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Method Validation Reports on Rules Proposals for the International Rules for Seed Testing 2018 Edition

Validation study to support the germination method for introducing a new species (*Brassica carinata* A. Braun) into the ISTA Rules to support B.1.1.

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Abstract
There is a need to introduce *Brassica carinata* A. Braun into the ISTA Rules to allow the issue of ISTA International Certificates for seed export and national trading. *B. carinata* is an old world Brassica species that has more recently been the subject of breeding programs to develop new varieties that can produce high quality oils for use as biofuels. New varieties have been developed in both N. America and Europe. The validation study used three seedlots from N. America and three from Europe and tested four germination methods in six ISTA accredited laboratories.

The germination methods chosen for testing were based on existing ISTA germination test methods used for other Brassica species and the experience of laboratories already testing *B. carinata*. The results of the statistical analysis concluded the data collected was fit for analysis. Repeatability and reproducibility analysis was used to select the most suitable germination method.

The between paper (BP) method at a constant 20**°**C had acceptable, and the best, repeatability and reproducibility values. BP at an alternating 20<=>30°C also had acceptable repeatability and reproducibility values and gave the same averaged mean normal germination (92%). Therefore the recommendation from the ISTA Germination Technical Committee is to include both BP 20°C and BP 20<=>30°C as germination methods for *B. carinata* in the ISTA Rules.

Introduction

Brassica carinata A. Braun is a species belonging to the same genus as seven other Brassica species already listed in the ISTA Rules.

The interest in introducing *B. carinata* into the International Rules for Seed Testing (ISTA Rules) is due to the increasing use of the species as an oilseed crop. The end-use is for non-food purposes, such as production of biofuel. In particular, the oil from *B. carinata* seems to have excellent characteristics as aviation fuel for jet engines. Research and varietal development, as well as open field cultivation, are taking place in different countries, such as Canada and Italy. In Italy, a catalogue for the voluntary varietal registration of new varieties of *Brassica carinata* was established in 2009.

Comparative test protocol

Seed source

Six seed samples of *Brassica carinata* were tested (three from Canada, three from Italy).

Test method

Although *Brassica carinata* is a new species to be included in the ISTA Rules, standard ISTA methods already exist for seven other *Brassica* species already in the ISTA Rules. The following four test methods were selected for comparative testing in six ISTA accredited laboratories that volunteered to participate in the study.

1) TP; 20 <=>30°C (first count 5 days; final count: 7 days). Additional treatment: None
2) TP; 20°C (first count 5 days; final count: 7 days). Additional treatment: None
3) BP; 20 <=>30°C (first count 5 days; final count: 7 days). Additional treatment: None
4) BP; 20°C (first count 5 days; final count: 7 days). Additional treatment: None

For each test and sample 400 seeds were tested in 4 replicates of 100 seeds. Seedling evaluations were based on the ISTA seedling group A-2-1-1-1 and the participants provided a description of any abnormal seedlings. When 5% or more of fresh seeds were present, their potential to germinate was determined by tetrazolium testing. The seed determined to have the potential to germinate were reported as fresh. The seed determined not to have the potential to germinate were reported as dead.

Statistical analysis

The comparative test was a total of 24 germination tests completed by each of the six participant laboratories. The data received from the participants was checked for completeness and accuracy.

Statistical analysis: possible outliers were assessed using side-by-side boxplots and using replicate tolerance checks (ISTA Rules Chapter 5: Table 5B). The performance of the methods was then assessed using the estimation of repeatability and reproducibility variances.

Data exploration with side-by-side boxplots

Figure 1: Boxplots for the six seedlots grouped across methods and laboratories.

