. Grégoire according to ISO 5725-2 to calculate h-values and k-values. The statistical tool is available for download at the ISTA website:

http://www.seedtest.org/upload/cms/user/ISO572511.zip

Results

Establishment of a relationship between field emergence and conductivity readings

There was a significant negative correlation (r = -0.791**) between conductivity and the field emergence of the eleven seed lots (figure 1; Appendix 2); standard germination was not correlated with emergence (r = 0.326; P <0.328). When only the six seed lots used for the comparative test were considered there was also a significant negative correlation (r = -0.908*) between conductivity and field emergence. Again, standard germination was not correlated with emergence (r = 0.557; P < 0.250)

Comparative test

Box plot analysis revealed differences between the average EC readings for the six seed lots (figure 2A) with few outside values. There were small differences in the average values obtained by the four laboratories (figure 2B). No seed lot x lab interaction was exhibited in the side-by-side box plots (figure 2C).

The means for the seed lots following the conductivity test showed clear and significant differences in seed leachate conductivity and hence vigour (table 1). Overall seed lots of E and F had the highest conductivity of measurements, i.e. lowest vigour, followed by lots C and A, while lots B and D had the lowest conductivity indicating the highest vigour.

Application of the tolerances for conductivity from Chapter 15B of the ISTA Rules (ISTA, 2017) showed that, the replicate data (Appendix 1) for each lot in each laboratory were in tolerance with one another. There were small, but significant, differences in the overall means from the four laboratories (table 1). However, the lot means from individual labs were in tolerance.

The Coefficient of Variation (CV) for the comparative test was 7.3% a value comparable with that reported (5.9%) for the method validation of conductivity for *Cicer arietinum* (Kabuli type) (Khajeh-Hosseini *et al.,* 2014) although the value was higher. The CV was calculated by dividing the standard deviation by the overall mean conductivity of the four labs (table 1) multiplied by 100.

Calculation of the z-scores (table 2) revealed that all data fell within the values that are acceptable within ISTA proficiency tests i.e. +2.00 to -2.00.

Repeatability and reproducibility were analysed with the statistical tool developed by S. Grégoire, based on ISO 5725-2; this allows the calculation of h- and k-values. The h-values show the tendency for a laboratory to give over-estimations or under-estimations compared to the mean of all the results available whereas the k-values give a measure of the variability of the repeats. Higher values indicate greater under- or over-estimations (h-values) or greater variability between replicates (k-values).

No significant h-values were found (figure 3), indicating that the measurements were not over or underestimated. There were only one significant k-value, namely for lot A in lab 3 (figure 4) indicating that there was greater variability between replicates.

Repeatability and reproducibility values are affected by the examined species and the seed quality of the lots tested, with low vigour seeds often having higher values. It is therefore not possible to compare directly the data from comparative tests using different seed lots. However, the values obtained for repeatability and reproducibility (table 3) were lower than those obtained previously for *Cicer arietinum* (Kabuli type) (Khajeh-Hosseini *et al.,* 2014).

Discussion

Field emergence trials clearly established that the conductivity test identifies differences in vigour of Desi type chickpea. Differences in conductivity of six seed lots were consistently identified in each of four laboratories. The test was both repeatable within laboratories and reproducible in different laboratories. In addition, the replicates within the laboratories and the mean values obtained for each lot in different laboratories all fell within tolerance, using the tolerance tables in the ISTA Rules (ISTA, 2015). This provides evidence in support of the addition of *Cicer arietinum* (Desi type), to the ISTA Rules as a species for which the conductivity test can be applied.

The conductivity test therefore applies to both the Desi and Kabuli type chickpea which are distinct types of the cultivated *Cicer arietinum*. The two types have been produced as a result of dispersal of

chickpea from its centre of origin in south-east Turkey, exposure of the crop to different ecological conditions during evolution and subsequent selection by man (Smartt and Simmonds, 1995). The white seeded Kabuli type give a higher range of conductivity values (Khajeh-Hosseini *et al.,* 2014) than the range seen here for the coloured Desi type. Similar contrasts in conductivity readings have been seen between cultivars of other grain legumes having coloured and white seed coats (Powell *et al*., 1986; Abdullah *et al*., 1991). Comparisons of the conductivity values obtained from different seed lots of chickpea are therefore recommended only within one type of chickpea and not between lots of one type with those of the other type.

Acknowledgements

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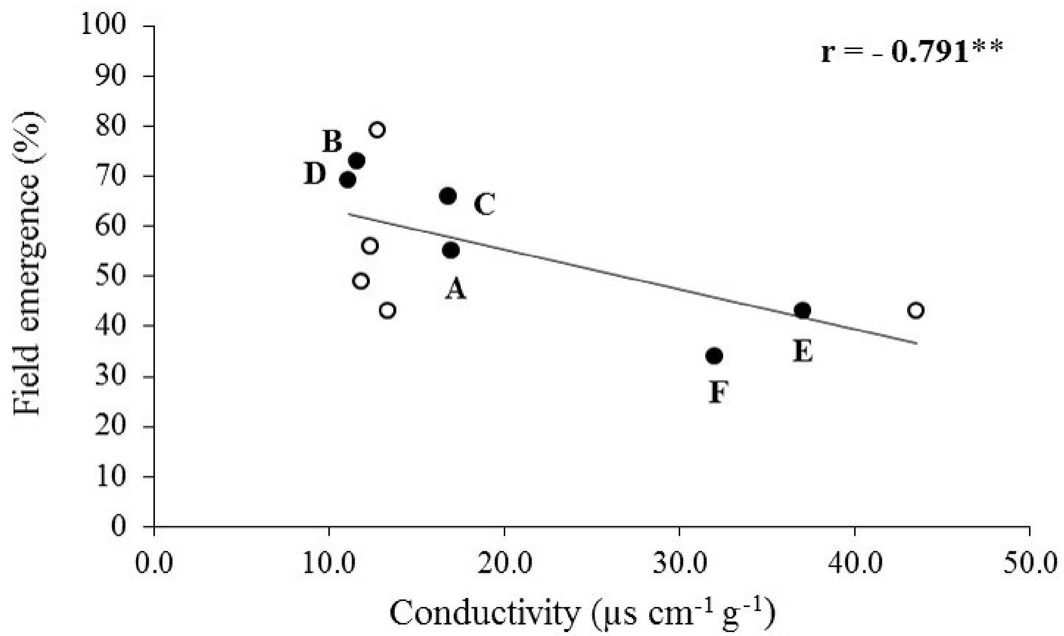

Figure 1: Relationship between conductivity and field emergence of eleven seed lots of Desi chickpea. Black circles are the seed lots selected for the comparative test.

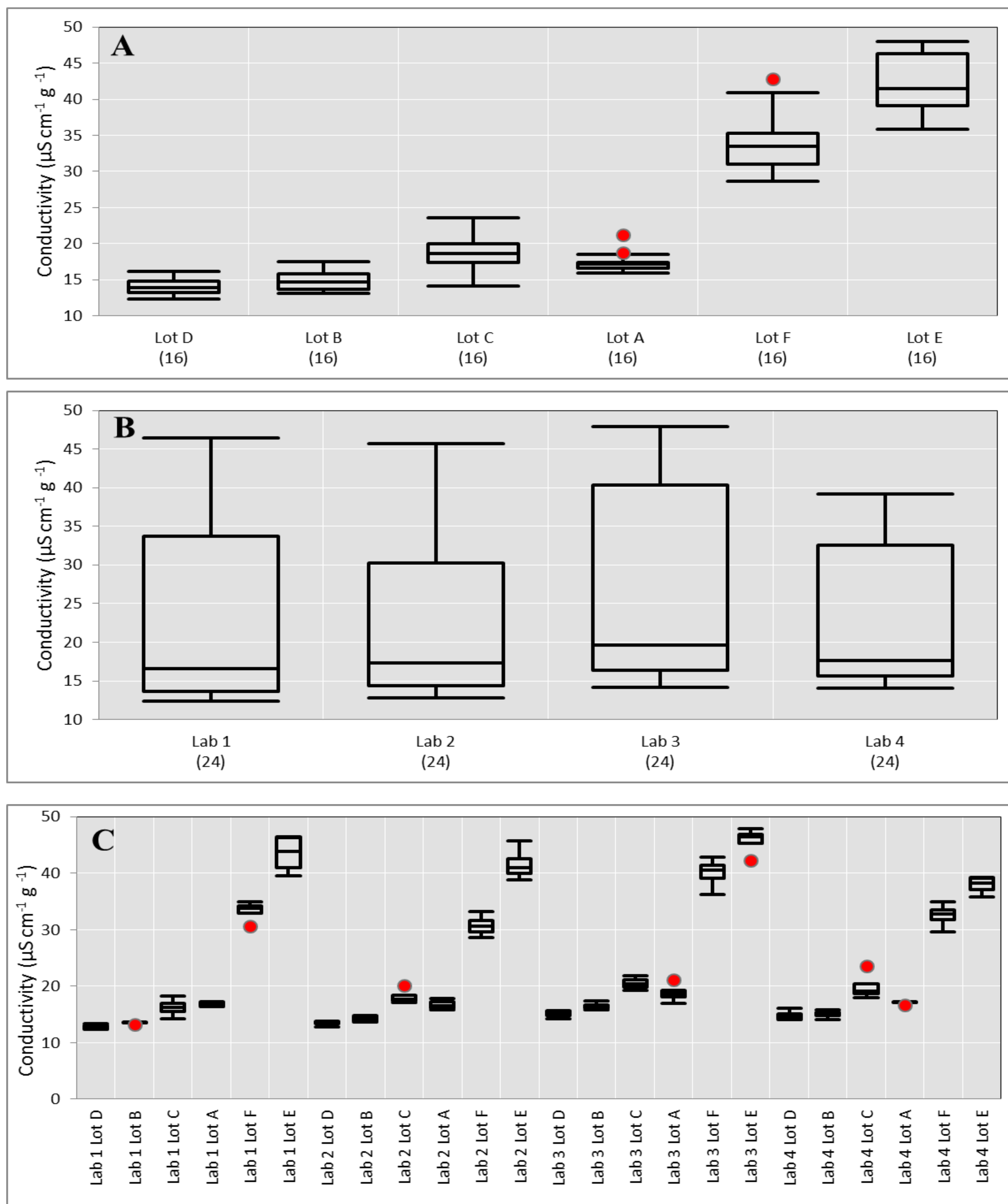

Figure 2: Box plot comparisons of the EC data from six seed lots of chickpea (*Cicer arietinum*), Desi type: (A) seed lots, (B) laboratories and (C) seed lot x laboratory

Table 1: Comparison of laboratory and seed lot means of six lots of chickpea (Desi type) tested by four laboratories using the conductivity test for 24 hours

Lab	Lot						Lab means
	A	B	C	D	E	F	
Lab 1	16.83^{IK}	13.48^{MN}	16.21^{JL}	12.80^{N}	43.40^{B}	33.31^{E}	22.67 ^{b}
Lab 2	16.70^{IK}	14.21^{LN}	18.15^{HJ}	13.48^{MN}	41.64^{BC}	30.74^{F}	22.49 ^{b}
Lab 3	18.86^{GI}	16.47^{JL}	20.50^{G}	15.15^{KM}	45.79^{A}	40.03^{CD}	26.13 ^{a}
Lab 4	17.11^{IK}	15.04^{KN}	20.00^{GH}	14.82^{KN}	37.89^{D}	32.56^{EF}	22.90 ^{b}
Lot means	17.38 ^{d}	14.80 ^{e}	18.72 ^{c}	14.06 ^{e}	42.18 ^{a}	34.16 ^{b}	

For lot and lab means, different lower case letters indicate that values are significantly different using LSD at the 5% level.
Within a row (laboratory), different upper case letters indicate that values (lots) are significantly different using LSD at the 5% level.