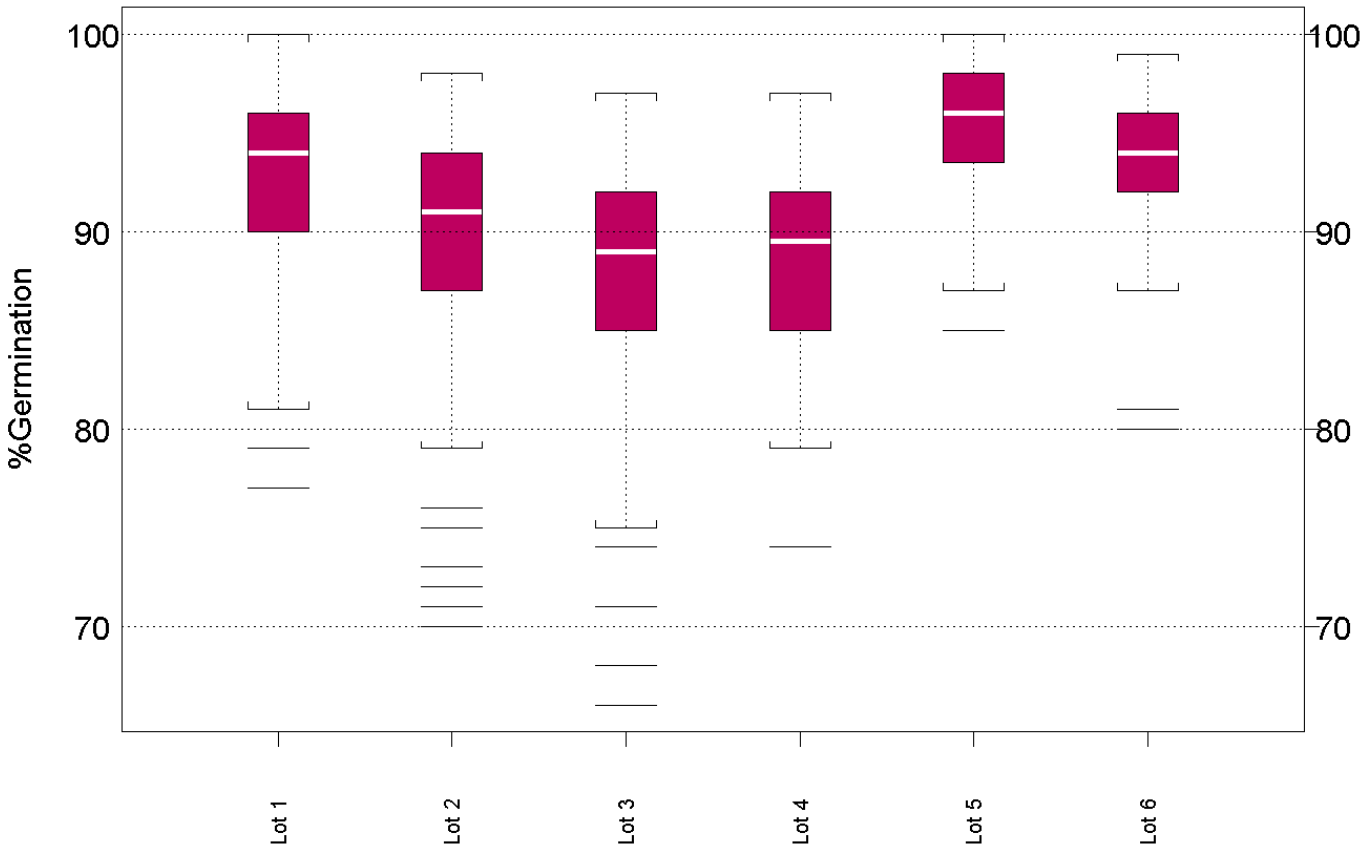


Figure 2: Boxplots for the six laboratories grouped across lots and methods.

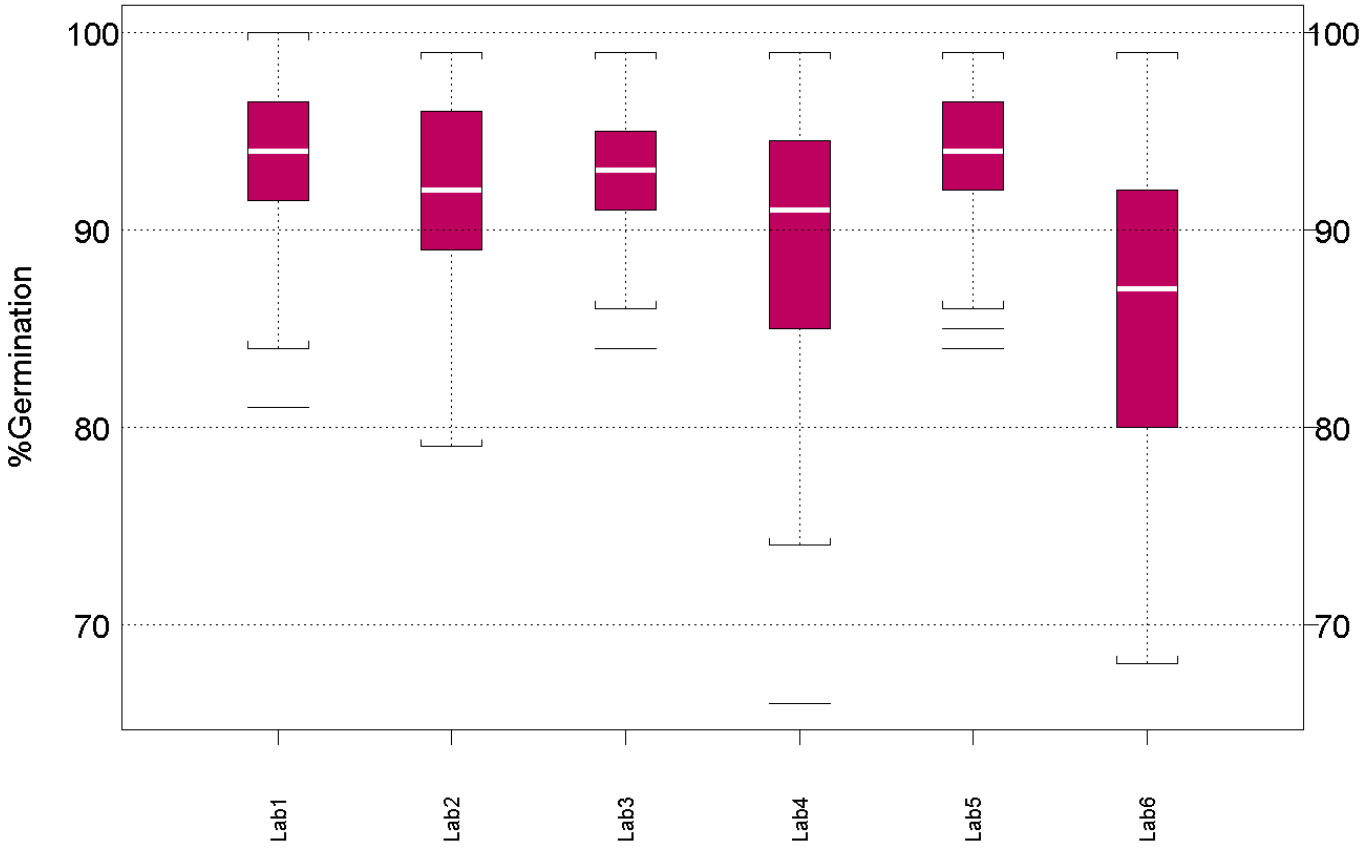


Figure 3: Boxplots for the laboratory x seedlots grouped across methods.