Table 2: Means, standard deviations (SD) and z-scores for six seed lots of chickpea (Desi type) tested by four laboratories using the conductivity test for 24 hours

Lot						
Lab	**A**	**B**	**C**	**D**	**E**	**F**
	a) means					
Lab 1	16.83	13.48	16.21	12.8	43.4	33.31
Lab 2	16.7	14.21	18.15	13.48	41.64	30.74
Lab 3	18.86	16.47	20.5	15.15	45.79	40.03
Lab 4	17.11	15.04	20	14.82	37.89	32.56
Mean	17.38	14.80	18.72	14.06	42.18	34.16
SD = $S_{\bar{x}}$	1.0047	1.2828	1.9521	1.1090	3.3275	4.0594
b) Z-scores						
Lab 1	-0.542	-1.029	-1.283	-1.138	0.367	-0.209
Lab 2	-0.672	-0.460	-0.289	-0.525	-0.162	-0.842
Lab 3	1.478	1.302	0.914	0.981	1.085	1.446
Lab 4	-0.264	0.187	0.658	0.683	-1.289	-0.394

Table 3: Values for repeatability and reproducibility of results from the conductivity test on *Cicer arietinum* (Desi type)

Lot	Repeatability (S_r)	Reproducibility (S_R)
A	1.0124	1.3334
B	0.5786	1.3772
C	1.7208	2.4559
D	0.6627	1.2487
E	2.6864	4.0601
F	2.1987	4.4838

Table 4: Mean Conductivity and tolerance ranges (4 replicates x 50 seeds) for six lots of chickpea (*Cicer arietinum*), Desi type,

Lot		Lab 1	Lab 2	Lab 3	Lab 4
A	Maximum tolerance range	4.6	4.6	5.1	4.8
	Observed range	0.9	2.0	4.2	0.6
	Mean	16.8	16.7	18.9	17.1
B	Maximum tolerance range	3.8	4.1	4.6	4.3
	Observed range	0.4	1.0	1.7	1.7
	Mean	13.5	14.2	16.5	15.0
C	Maximum tolerance range	4.8	5.1	5.5	5.5
	Observed range	4.1	3	2.6	5.5
	Mean	16.2	18.2	20.5	20.0
D	Maximum tolerance range	3.6	3.8	4.3	4.1
	Observed range	0.9	1	1.5	2.1
	Mean	12.8	13.5	15.2	14.8
E	Maximum tolerance range	11.3	10.8	11.8	9.8
	Observed range	6.9	6.8	5.6	3.4
	Mean	43.4	41.6	45.8	37.9
F	Maximum tolerance range	8.8	8	10.5	8.5
	Observed range	4.3	4.6	6.5	5.3
	Mean	33.3	30.7	40.0	32.5

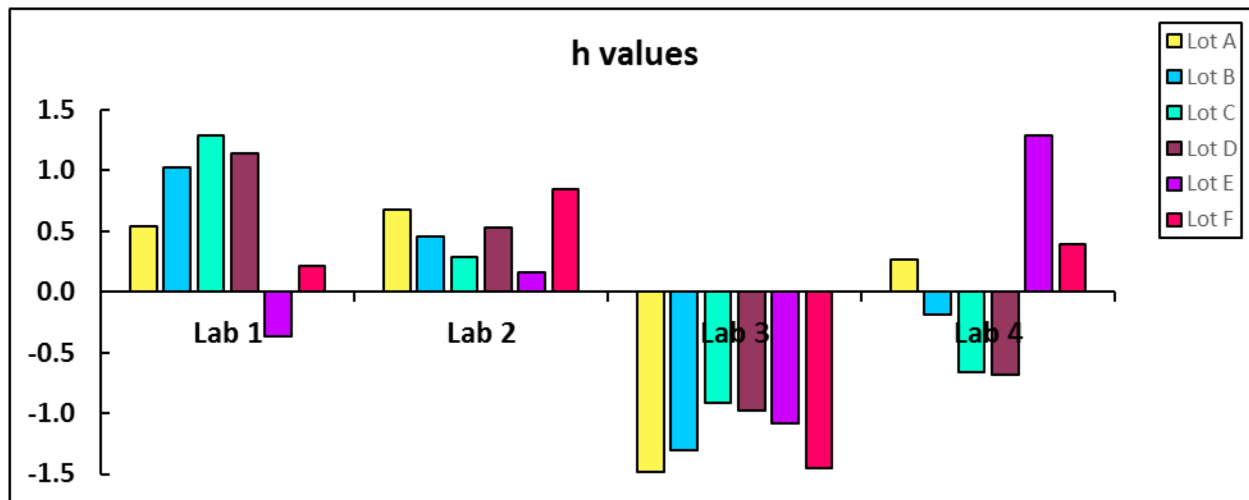

Figure 3: h-values for six seed lots of *Cicer arietinum* (Desi type) tested using the conductivity test for 24 hours in four laboratories.

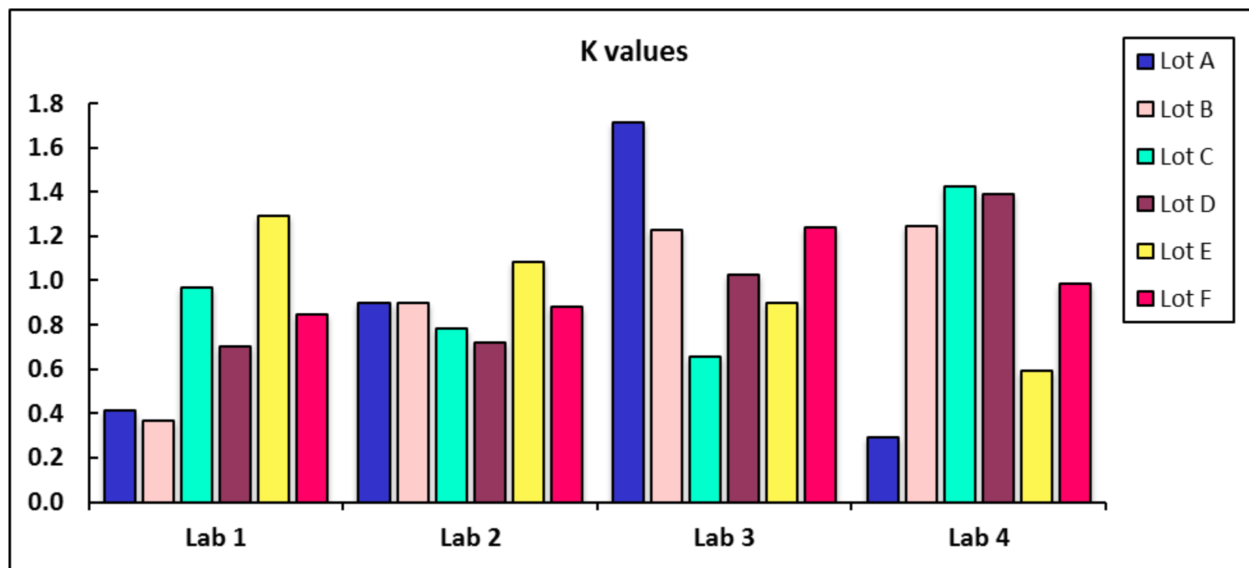

Figure 4: k-values for six seed lots of *Cicer arietinum* (Desi type) tested using the conductivity test for 24 hours in four laboratories.

Appendix 1: Data for each replicate conductivity reading for each of six lots of chickpea (*Cicer arietinum*), Desi type, taken in each of four laboratories.

Lot	Rep	Lab			
		Lab 1	Lab 2	Lab 3	Lab 4
A	1	17.19	16.10	18.57	17.27
	2	17.16	16.97	18.69	17.28
	3	16.34	15.87	21.19	16.66
	4	16.62	17.87	17.00	17.21
	Mean	16.83	16.70	18.86	17.11
	SD	0.42	0.91	1.73	0.30
B	1	13.60	13.71	17.45	14.07
	2	13.17	14.57	16.16	15.21
	3	13.56	14.75	15.80	15.05
	4	13.61	13.83	16.48	15.81
	Mean	13.48	14.21	16.47	15.04
	SD= S	0.21	0.52	0.71	0.72
C	1	16.48	17.14	20.86	18.04
	2	15.98	20.12	21.89	19.41
	3	14.17	17.54	19.29	18.98
	4	18.21	17.79	19.94	23.57
	Mean	16.21	18.15	20.50	20.00
	SD	1.67	1.34	1.13	2.45
D	1	13.30	13.78	15.70	14.76
	2	12.38	13.79	14.21	14.08
	3	13.09	13.56	15.09	14.30
	4	12.43	12.78	15.60	16.13
	Mean	12.80	13.48	15.15	14.82
	SD	0.47	0.48	0.68	0.92
E	1	41.43	40.40	46.58	35.80
	2	46.26	38.89	42.32	39.19
	3	39.52	41.60	46.31	39.09
	4	46.39	45.67	47.94	37.50
	Mean	43.40	41.64	45.79	37.89
	SD	3.46	2.91	2.42	1.60
F	1	33.91	31.15	40.95	32.49
	2	35.01	29.99	42.78	32.94
	3	30.66	28.62	36.30	34.94
	4	33.67	33.18	40.10	29.69
	Mean	33.31	30.74	40.03	32.52
	SD	1.86	1.93	2.73	2.16

Appendix 2: ANOVA table of field emergence.

Source of variation	d.f	Sum of Squares	Mean Square	F-value	P-value
Replicate	3	9.3409091	3.1136364	1.29	0.2958
Seed lot	10	476.6818182	47.6681818	19.75	<.0001
Error	30	72.4090909	2.4136364		
Total	43	558.4318182			

Evaluation of the ISTA germination methods for *Raphanus sativus* to support C.5.1.

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Summary

The study was conducted in order to evaluate the two ISTA germination methods 20 °C and 20<=>30 °C for *Raphanus sativus* in order to find out whether they both work well and should still be included in the rules or if one of them should be removed or not used in certain circumstances. 2 laboratories analysed 6 seed lots using both temperature regimes and the substrates PP, TP and S, prechilling was used as pretreatment. Statistical analyses showed that 20 °C PP and 20 °C TP resulted in a significantly lower percentage of normal seedlings for two of the lots. These two lots contained dormant seeds and the conclusion is that constant temperature 20 °C using substrates PP and TP was not able to break dormancy leading to many fresh seeds and therefore a lower percentage of normal seeds. 20 °C on sand gave good results even with dormant seed lots. For non dormant seed lots both temperature regimes work well. It is therefore suggested to give an advice in the ISTA rules to use 20<=>30 °C or sand for dormant seed lots.

Introduction

Black radish, *Raphanus sativus*, has in the ISTA rules two different allowed temperatures: 20<=>30 and constant 20 °C and 3 different allowed substrates: BP, TP and S. BP can be replaced by PP. The recommendation for breaking dormancy is to use prechilling.

The study was performed in order to examine whether or not both 20<=>30 and 20 °C should still be in the ISTA rules for germination of *Raphanus sativus* or if 20 °C should be removed or not used in certain cases. The reason to doubt the constant 20 °C temperature regime was the testing of four seed lots of *Raphanus sativus*, variety Lungo Bianco, at the seed testing station in Sweden using TP constant 20 °C. The germination of the lots were between 35 and 69%. The same lots using alternating 20<=>30°C resulted in germinations between 86 and 95%. Prechilling were used in both temperature regimes. These results were later verified when the seed testing station Agroscope Zurich in Switzerland retested the 4 samples and 1 reference sample using both 20 °C and 20<=>30°C on three different substrates, BP, TP and PP. The retest confirmed the poor result using 20 °C (all due to fresh seeds) over all substrates but worst with TP. Prechilling for 3 days at 8-10 °C didn´t help breaking dormancy.

To complement these results the germination committee decided to perform this study using 6 different seed lots of *Raphanus sativus.* Christine Herzog from Agroscope Zurich in Switzerland and Pernilla Andersson from the official seed testing station in Sweden wrote the test plan and organized the study.