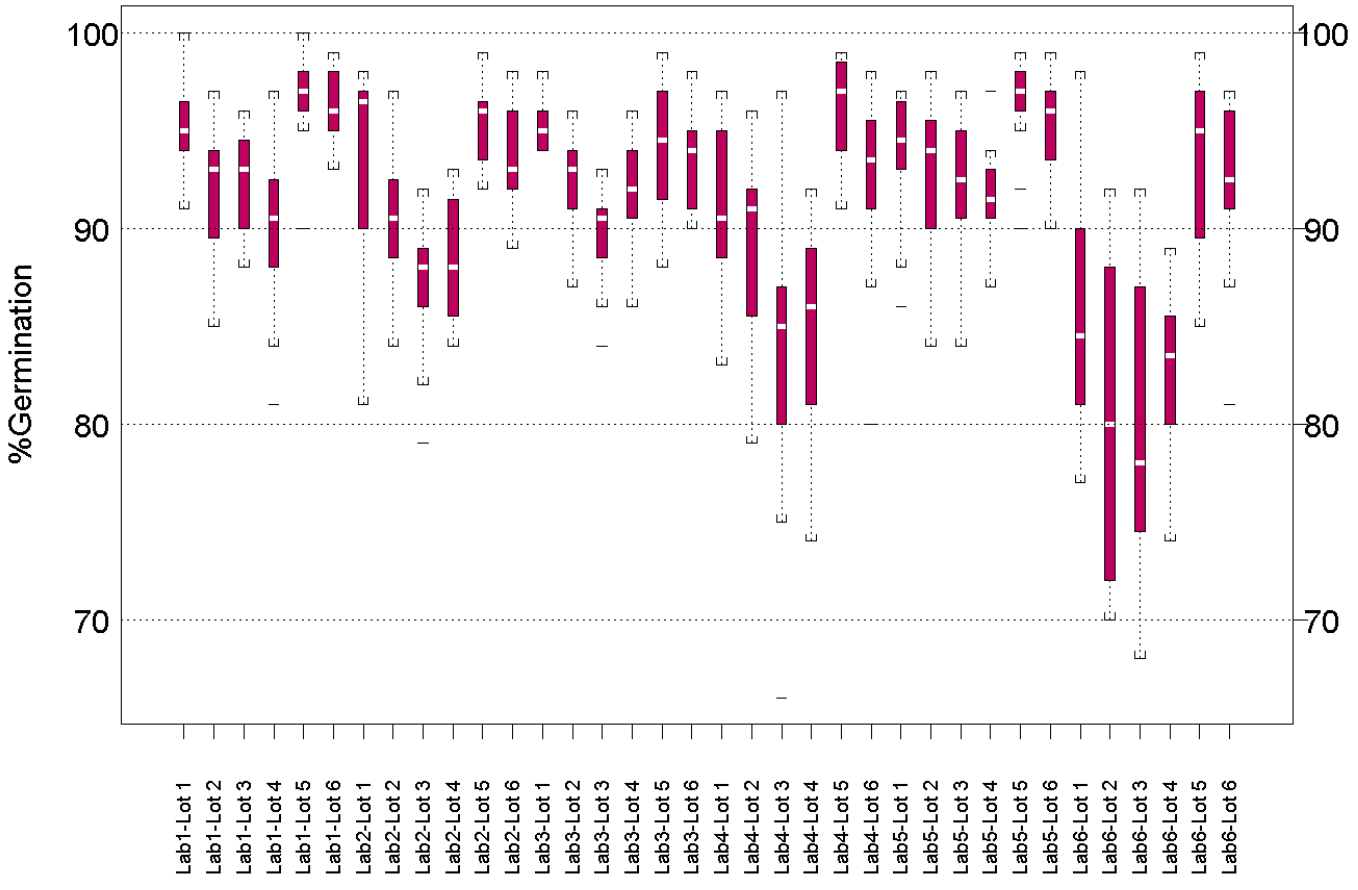


Figure 4: Boxplots for the methods grouped across lots and laboratories.