Material and methods

Seed material

Lot	Latin name	Common name	Origin
1	*Raphanus sativus* var. *niger*	Black radish	New Zeeland
2	*Raphanus sativus* var. *niger*	Black radish	New Zeeland
3	*Raphanus sativus* var. *sativus*	Radish	France
4	*Raphanus sativus* var. *sativus*	Radish	France
5	*Raphanus sativus* var. *oleiformis*	Oil seed radish	Germany
6	*Raphanus sativus* var. *oleiformis*	Oil seed radish	Germany

Table 1. Seed lots used in the Radish project

The intention was to include seed lots with as well as without dormancy and lots 1 and 2 were expected to contain seeds with some degree of dormancy.

Participant laboratories

The lots were analysed at two different laboratories, one in Sweden and the other in Switzerland. Both labs are familiar with testing *Raphanus sativus*.

Pretreatment

All lots were analysed with prechilling at between 8-10 degrees.

Germination methods

For each test and sample, 400 seeds were analysed in replicates of 50 seeds. The substrates Top of paper (TP), Pleated paper (PP) and Sand (S) were used in both 20<=>30°C as well as constant temperature 20 °C.

Germination counts were made after 3 days prechilling and then 4, 7 and 10 days in 20 or 20<=>30°C.

The evaluation of the seedlings were made according to seedling type E and seedling group A-2-1-1-1 in ISTA handbook of seedling evaluation. In cases of 5% or more fresh seeds the seeds were evaluated as fresh or dead by using Tetrazolium.

Statistical analyses

Statistical analyses was performed by Jean-Louis Laffont, head of the ISTA Statistics Committee, by using the new tool from the ISTA Statistics Committee 'ISTAgermMV'. The figures with the boxplots (per lot, per method, per lab, and the full combination between these factors) as well as the data checking, repeatability/reproducibility and the mixed model analyses were generated from this statistical tool.

Results

Ungerminated seeds in the lots

Both lots 1 and 2 produced a lot of fresh seeds when the methods TP 20 °C and PP 20 °C were used. The other methods, 20 °C in sand as well as alternating temperature 20<=>30°C, regardless of substrate, produced no or very few fresh seeds. Lots 3-6 produced no or very few fresh seeds regardless of method (Table 2).

Lot \ Method	TP 20 °C	PP 20 °C	S 20 °C	TP 20<=>30°C	PP 20<=>30°C	S 20<=>30°C
1	34	24	1	4	1	0
2	40	34	1	7	1	0
3	0	1	0	0	1	0
4	0	0	0	0	0	0
5	0	0	0	0	1	0
6	0	1	0	0	0	0

Table 2. Percentage of fresh seeds in the lots depending on method (the percentage is the mean value between the two labs).

Germination results by seed lot

Table 3 shows the lowest and highest result of normal seedlings for a lot among the results obtained from both laboratories and among all methods. Figure 1 presents the median values of the percentage of normal seedlings per lot, for both laboratories and all methods. From Table 3 and Figure 1 it is clear that there is a difference in normal seedlings depending on the method used, at least for lots 1 and 2.

Lot nr	Normal seedlings lowest result (method used in brackets)	Normal Seedlings Highest result (method used in brackets)	Variation between methods and laboratories
1	36 % (TP 20°C)	97 % (PP 20<=>30°C)	61%
2	43% (TP 20°C)	98% (PP 20<=>30°C)	55%
3	74% (S 20<=>30°C)	88% (TP 20°C)	14%
4	89% (PP 20°C, PP 20<=>30°, S 20°C)	96% PP 20<=>30°	7%
5	71% (TP 20°C)	93% (PP 20°C)	22%
6	83% (PP 20<=>30°)	92% (TP 20<=>30°, TP 20°C)	9%

Table 3. Differences in the result of normal seedlings for the lots depending on method and laboratory

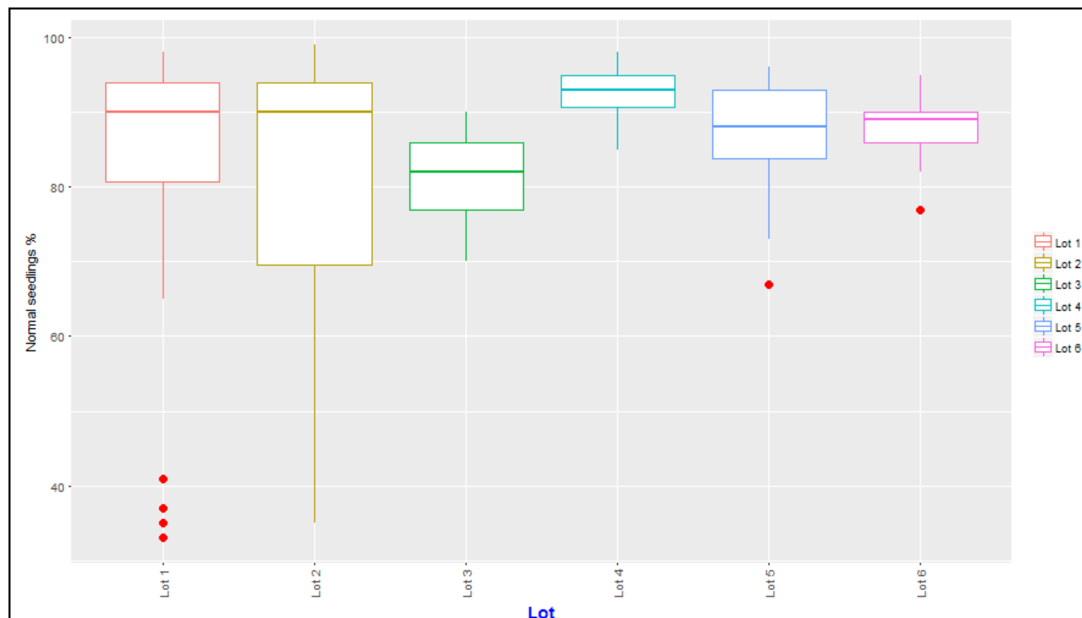

Figure 1. Percentage of normal seedlings per lot for all methods and both laboratories. Red dots = outliers.

Germination results by method

Figure 2 presents the median values of the percentages of normal seedlings per method, for all lots and both laboratories. PP20°C gave 85% of normal seedlings, PP20<=>30°C gave 92%, S 20°C gave 90%, S 20<=>30°C gave 90%, TP 20°C gave 86% and TP20<=>30°C gave 89% of normal seedlings. The variation in results are highest with the method TP 20°C followed by PP 20°C.

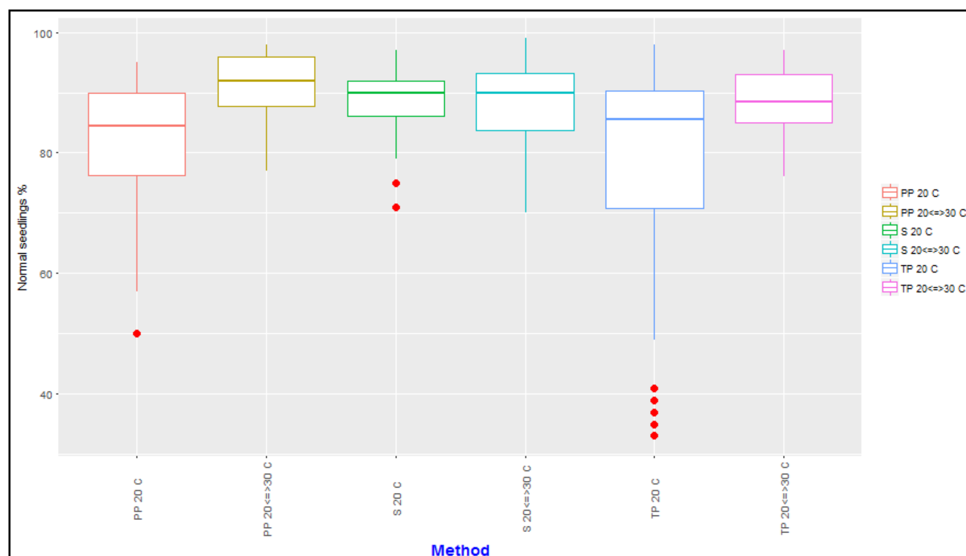

Figure 2. Median values of the percentages of normal seedlings per method for all lots and both laboratories. Red dots = outliers.

Germination results by method x lot

Figure 3 shows the percentage of normal seedlings for both laboratories per lot and per method. The median value in the side by side boxplots shows that the germination method played a role for the germination result. For lot 1 and 2 the percentage of normal seedlings were lower when using TP 20°C or PP 20°C compared to when using other methods. The variation between replicates and laboratories were highest when using TP 20°C for lots 1 and 2 as well as for lot 5. For the other lots the method plays a less important role for the results obtained.

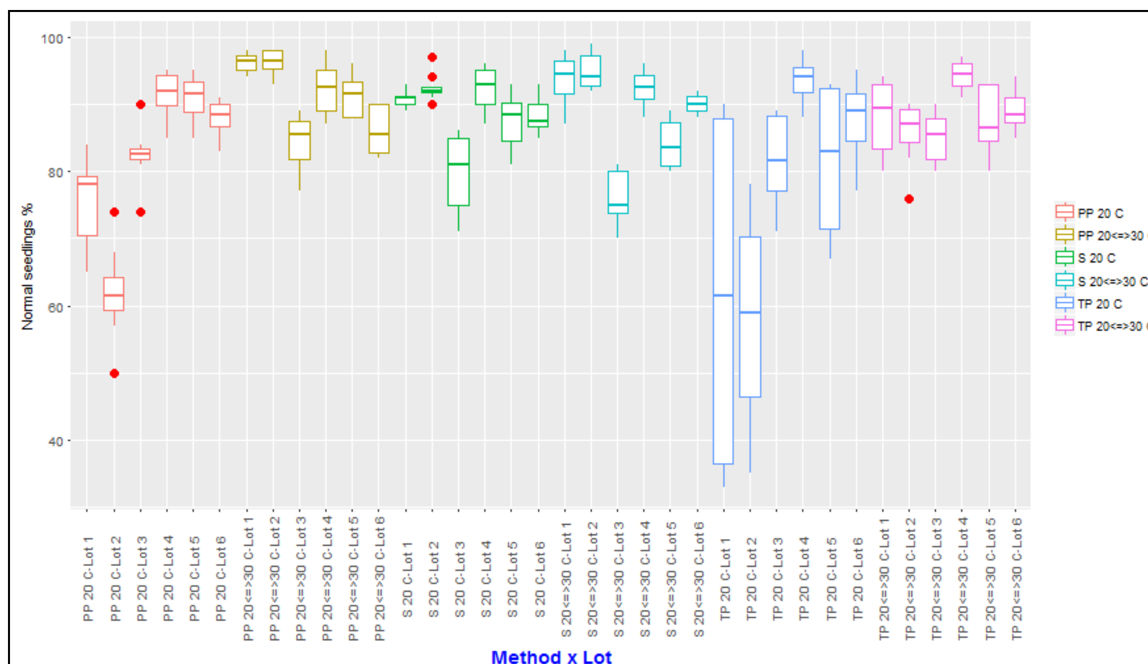

Figure 3. Percentage of normal seedlings for both laboratories per lot and per method. Red dots = outliers.

Germination results by method and laboratory

Figure 4 shows the variability per method and laboratory for all lots. For both laboratories, although more clear in laboratory 1, the variability in germination results was higher using TP 20°C as well as PP 20°C compared to the other methods.

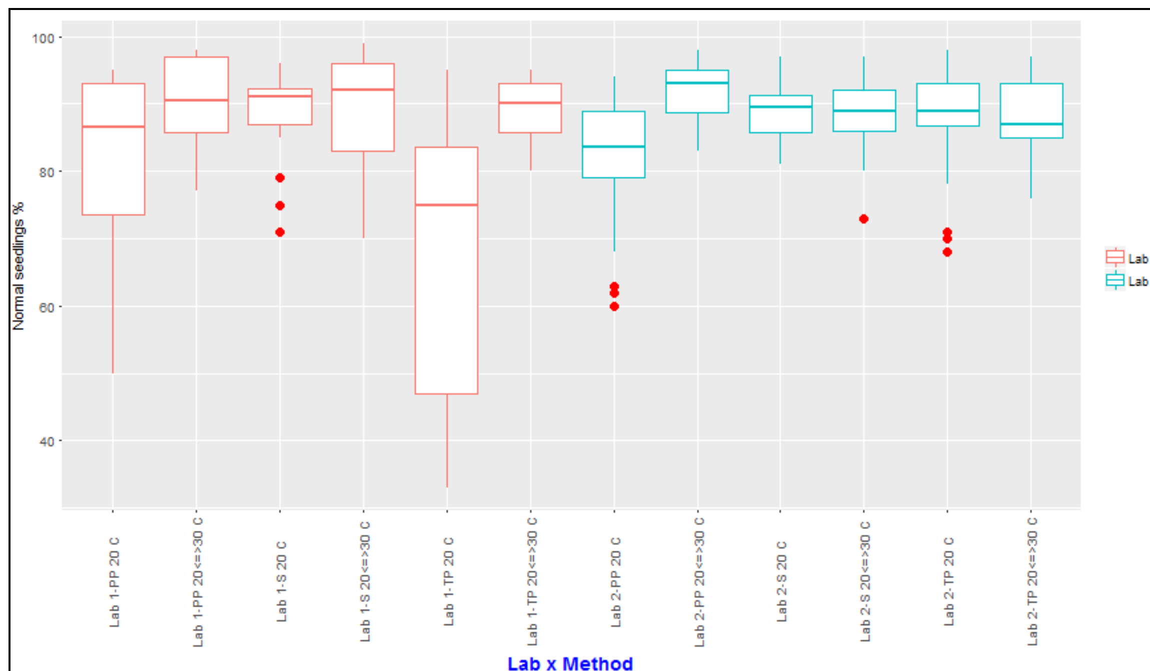

Figure 4. Percentage of normal seedlings for all samples per laboratory and per method. Red dots = outliers.

Results of data checking

Data checking has been performed according to ISTA rules by computing tolerances for germination test replicates. Only one result was found out of tolerance:

Method	Lot	Lab	Mean	# Reps	Range	Tol	Out of Tol
PP 20 C	Lot 2	Lab 1	60	4	24	19	OUT

The result has anyway been included in the statistical analysis.

Repeatability/Reproducibility

Results are shown below, however, as the number of laboratories is only 2 it is difficult to assess the reproducibility.

Method	$\overline{p}_{...}$	S_r	f_r	S_R	$\sqrt{\hat{\sigma}^2_{Lab}}$	$\sqrt{\hat{\sigma}^2_{Lot \times Lab}}$
PP 20 C	82	4.3	1.11	5.41	0	3.28
PP 20<=>30 C	91	2.64	0.92	3.79	0	2.72
S 20 C	89	2.23	0.70	4.26	0	3.63
S 20<=>30 C	89	2.62	0.82	3.56	0	2.40
TP 20 C	77	4.2	1.00	18.69	13.41	12.33
TP 20<=>30 C	88	2.59	0.80	5.28	0	4.61

$\overline{p}_{\cdots}$ is the overall average percentage of germinated seeds
S_r: is the **repeatability** standard-deviation (inter-lab variation)
f_r is an estimate of the dispersion parameter
S_R is the **reproducibility** standard-deviation (intra-lab variation)

A high value in the column $\sqrt{\hat{\sigma}^2_{Lab}}$ reflects low reproducibility due to big differences across labs and a high value in the column $\sqrt{\hat{\sigma}^2_{Lot\times Lab}}$ reflects low reproducibility due to differences in the way labs are measuring lots. TP 20°C has a high value in both these columns. The reason for this is that for TP 20°C one of the labs got a more or less lower result for all lots while the labs got very similar results between each other using the other germination methods.

Discussion

The study shows that, for Lots 1 and 2, two of the methods used, TP 20°C and PP 20°C, give significant lower percentage of normal seedlings compared to the other methods. They have also poor repeatability compared to the other methods and PP 20°C has poor reproducibility. We can however not conclude so much about repeatability and reproducibility as there are only 2 laboratories in the study.

The reason for the low percentage of normal seedlings in lots 1 and 2 when using TP 20°C and PP 20°C is dormancy. Both lots 1 and 2 produced a lot of fresh seeds when these methods were used. The other methods, 20 °C in sand as well as alternating temperature 20<=>30°C, regardless of substrate, were able to break the dormancy in these lots and therefore gave a higher percentage of normal seedlings.

For Lots 3-6, without dormancy, the difference in percentage of normal seedlings between methods was much less and for these lots all methods worked well.

Prechilling was not able to break dormancy in this study. The prechilling was performed at 8-10 degrees for 3 days. The effect of prechilling at a lower temperature (5-7 degrees) or the effect of a longer prechilling period (7-14 days) was not evaluated in this study.

The fresh seeds in the lots were evaluated by TZ and most of them were indeed fresh with the potential to germinate. Fresh seeds should be included in the germination percentage when reporting to a customer and if this is done one could argue that all methods work well. There is however a risk that the laboratory misjudge the fresh seeds as being dead and then reports a too low germination result to the customer.

General conclusion

As both temperature regimes work well for non dormant seed lots we do not suggest maintaining 20°C and 20⇔30°C in the rules. However, as this study has shown that the 20 °C germination method for *Raphanus sativus* is not able to break dormancy as efficient as alternating temperature 20<=>30°C in TP and PP, and as sand gives better results when seeds are dormant whatever the temperature, we propose to add an additional advice for *Raphanus sativus* in Table 2A part 1. The advice would be to recommend 20⇔30°C or sand, when seeds are known to be or are expected to be dormant.

Acknowledgements

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Thanks to the technical reviewers Harry Nijenstein and Augusto Martinelli and to everyone else in the germination committee, especially Sylvie Ducornau, for help and guidance during the project.

Thanks to Jean-Louis Laffont, chair of the statistical committee, for running the statistical analyses.

Validation study of seed germination test of *Spinacia oleracea* to support C.5.3.

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Summary

This project started in 2013 and was motivated by the assumption that the lack of clear root criteria to assess spinach seedlings led to germination results variability between laboratories, either of seed companies or institutes. So far, for a spinach seedling to be considered as normal, it should comply with the following definition: 'primary root with limited damage (e.g. not affecting the conductive tissue) or slight growth retardation'. However, the maximum growth retardation tolerated was not clearly defined for *Spinacia oleracea*. This lack of clear definition caused the root length to be evaluated in different ways by laboratories. The objective of this validation study was to assess if introducing a root length criterion for spinach seedlings would decrease the results variation between laboratories. The new criterion is intended to be included into 'Chapter 5: The germination test', paragraph '5.2.7 Normal seedlings' in the description of '5.2.7.2 Slight defects'.

Six ISTA laboratories from six different countries participated in this study, additionally to the test organizer (not ISTA accredited). Samples from six seed lots were distributed to each one of them, together with the test plan. The following methods were included in the test plan, in accordance to the current ISTA rules:

1. **VRT1**: germination test used routinely by the participating ISTA lab (PP or BP, 10 or 15 °C, optional for breaking dormancy: prechill). No specific instructions were given regarding the main root length and laboratories were asked to use their routine protocol for normal seedling assessment.
2. **VRT2**: germination test used routinely by the participating ISTA lab (PP or BP, 10 or 15 °C, optional for breaking dormancy: prechill). Specific instructions were given regarding the main root length: to be considered as normal, the length of the primary root of the seedling had to be equal or greater than the half of the length of the hypocotyl (root:shoot ratio of 0,5).

The statistical analysis could be performed using all the results obtained by all the participants and showed the repeatability and reproducibility for the two methods. Based on the results of that analysis, the criterion proposed to be included in the ISTA Rules for *Spinacia oleracea* is: the length of the primary root has to be equal or greater than the half of the length of the hypocotyl.

INTRODUCTION

The project about the evaluation of short roots in spinach was initiated in November 2011 with a meeting in The Netherlands between major seed companies. The discussion concerned an unexpected issue experienced with a seed lot of Enza Zaden produced in 2009, which showed a high percentage of seedlings with their root displaying a growth delay. ISTA germination tests done by the Naktuinbouw gave a Total Germination (TG – normal seedlings) superior to 85 % for this seed lot while field emergence experienced by Enza Zaden's customers was only of 50 %. Consequently, it seemed that there were some discrepancies between ISTA criteria and practical criteria used by seed companies. However, the fact that root growth delay caused the low emergence in the field had not been proven to this point.

The Naktuinbouw evaluated the germination methods used by nine seed companies which volunteered to participate, in order to identify the possible origin for germination results variability between laboratories (data not shown). The main conclusion was that one germination protocol and clear criteria for root length would help reducing the deviation in germination results between laboratories. Nevertheless, this conclusion was only based on the questionnaires sent to the participants and not on experimental results. A Nal-proficiency test of 4 lots of Spinach produced in 2012 was done in 2013, led by the Naktuinbouw. The outcome was that the differences in the test results were mainly caused by the percentage of abnormal seedlings. The differences were likely caused by the interpretation of the development in the root system. The conclusion was that to solve this evaluation problem, it would be advisable to give a clear description of the abnormalities of the root system e.g. root length.

Consequently, Enza Zaden continued this study in 2014-2015 by organizing three ring tests between ten laboratories of nine seed companies and the Naktuinbouw. Three seed lots were used: lots 1 and 2 were provided by Enza Zaden and lot 3 by Pop Vriend. These lots were selected for their various percentages of seedlings with root growth delay. The three ring tests aimed at defining the cause of the variation between laboratories. The impact of the germination method and of the root length criterion on results variability was assessed successively. Further experiments were also done by Enza Zaden in 2015 and 2016:

- **Impact of temperature and water quantity** on germination rate and root growth on six lots (three new lots provided by Enza Zaden in addition to the three lots initially tested, lots 1, 2 and 3)
- **Correlation between paper and soil germination results** on the six lots selected for the ISTA validation study (the three initial lots and three new lots provided by Enza Zaden, Pop Vriend and Bayer for lots 4, 5 and 6 respectively)
- **Correlation between root length and seedling development**, assessed by seedlings fresh weight during a soil germination test under optimal conditions (lots 1 to 6)
- **Impact of Thiram disinfection on the occurrence of root growth delay**, on lots 1 to 6; PP15, 2 x 100 seeds, assessment 14 days after sowing

The results of the experiments listed here-above (not presented in this report) highlighted the importance of having a root criterion during spinach seedlings evaluation in laboratory germination tests. One of the main conclusions of these experiments was that for germination tests done in paper at 15 °C, the most accurate root:shoot ratio to consider was 0,5. Using a threshold of 1 led to an under-estimation of the number of normal seedlings during the paper germination test compared to the soil germination test. As well, it was shown that the superficial disinfection of seeds with Thiram did not lead to a significant difference of normal seedlings percentage, compared to non-disinfected seeds, and that the percentage of seedlings with a root growth delay was similar. This root:shoot threshold of 0,5 was then validated by the GERCOM members during the ISTA meeting in Tallinn in June 2016, to be used in the upcoming validation study. It was concluded from the ring tests results from non-ISTA accredited laboratories that using a uniform root criterion decreased the

germination results variability between laboratories. These results are presented in this report. However, as no laboratory was ISTA accredited except for the Naktuinbouw, the ISTA Germination Committee (GERCOM) members required to repeat the study with ISTA accredited laboratories to confirm the conclusions, leading to the final Validation Study.