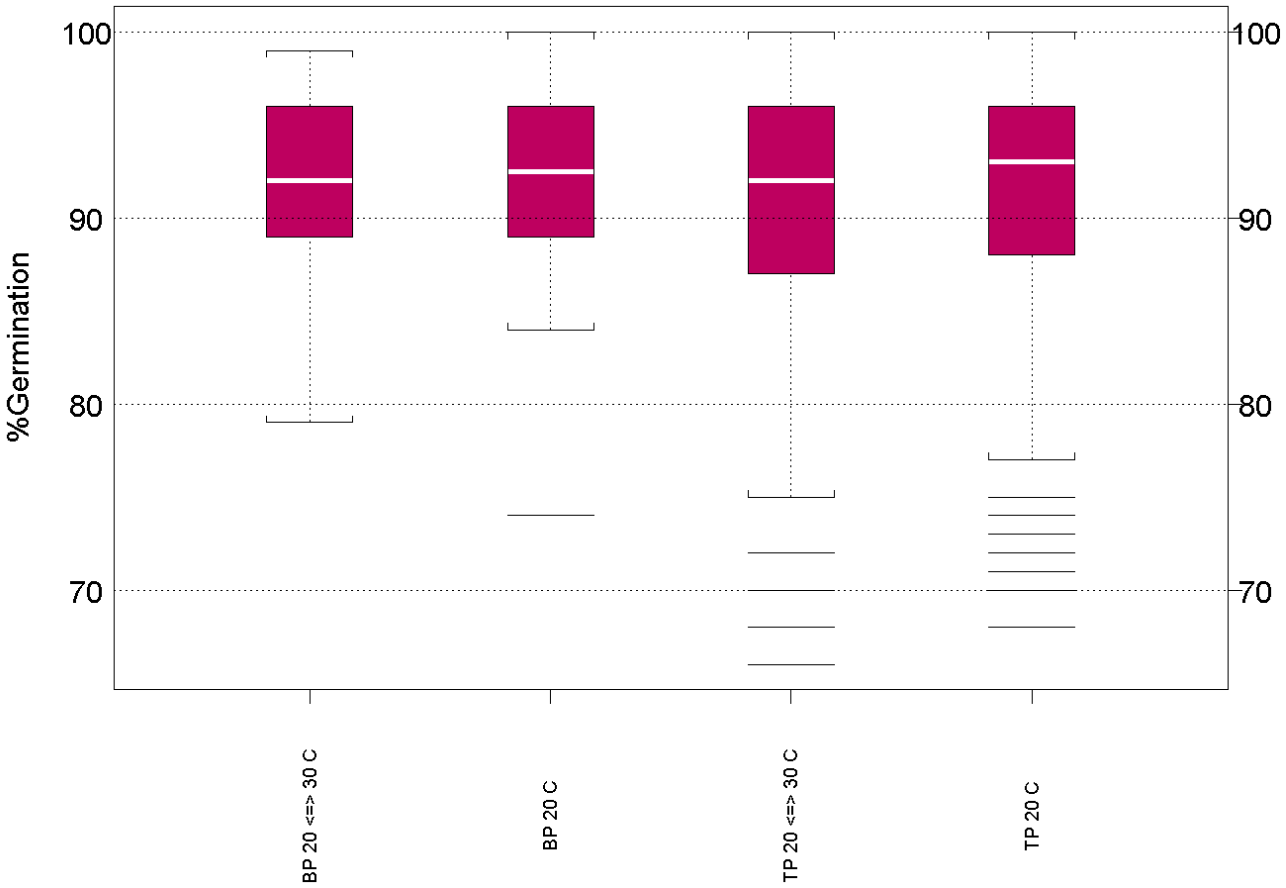
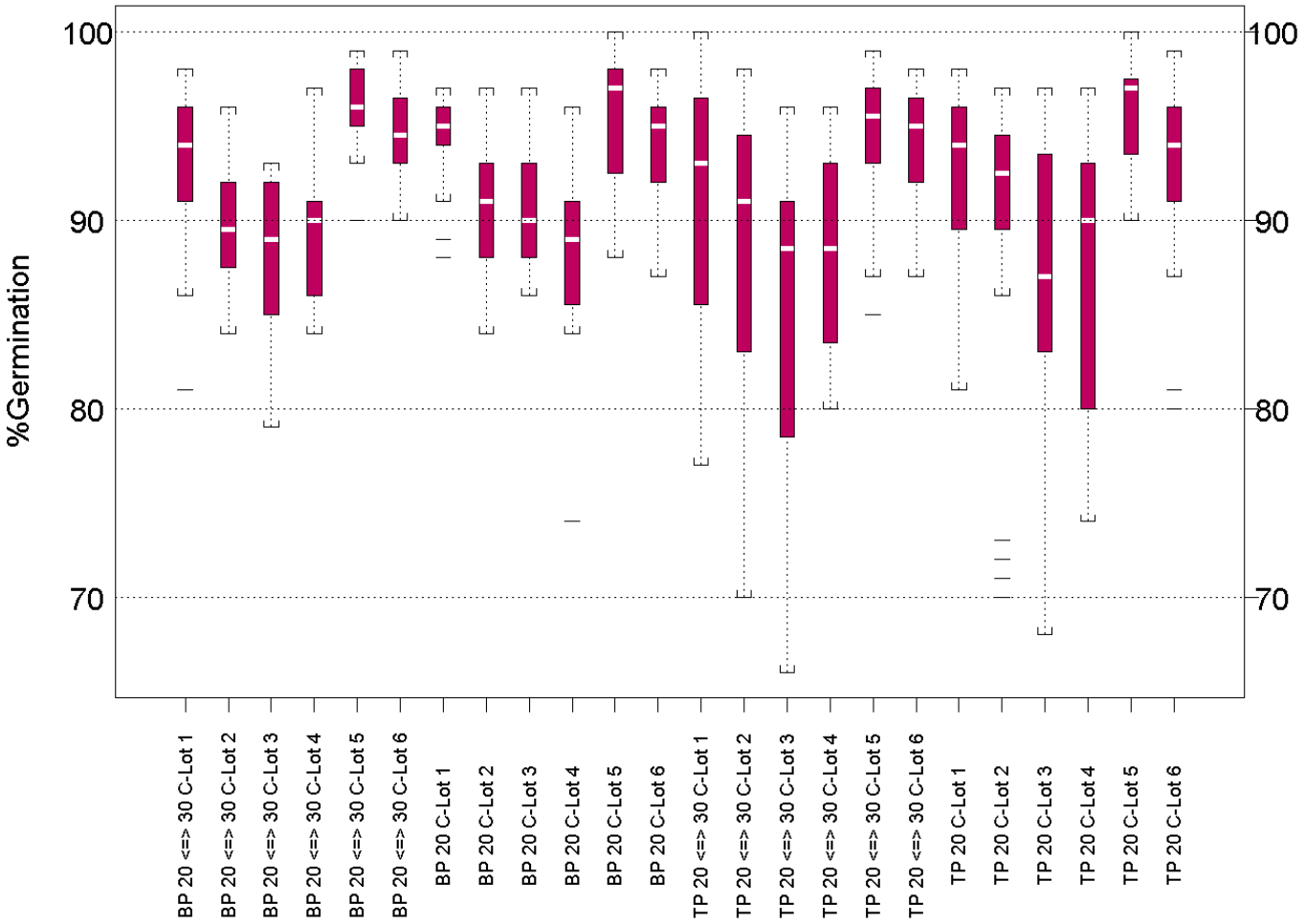