MATERIAL AND METHODS

Ring tests – not ISTA accredited labs

- **Seed material**

Three samples of untreated seeds of *Spinacia oleracea* from three different varieties were used in the experiments, two originated from Enza Zaden and the last one from Pop Vriend (**Table 1**), so a possible variety effect was taken in consideration. The three samples were drawn from commercial lots intended for marketing.

Table 1. Batches available for the Spinach Short Root Project ring tests in 2014 (organized by Enza Zaden) with their corresponding initial quality as assessed by the supplying companies; TG = Total Germination, SR = Short Root and NG = Not Germinated seed

Lot	Seed size (mm)	Supplier	Production year	TG (%)	SR (%)	Other abnormal seedlings (%)	NG (%)
1	2,75-3,50	Enza Zaden	2009	37	59	3	1
2	2,50-3,50	Enza Zaden	2013	59	13	18	10
3	2,75-3,50	Pop Vriend	2012	68	21	8	3

- **Participant laboratories**

1. **BEJO ZADEN B.V.** (Netherlands); Contact: Richard Hoogeboom, r.hoogeboom@bejo.nl
2. **ENZA ZADEN** (Netherlands); Contact: Lucile Daron, l.daron@enzazaden.fr
3. **NAKTUINBOUW** (Netherlands); Contact: Marcel Toonen, m.toonen@naktuinbouw.nl
4. **GERMAINS SEED TECHNOLOGY** (Netherlands); Contact: Jurre Kleine Schaars, jkschaars@germains.com
5. **INCOTEC** (Netherlands); Contact: Petra Bakker, petra.bakker@incotec.com
6. **JENSEN SEEDS A/S** (Denmark); Contact: Annitta Christensen, an@jensen-seeds.dk
7. **BAYER-NUNHEMS NL** (Netherlands); Contact: Mieke Roost, mieke.roost@bayer.com
8. **BAYER-NUNHEMS USA** (United States of America); Contact: Elizabeth Bada, Elizabeth.bada@bayer.com
9. **POP VRIEND** (Netherlands); Contact: Sina Sietses, SSietses@popvriendseeds.nl
10. **VIKIMA SEEDS A/S** (Denmark); Contact: Brit Malec, brm@vikima.com

All these laboratories had experience with spinach germination tests. The number given in this list does not correspond to the lab number in the results chapter, as results were to be reported anonymously.

Table 2. List of companies participating in the 2014-2015 Spinach Short Root ring tests and their level of accreditation; NAL = Naktuinbouw Authorized Laboratories

Company	ISTA rules in routine tests	ISTA accredited	Remark
Bejo Zaden	yes	no	NAL accredited
Enza Zaden	yes	no	NAL accredited
Germains Seed Technology	yes	no	NAL accredited
Incotec	yes	no	NAL accredited
Jensen Seed A/S	yes	no	
Bayer (Nunhems) NL	yes	no	NAL accredited
Bayer (Nunhems) USA	yes	no	NAL accredited
Pop Vriend	yes	no	
Vikima Seed A/S	yes	no	
Naktuinbouw	yes	yes	

- **Additional treatments**

No additional treatments were recommended and no laboratory used a pre-chilling treatment before the germination test. Three laboratories disinfected the seeds with Thiram before germinating them.

- **Germination method**

The germination method recommended in the International Rules for seed testing (2014) is:

- Substrate: Top of Paper (TP) or Between Paper (BP)
- Temperature (°C): 15 or 10
- First count: 7 days
- Final count: 21 days
- Recommendations for breaking dormancy: Pre-chill
- Additional directions or advice: none

Table 3 presents the detailed protocol for all participating laboratories. The laboratories are not listed in the same order as in the previous paragraph, as results were to be reported anonymously.

Table 3. Germination method details for the 10 laboratories participating in the 1st ring test (2014); PP = Pleated Paper

Laboratory	Method	Temperature (°C)	Water supply (mL)	Light regime (light/dark) (h)	**Germination assessment** (days after sowing)	**Short root criterion** (root:shoot threshold or root length)	Remark
A	PP	15	30	0/24	6-8-12	0,5	No standard treatment
B	PP	15	40	8/16	7-14	1	Thiram treated
C	PP	15/20	35	8/16	14	1 cm	No standard treatment
D	PP	10	50	0/24	4-14	50% of average root length	Thiram treated
E	PP	15	40	10/14	7-14-21	0,5	No standard treatment
F	BP	15	12.9	8/16	7-10-14-21	0,5	Thiram treated
G	BP	15	unknown	8/16	7-10-14-21	0,5	Thiram treated
H	PP	10	25	16/8	7-21	1	Thiram treated
I	PP	10	40	8/16	10-21	0,25	No standard treatment
J	PP	15	40	12/12	7-10-14-21	0,5	No standard treatment

Hereafter are the instructions given for each ring test regarding germination method and root criterion, which are summarized in **Table 4**:

- Ring Test 1 (RT1): no instructions for either the germination method nor the root criterion
- Ring Test 2 (RT2): use of germination method PP or BP at 10 °C, two repetitions of 100 seeds, only assessment after 14 days, 12h light, no seed disinfection, no instruction for root criteria
- Ring Test 3 (RT3): same germination method as RT2 and use of threshold root:shoot >= 1 to consider a seedling as normal (considering that the hypocotyl and cotyledons development is otherwise normal)

Table 4. Instructions given for the germination method and root criterion for the 1st, 2nd and 3rd ring tests (2014-2015)

Ring test no.	**Germination method**	**Repetitions * no. seeds**	**Disinfection**	**Temperature** (°C)	**Water supply** (mL)	**Light regime** (light/dark)	**Germination assessment** (days after sowing)	**Root:shoot threshold**
1	Variable	4 * Variable	Variable	Variable	Variable	Variable	Variable	Variable
2	PP	2*100	none	10	40	12h/12h	14	Variable
3	PP	2*100	none	10	40	12h/12h	14	1

Based on preliminary experiments (data not shown), we found that the best temperatures regarding the number of normal seedlings were 10 °C or 15 °C, but as the percentage of not germinated seeds (NG) was higher for 15 °C, we concluded that 10 °C was the optimal temperature.

As well, the optimal water amount was identified to be 40 mL, as it led to the highest percentage of normal seedlings. In order to ease the seedlings assessment, we decided to have a 12h/12h light/dark regime, as the light coloured the hypocotyl in pink (**Figure 1**), which facilitated the distinction between hypocotyl and root and so, eased to assess the ratio between the two. As well, we decided to limit the germination period to 14 days, so that the seedlings would not be too big and would be easy to assess.

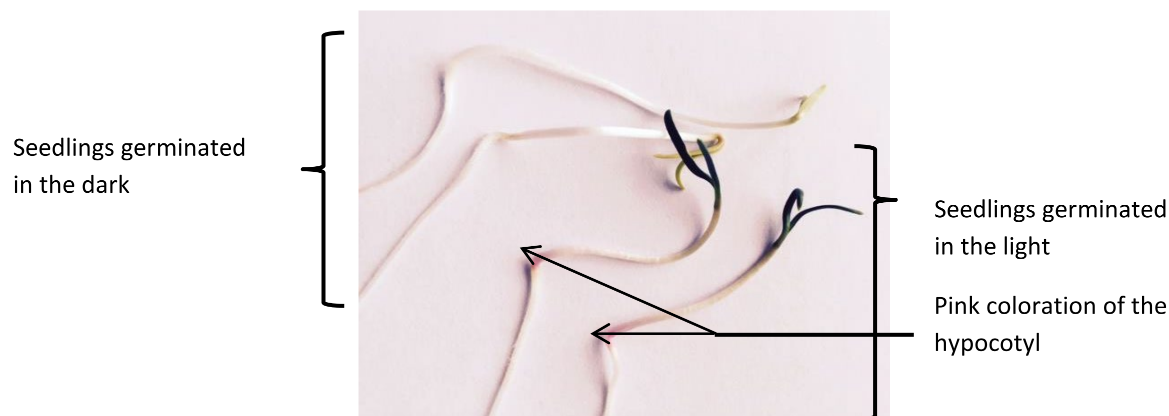

Figure 1. Comparison between seedlings germinated in the dark (left, two seedlings) and in the light (right, two seedlings) where the pink hypocotyl is visible, 21 days after sowing, Pleated Paper at 15 °C; source: Naktuinbouw, Antoon Grim

Preliminary experiments indicated that for the germination test at 10 °C (Pleated Paper at 10 °C = PP10), using a root:shoot ratio of 0,5 led to an over-estimation of the percentage of normal seedlings (data not shown), while the ratio of 1 gave more accurate results compared to results of normal seedlings at 15 °C in paper and in soil. However, for PP15, using the ratio of 1 led to an under-estimation of the percentage of normal seedlings. Consequently, we chose to use the root:shoot ratio threshold of 1 in our ring tests, as the germination method was set to be PP10.

Validation study – ISTA accredited labs

- **Seed material**

Six samples of untreated seeds of *Spinacia oleracea* were used in the study, three originated from Enza Zaden, two from Pop Vriend and one from Bayer (**Table 5**). The six samples were drawn from commercial lots intended for marketing. There were all from different varieties, so a possible variety effect was taken in consideration. As there were not enough seeds left from lot 2 to perform two repetitions of 100 seeds for two independent samples, only one sample of 200 seeds was sent to each laboratory for both VRT1 and VRT2.

Table 5. Batches available for the Spinach validation study in 2016 (organized by Enza Zaden) with their corresponding initial quality as assessed by the supplying companies; TG = Total Germination, SR = Short Root and NG = Not Germinated seed

Lot	Seed size (mm)	Supplier	Production year	TG (%)	SR (%)	Other abnormal seedlings (%)	NG (%)
1	2,75-3,50	Enza Zaden	2009	37	59	3	1
2	2,50-3,50	Enza Zaden	2013	59	13	18	10
3	2,75-3,50	Pop Vriend	2012	68	21	8	3
4	2,50-3,50	Enza Zaden	2012	80	7	6	7
5	2,60-3,50	Pop Vriend	2015	80	12	4	4
6	x	Bayer	2015	88	7	0	5

- **Participant laboratories**

Six ISTA accredited laboratories which had experience with spinach germination tests participated, additionally to the test organizer (ENZA ZADEN, not ISTA accredited). The number given in this list does not correspond to the lab number in the results chapter, as results were to be reported anonymously.

1. **GEVES** (France); Contact: Sylvie Ducournau, Sylvie.ducournau@geves.fr

2. **LANDWIRTSCHAFTLICHES TECHNOLOGIE ZENTRUM AUGUSTENBERG - LTZ** (Germany); Contact: Andrea Jonitz, Andrea.Jonitz@ltz.bwl.de
3. **NAKTUINBOUW** (Netherlands); Contact: Marcel Toonen, m.toonen@naktuinbouw.nl
4. **ARO VOLCANI CENTER** (Israël); Contact: Miriam Dekalo Keren; meryam@volcani.agri.gov.il
5. **CREA** (IT); Rita Zecchinelli, rita.zecchinelli@crea.gov.it
6. **SGS BROOKINGS** (USA); Sarah Dammen, sarah.dammen@sgs.com
7. **ENZA ZADEN (**Netherlands); Lucile Daron, l.daron@enzazaden.fr

Additional treatments

No additional treatments were recommended and only one laboratory used a pre-chilling treatment of 5 days at 5 °C (LTZ).

Germination method

Table 6 summarizes the different parameters of the routine germination methods per laboratory, used during the first validation test.