Figure 5: Boxplots for the methods x seedlots grouped across laboratories.



Results of data checking

Data checking was performed according to the ISTA rules by computing tolerances for germination test replicates. Three test results out of the 144 tests were out of tolerance:

	%Normal seedlings
BP 20<=>30°C	**1**
BP 20°C	**0**
TP 20<=>30°C	**1**
TP 20°C	**1**

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 $f_r = \sqrt{\frac{m\,\hat{\sigma}^2}{\overline{p}_{...}(100 - \overline{p}_{...})}}$ 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 this indicates overdispersion because the data have larger variance than expected under the assumption of a binomial distribution.

Repeatability (S_r) results:

BP 20<=>30°C

$\overline{p}_{...}$	S_r	f_r
91.72	2.92	1.06

BP 20°C

$\overline{p}_{...}$	S_r	f_r
92.27	2.61	0.98

TP 20<=>30°C

$\overline{p}_{...}$	S_r	f_r
90.14	2.93	0.98

TP 20°C

$\overline{p}_{...}$	S_r	f_r
90.62	3.04	1.04

Repeatability standard deviations are acceptable for all four methods, i.e. the dispersion factor (f_r *)* is close to 1.

Reproducibility (S_R) results

BP 20<=>30°C

$\overline{p}_{...}$	S_R	$\hat{\sigma}^2_{Lab}$	$\hat{\sigma}^2_{Lot\times Lab}$
91.72	3.58	1.09	1.77

BP 20°C

$\overline{p}_{...}$	S_R	$\hat{\sigma}^2_{Lab}$	$\hat{\sigma}^2_{Lot\times Lab}$
92.27	3.53	1.59	1.77

TP 20<=>30°C

$\overline{p}_{...}$	S_R	$\hat{\sigma}^2_{Lab}$	$\hat{\sigma}^2_{Lot\times Lab}$
90.14	6.64	5.05	3.15

TP 20°C

$\overline{p}_{...}$	S_R	$\hat{\sigma}^2_{Lab}$	$\hat{\sigma}^2_{Lot\times Lab}$
90.62	6.75	5.11	3.22

For the same substrate, reproducibility standard-deviations are similar but are higher for the TP substrate.