Table 6. Germination methods parameters for the six ISTA laboratories participating in the validation study (2016)

Laboratory	**Method**	**Temperature** (°C)	**No. independent samples**	**Repetitions * no. seeds**	**Total no. seeds tested ***	**Water supply** (mL)	**Light regime (light/dark)** (h)	**First-Final germination assessment** (days after sowing)	**Root:shoot threshold**
GEVES	PP	15	2	2 * 100	400	32	8h/16h	7-21	1:2
LTZ	PP	15	2	4 * 50	400	35	8h/16h	7-14	2:3
NAKTUINBOUW	PP	15	2	2 * 100	400	40	12h/12h	7-21	1:2
ARO VOLCANI	BP	15	2	4 * 50	400	Not exact amount: spraying several times during the test	0h/24h	7-21	No defined threshold: if no change in root length during test, seedling classified as abnormal
CREA	BP	15	2	2 * 100	400	7	0h/24h	6-21	1:2
SGS BROOKINGS	BP	15	2	2 * 100	400	Towels soaked and excess water removed	8h/16h	7-21	1
ENZA ZADEN	PP	15	2	4 * 50	400	40	8h/16h	7-14	1

*Except for Lot 2 for which only 200 seeds were tested per VRT

The third ring test of the 2014-2015 experiments, with a defined root criterion, was done under fixed conditions. This did not reflect the diversity of germination methods that occur in practice, as listed in **Tables 3 and 6**. That is why during the validation study, it was decided to let the laboratories use their routine germination protocols, to be more representative of real practices. The aim was to evaluate if adding a root criterion under such a variety of conditions would indeed decrease results' variability between laboratories.

Hereafter are the instructions given for each validation ring test (VRT) regarding germination method and root criterion, which are summarized in **Table 7**:

- Validation Ring Test 1 (VRT1): no instructions for either germination method nor root criterion

- Validation Ring Test 2 (VRT2): no instructions for the germination method but use of root:shoot >= 0,5 to consider a seedling as normal (considering that the hypocotyl and cotyledons development is otherwise normal).

Table 7. Instructions given for the germination method and root criteria for the 1st and 2nd validation ring tests (2016)

Ring test no.	**Germination method**	**Repetitions and no. of seeds**	**Pre-treatment**	**Germination temperature** (°C)	**Water supply** (mL)	**Light regime** (light/dark)	**Germination assessment** (days after sowing)	**Root:shoot threshold**
1	Variable	Variable	Variable	15	Variable	Variable	Variable	Variable
2	Variable	Variable	Variable	15	Variable	Variable	Variable	0,5

As mentioned in the introduction and in the previous chapter, we chose a root:shoot ratio of 0,5 for the VRTs (PP15) as using a ratio of 1 led to an under-estimation of the percentage of normal seedlings, when compared with germination tests done at 10 °C and in soil at 15°C. This decision was validated by the GERCOM members in 2016. Only normal seedlings were reported during the first counting assessment, while the number of seedlings with a short root was reported during the final assessment, as is done routinely by the laboratories.

Statistical analysis

For all ring tests and validation ring tests, the data obtained was cleaned by removing the results from laboratories which did not respect the given instructions. This led to the exclusion of laboratories 2 and 8 for RT1, RT2 and RT3. Reporting the results is done anonymously in this report, as agreed upon with the seed companies.

Ring tests RT1, RT2 and RT3 and Validation ring tests VRT1 and VRT2

The new tool from the ISTA Statistics Committee 'ISTAgermMV', designed for the analysis of the data from Method Validation studies, was used to produce the boxplots for VRT1 and VRT2 (analysis done by Jean-Louis Laffont, head of the ISTA Statistics Committee): per lot, per method, per lab, and the full combination between these factors. Data checking, repeatability/reproducibility and the mixed model analyses were also outputs of this statistical tool. In this report, only the most relevant charts were included, but the complete statistical reports (as communicated by M. Laffont) are made available to the ISTA committee if they were to be needed for re-evaluation or if additional information was necessary. As well, the raw data of all experiments is made available to ISTA if needed for follow-up or re-analysis.

RESULTS

Ring tests – not ISTA accredited labs

A wide range of phenotypes was encountered during the germination tests. **Figures 2, 3 and 4** give some examples of what was considered as 'short root' seedlings and 'other abnormal' seedlings. The classification was uniform overall, as shown in the pictures.

Due to a technical error, results of Lab 7 were saved for only one repetition of 100 seeds for RT1, instead of 4 repetitions of 100 seeds. These data were kept in the analysis and the statistical software adjusted accordingly to take this exception into account.

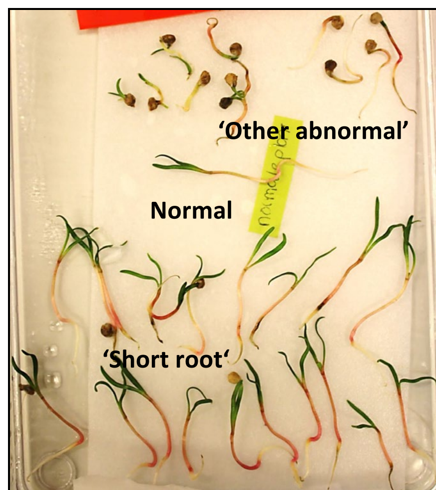

Figure 2. Examples of seedlings from lot 2, classified as 'other abnormal' (top of picture), 'normal' (middle of picture) and 'short root' (bottom of picture), 3rd ring test, 2015, Enza Zaden

Figure 3. Examples of seedlings from lot 2, classified as 'short root' (top of picture) and 'other abnormal' (bottom of picture), 3rd ring test, 2015, Incotec

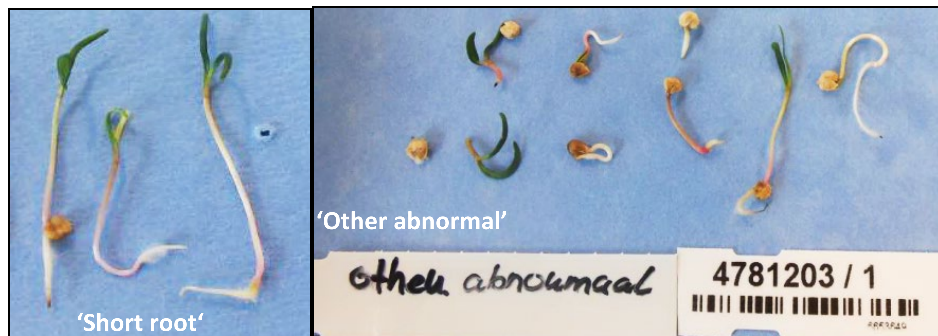

Figure 4. Examples of seedlings from lot 2, classified as 'short root' (left picture) and 'other abnormal' (right picture), 3rd ring test, 2015, Bayer

- **Germination results by seed lot**

Figure 5 presents the median values of the percentage of normal seedlings per lot, for all ring tests and all laboratories (excluding lab 2 and lab 8 as explained in the Material and Methods). Lot 1 gave 62,0 % of normal seedlings, lot 2 gave 72,7 % of normal seedlings and lot 3 gave 76,0 % of normal seedlings. The highest variability was found for lot 1, as shown in the side-by-side boxplots, while the variation for lots 2 and 3 was similar.

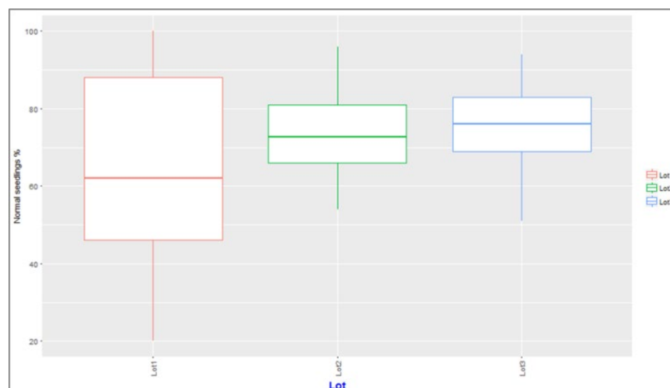

Figure 5. Percentage of normal seedlings per lot for all methods and all laboratories; into brackets = number of values

- **Germination results by method**

Figure 6 presents the median values of the percentages per method, for all lots and the eight laboratories. RT1 gave 81,0 % of normal seedlings, RT2 gave 78,0 % of normal seedlings and RT3 gave 60,0 % of normal seedlings. The drop in percentage of normal seedlings for RT3 can be explained by the fact that the root:shoot threshold was instructed to be 1, while for RT1 and RT2, 60 % of the laboratories were using a ratio equal to or less than 0,5 (**Table 3**; 5 labs using 0,5 and 1 lab using 0,25). Consequently, more seedlings were categorized as having a short root in RT3, leading to a higher percentage of abnormal seedlings.

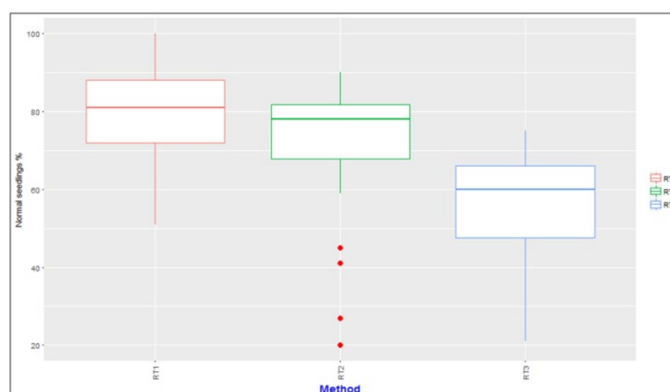

Figure 6. Percentage of normal seedlings per method for all lots and eight laboratories; red dots = outliers; into brackets = number of values

- **Germination results by method x laboratory**

As shown in **Figure 7**, there was a significant decrease of the percentage of normal seedlings for RT3, due to the change of root:shoot threshold from 0,5 to 1 for most laboratories, except those already using 1 as

threshold for RT1 and RT2 (laboratories 7 and 10). The data shows certain variability between laboratories but it did not lead to the exclusion of any result for further analysis.

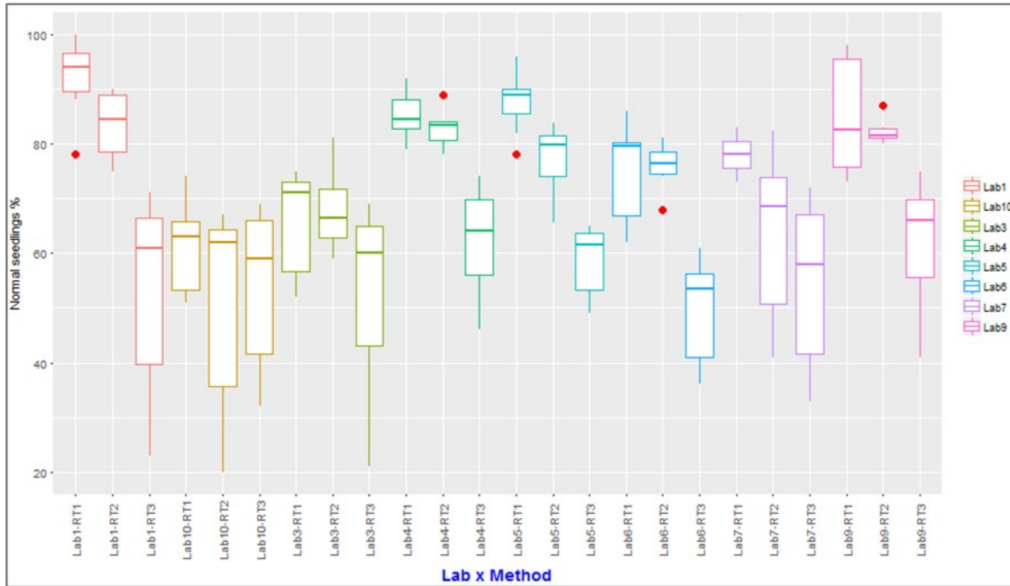

Figure 7. Percentage of normal seedlings for all samples per laboratory and per method; red dots = outliers; into brackets = number of values

○ Germination results by laboratory x lot

Figure 8 shows the data grouped per lot and per laboratory. The variability between laboratories shown in **Figure 7** is confirmed, for all lots. The least variation was found for lot 3 and the highest variation for lot 1, as expected. Indeed, it is known that batches with high quality have less variability than those with low quality, which is well illustrated here.

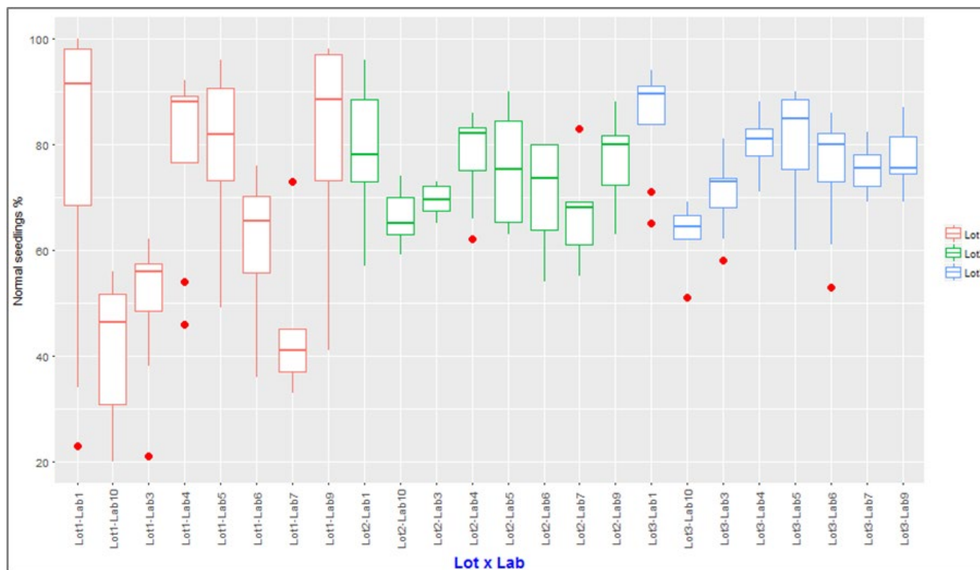

Figure 8. Percentage of normal seedlings for all methods, per lot and per laboratory; red dots = outliers; into brackets = number of values

- **Germination results by method x lot**

Figure 9 shows the data of the percentage of normal seedlings for all laboratories per lot and per method. As graphically shown by the median value in the side-by-side boxplots, the variation was smaller for RT2 compared to RT1 for lots 2 and 3, illustrating that the diversity of germination methods also played a role in the results variability. The variation was also smaller for all lots for RT3 compared to RT1. The variation was smaller for RT3 compared to RT2 for lots 1 and 2, demonstrating that **defining a clear root length criterion decreased the results variability between laboratories**, for two of the three lots tested.

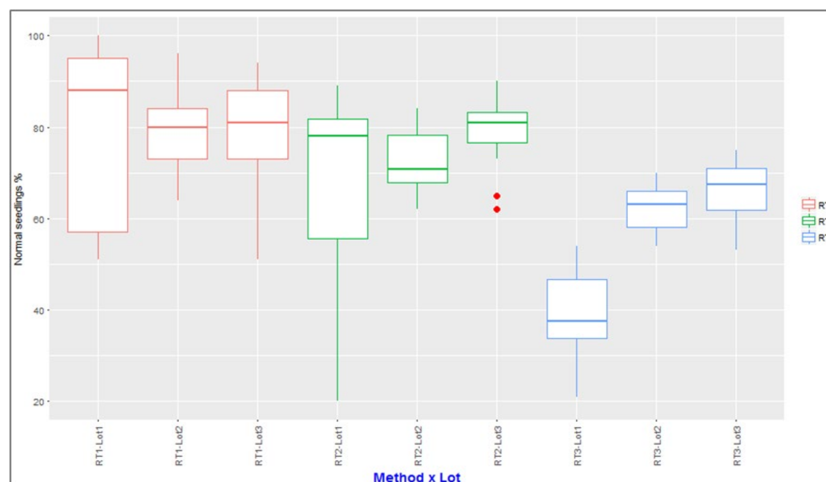

Figure 9. Percentage of normal seedlings per method and per lot; red dots = outliers; into brackets = number of values

- **Results of data checking**

Data checking has been performed according to ISTA rules by computing tolerances for germination test replicates. Only one result was found out tolerance:

Method	Lot	Lab	Mean	# Reps	# seeds/rep	Range	Tolerance	Out of Tolerance
RT3	Lot1	Lab3	30	2	100	17	14	OUT

- **Repeatability/Reproducibility**

For each method, the following linear mixed model has been fitted: $y_{ijk} = \mu + \alpha_i + b_j + (\alpha b)_{ij} + e_{ijk}$

y_{ijk} is the observed percent of normal seedlings in rep k of lot i and lab j

µ is the intercept

α_i is the fixed effect of lot i

b_j is the random effect of lab j; b_j ~ i.i.d. N $(0,\sigma^2_{Lab})$

$(\alpha b)_{ij}$ is the random interaction effect between lot i and lab j $(\alpha b)_{ij}$ ~ i.i.d. N $(0,\sigma^2_{Lot\times Lab})$

e_{ijk} are the residuals; e_{ijk} ~ i.i.d. N $(0,\sigma^2)$

Repeatability standard-deviation is then given by $S_r = \sqrt{\hat{\sigma}^2}$ and reproducibility standard-deviation by $S_R = \sqrt{\hat{\sigma}^2 + \hat{\sigma}^2_{Lab} + \hat{\sigma}^2_{Lot \times Lab}}$.

The dispersion factor is calculated as $f_r = \sqrt{\dfrac{m\,\hat{\sigma}^2}{\overline{p}_{\dots}(100-\overline{p}_{\dots})}}$ where $\overline{p}_{\dots}$ (100- $\overline{p}_{\dots}$) is the overall average percentage of normal seedlings and m is the number of seeds per rep (m = 100 in this study). If f_r > 1, one speaks of over dispersion because the data have a larger variance than expected under the assumption of a binomial distribution.

Results (including 'out of tolerance' values):

Method	$\overline{p}_{\dots}$	S_r	f_r	S_R	$\sqrt{\hat{\sigma}^2_{Run}}$	$\sqrt{\hat{\sigma}^2_{Lot \times Run}}$
RT1	79	**4.23**	**0.96**	**13.04**	9.86	7.40
RT2	73	**3.60**	**0.81**	**14.61**	9.94	10.09
RT3	56	**4.99**	**1.00**	**7.42**	3.21	4.46

. $\overline{p}_{\dots}$ is the overall average percentage of germinated seeds
. S_r: is the **repeatability** standard-deviation (intra-lab)
. f_r is an estimate of the dispersion parameter
. S_R is the **reproducibility** standard-deviation (inter-lab)

Though the repeatability standard-deviation and the dispersion factor increased for RT3, all three methods are valid regarding repeatability as the dispersion factor was close or equal to 1. We could observe a drastic decrease of the reproducibility standard-deviation from 14,61 for RT2 to 7,42 for RT3, meaning that **the use of a fixed root criterion increased the uniformity of evaluation between laboratories**.

Conclusions Ring Tests 2014-2015

The ring tests have demonstrated that the germination results variation between laboratories was mainly caused by the diversity of root length criteria. Indeed, when fixing the germination method (RT2) the repeatability standard deviation and the dispersion factor decreased, meaning that the results were more uniform within each lab, though the reproducibility standard deviation slightly increased, meaning that the uniformity did not improve between labs.
When comparing RT3 with RT2 (thus ignoring the impact of the factor 'germination method'), the repeatability standard deviation and the dispersion factor increased, meaning that the addition of a root criterion for the assessment of spinach seedlings did not improve the uniformity within labs but the **reproducibility standard-deviation decreased** a lot, meaning that the **evaluation was much more uniform between labs**. This is illustrated by the side-by-side boxplots of **Figure 10** (per lot and per method), where we can see that RT3 led to a much more uniform evaluation for two of the three lots tested (lots 1 and 2).

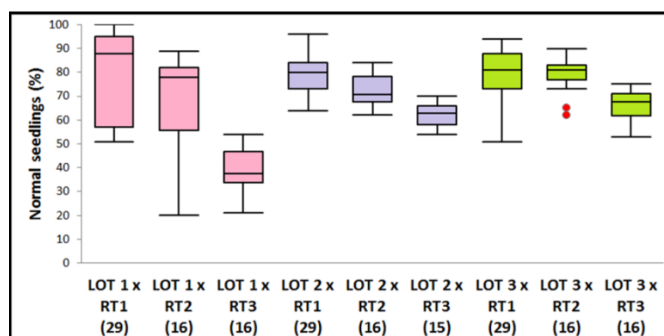

Figure 10. Percentage of normal seedlings per lot and per method; red dots = outliers; into brackets = number of values

Validation study – ISTA accredited labs

Though Enza Zaden's germination laboratory was not ISTA accredited, its results were included in the analysis as this laboratory has a lot of experience in spinach germination tests. Thus, including these results brings additional and relevant information to the study.

Remark: The 'out of tolerance' results have been kept in the analysis so that repeatability and reproducibility estimates reflect the presence of these 'out of tolerance' results.

- **Germination results by seed lot**

Figure 11 presents the percentage of normal seedlings obtained for all methods, by all laboratories. As shown by the median value in the side-by-side boxplots, sample 1 gave 74,0 % of normal seedlings, sample 2 gave 83,5 %, sample 3 gave 80,0 %, sample 4 gave 85,5 %, sample 5 gave 83,5 % and sample 6 gave 89,4 %.

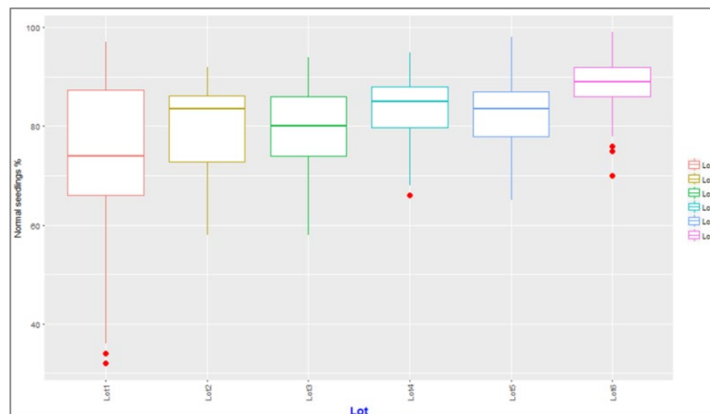

Figure 11. Percentage of normal seedlings for all methods and all laboratories, per lot; red dots = outliers

Germination results by method

Figure 12 shows the percentage of normal seedlings for all samples and all laboratories, per method. As graphically shown by the median value in the side-by-side boxplots, both VRT1 (red) and VRT2 (blue) gave 84,0 % of normal seedlings. Overall, the variation was smaller for VRT2, confirmed by the presence of several outliers for VRT1.

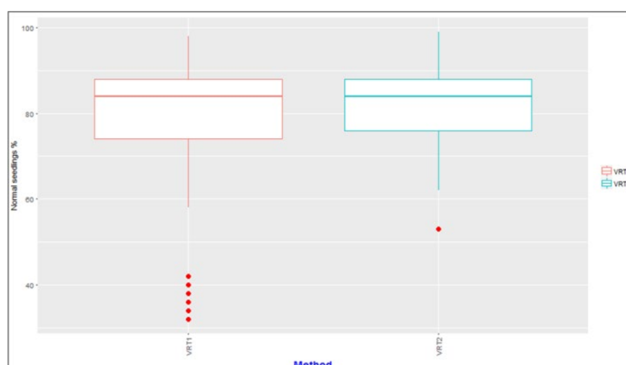

Figure 12. Percentage of normal seedlings for all samples and all laboratories, per method; red dots = outliers

Germination results by method x laboratory

Figure 13 shows the percentage of normal seedlings for all samples per method and per laboratory. The data shows certain variability between laboratories. However, this did not lead to the exclusion of any result for further analysis. This indicates that other factors than the root length criterion in the germination protocols also created variation between laboratories, as could be suspected when looking at the diversity of methods listed in **Table 6**.

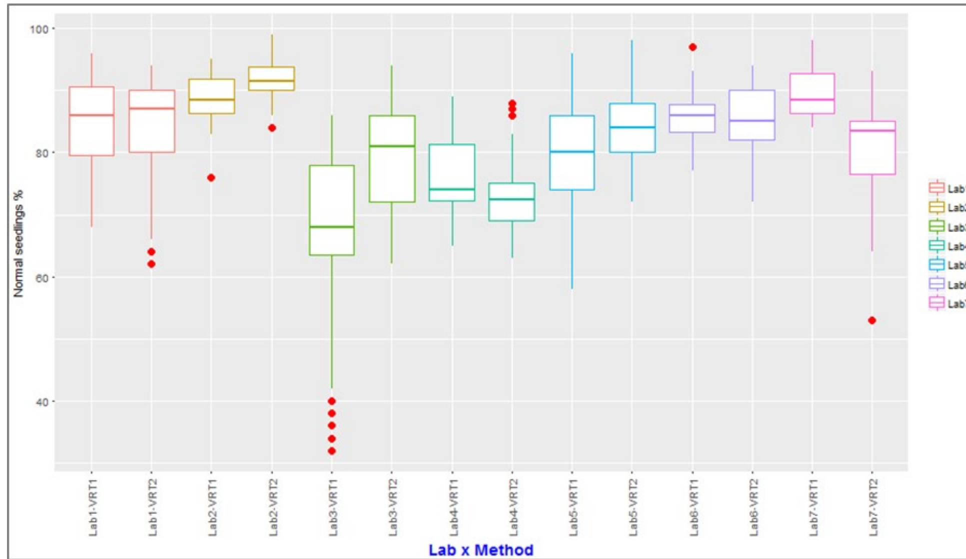

Figure 13. Percentage of normal seedlings for all samples per laboratory and per method (VRT1 and VRT2); red dots = outliers

- **Germination results by laboratory x lot**

Figure 14 shows the data grouped per lot and per laboratory. The variability between laboratories shown in **Figure 13** is confirmed, for all lots. The least variation was found for lot 6 and the highest variation for lot 1, as expected. Indeed, batches with high quality have less variability than those with low quality, which is well illustrated here. This did not lead to the exclusion of any result for further analysis.

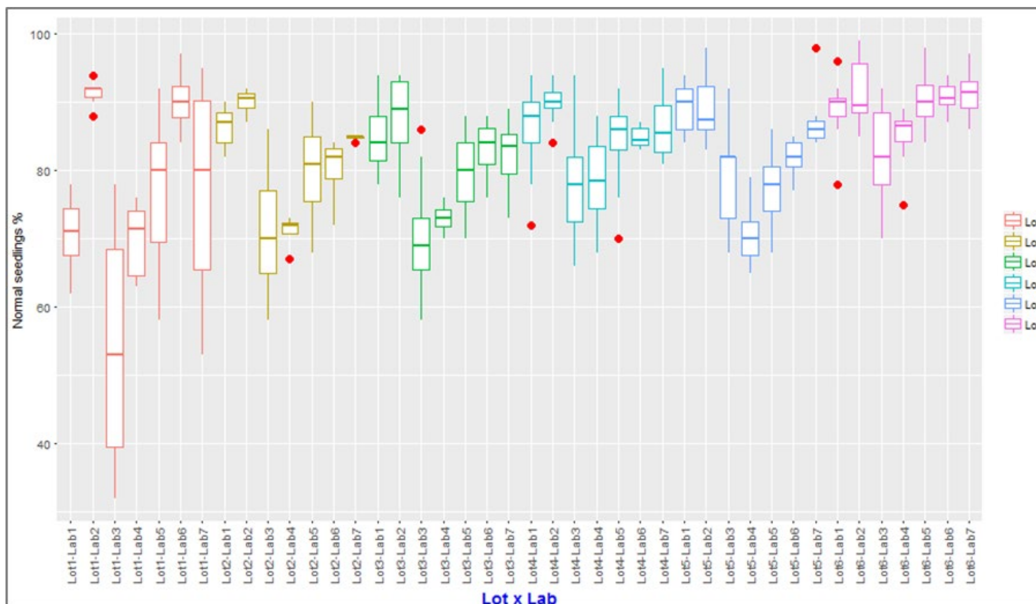

Figure 14. Percentage of normal seedlings for both methods, per lot and laboratory; red dots = outliers

- **Germination results by method x lot**

Figure 15 shows that the **variation decreased for all lots between VRT1 and VRT2,** though some outliers for lots 5 and 6 suggest that differences remained between laboratories for these lots.

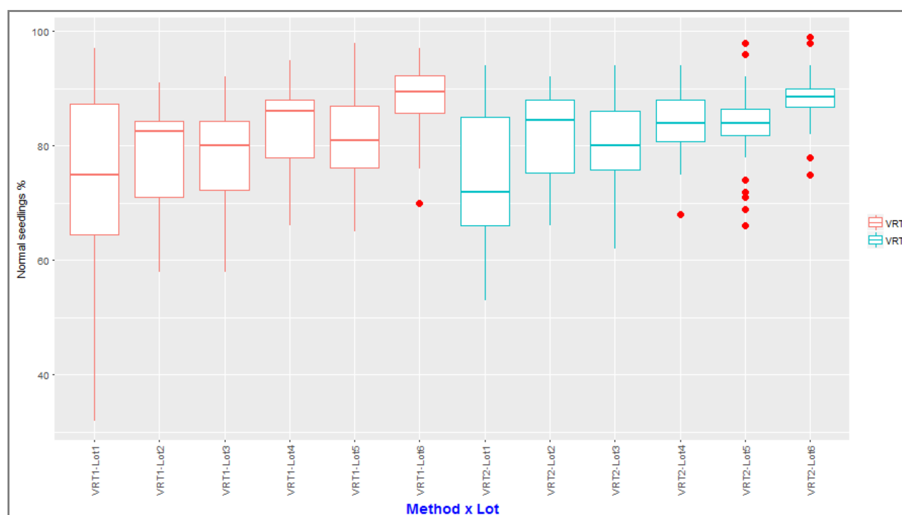

Figure 15. Percentage of normal seedlings per method and per lot; red dots = outliers

- **Results of data checking**

Data checking has been performed according to ISTA rules by computing tolerances for germination test replicates. Four results were found out of tolerance:

Method	Lot	Lab	Mean	# Reps	Range	Tolerance	Out of Tolerance
VRT1	Lot1	Lab6	90	4	13	12	OUT
VRT1	Lot3	Lab2	84	4	16	14	OUT
VRT2	Lot5	Lab2	93	4	12	10	OUT
VRT2	Lot6	Lab2	93	4	12	10	OUT

- **Repeatability/Reproducibility**

Results (including 'out of tolerance' values):

Method	$\overline{p}_{...}$	S_r	f_r	S_R	$\sqrt{\hat{\sigma}^2_{Run}}$	$\sqrt{\hat{\sigma}^2_{Lot \times Run}}$
VRT1	81	**4.96**	**1.07**	**11.07**	7.94	5.91
VRT2	82	**4.74**	**1.04**	**8.37**	5.14	4.61

. $\overline{p}_{...}$ is the overall average percentage of germinated seeds

. S_r: is the **repeatability** standard-deviation (intra-lab)

. f_r is an estimate of the dispersion parameter

. S_R is the **reproducibility** standard-deviation (inter-lab)

The dispersion factor slightly decreased for VRT2 (f_r = 1,04) compared to VRT1 (f_r = 1,07) and both methods are valid regarding repeatability as the dispersion factor was close to 1. The **repeatability improved** for VRT2, as the repeatability standard-deviation decreased (S_r VRT1 = 4,96 *vs* S_r VRT2 = 4,74), **as did the reproducibility,** as shown by the reproducibility standard-deviation decrease between VRT 1 (S_R = 11,07) and VRT2 (S_R = 8,37). The statistical results regarding VRT1 and VRT2 obtained with the statistical tool 'ISTAgermMV' are presented in the following tables.

Source of variation	Sum Sq	Mean Sq	NumDF	DenDF	F.value	Pr(>F)
Method	2,70	2.70	1	6.05	0,11	0,75
Lot	753.59	150.72	5	30.96	6.41	0.00
Method:Lot	21.83	4.37	5	31.24	0,19	0,97

Method	Lot	Estimate	Standard Error	Lower CI	Upper CI
VRT1		81,44	2,69	75,40	87,47
VRT2		82,33	2,69	76,29	88,36
	Lot1	75,58	2,83	69,42	81,74
	Lot2	80,55	2,89	74,32	86,78
	Lot3	80,02	2,83	73,87	86,18
	Lot4	84,10	2,83	77,94	90,25
	Lot5	82,24	2,83	76,08	88,40
	Lot6	88,80	2,83	82,64	94,96
VRT1	Lot1	75,14	3,33	68,22	82,06
VRT2	Lot1	76,02	3,33	69,09	82,94
VRT1	Lot2	79,33	3,43	72,25	86,40
VRT2	Lot2	81,77	3,43	74,69	88,84
VRT1	Lot3	79,51	3,33	72,58	86,43
VRT2	Lot3	80,54	3,33	73,62	87,47
VRT1	Lot4	84,43	3,33	77,50	91,35
VRT2	Lot4	83,77	3,33	76,84	90,69
VRT1	Lot5	81,38	3,33	74,45	88,30
VRT2	Lot5	83,11	3,33	76,18	90,03
VRT1	Lot6	88,84	3,33	81,92	95,76
VRT2	Lot6	88,76	3,33	81,84	95,68

Conclusions Validation Study

Overall, the additional root criterion did lead to a decrease of variation when considering the results of all lots and all labs for VRT2 compared to VRT1. When considering results per lot, there was a **decrease of variation**

for all lots for VRT2 compared to VRT1, though a few outliers suggested that some differences remained for lot 5 and lot 6. This decrease of variation was reflected by the decrease of the repeatability standard-deviation and of the reproducibility standard-deviation. In conclusion, the **addition of a root criterion** (with root:shoot ratio = 0,5) for the assessment of spinach seedlings led to a **more uniform evaluation between laboratories and within laboratories for all six lots tested**.

DISCUSSION

We observed a clear decrease of variation per lot between RT2 and RT3 during the 2014-2015 ring tests, as for VRT1 and VRT2 in 2016. This proved that a root criterion can improve the uniformity of results between and within laboratories. However, the decrease of variation observed between RT1 and RT2 for lots 2 and 3 suggests that part of the variation observed was caused by the various germination conditions between laboratories. As explained before, the most accurate root:shoot ratio in relationship to the percentage of normal seedlings under optimal conditions was 1 for tests done at 10 °C and 0,5 for tests done at 15 °C. Hence, some variation will likely remain between laboratories even after the root criterion will be implemented (root:shoot ratio of 0,5), due to the diversity of germination conditions.

GENERAL CONCLUSION

Results from both the ring tests (not ISTA accredited laboratories) and the validation study (ISTA accredited laboratories) showed that using a root criterion for the assessment of *Spinacia oleracea* seedlings led to a more uniform evaluation between laboratories and within laboratories. Consequently, we recommend including the root:shoot ratio of 0,5 in the ISTA rules in Chapter 5, under '5.2.7.Normal seedlings', '5.2.7. 2. Slight defect', as follows:

'**for *Spinacia oleracea*, the length of the primary root has to be equal to or greater than the half of the length of the hypocotyl**'

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