Conclusions

The between paper (BP) method at a constant 20°C had acceptable, and the best, repeatability and reproducibility standard-deviation values. BP at an alternating 20<=>30°C also had acceptable repeatability and reproducibility standard-deviation values and gave the same averaged mean normal germination (92%). Therefore the recommendation from the ISTA Germination Technical Committee is to include both BP 20°C and BP 20<=>30°C with a first count at 5 days and final count at 7 days, as germination methods for *B. carinata* in the ISTA Rules. No recommendations for breaking dormancy are proposed.

Acknowledgements
Thanks to Agrisoma Biosciences Inc., Canada and CREA-SCS Certification Office, Palermo, Italy for providing the seedlots for the study.

Thanks also to the staff at the following ISTA accredited laboratories for participating in the study: Agroscope, Switzerland; CFIA Saskatoon Laboratory, Canada; CREA-SCS Sede di Tavazzano-Laboratorio, Italy; GEVES-SNES, Station Nationale d'Essais de Semences, France; Monsanto Vegetable Seeds Inc., Oxnard, USA and 20/20 Seed Lab Inc, Nisku, Canada; co-ordinated at the laboratories by Christine Herzog, Steve Jones, Rita Zecchinelli, Sylvie Ducournau, David Johnston and Carey Matthiessen respectively. Thanks to the technical reviewers in the ISTA Germination Technical Committee (TCOM): Sarah Dammen and Augusto Martinelli.

In particular thanks to Sylvie Ducournau as Chair of the ISTA Germination (TCOM) for coordinating the activity, Nadine Ettel for her support during the method validation process, Rita Zecchinelli as test leader, Marie-Helene Wagner for the data summary, Steve Jones for preparing the final version of the report and special thanks to Jean-Louis Laffont as ISTA Statistics TCOM Chair for his ongoing support of the ISTA validation process and this validation analysis report in particular.

Validation study for blotter and malt agar methods for detecting *Leptosphaeria maculans (Phoma lingam)* on vegetable *Brassica* seeds to support C.7.1

Task 5.7 ISHI-Veg (ISF)/TESTA WP5/ISTA/GEVES

Abstract

The performance of methods for the detection of the seed-transmitted pathogen *Phoma lingam* on vegetable *Brassica* seeds was compared in 10 laboratories in an international comparative test organized by GEVES as part of the TESTA project. The methods include a malt agar and two blotter tests performed followed by a PCR and pathogenicity test on suspect colonies to identify *Leptosphaeria maculans* and *Leptosphaeria biglobosa*. The two blotter tests are differentiated by the method used to inhibit germination of seeds: one uses 2,4-D whereas the other deep freezes seed. A healthy seed lot and two naturally infected (low and medium levels of infection) seed lots were tested in three blind samples of 1,000 seeds each by the three methods. The blotter methods showed comparable results to the malt agar method. The deep freeze step was shown to be more efficient than 2,4-D in inhibiting seed germination. The detection methods showed high values of accordance (repeatability), concordance (reproducibility), sensitivity, specificity and accuracy for all three infection levels (health, low and medium). As 2,4-D is toxic and its use is not recommended for routine laboratories, the blotter test with the deep freeze step and malt agar methods are considered to be reliable methods for the detection of *Phoma lingam* on *Brassica* spp. seeds and are highly recommended for routine seed health testing. As the identification of the fungus is based on the way they grow on seeds and on the morphological characters of fruiting bodies, these methods can also be used on a variety of *Brassica* and related crops.

Contents

Introduction

Phoma lingam (teleomorph: *Leptosphaeria maculans*) is the causal agent of black leg, stem canker and dry rot in *Brassica* species and other crucifers. This pathogen is present in many regions around the world and causes serious economic damage (Fitt *et al*., 2008). Nevertheless, the epidemiology and severity differs between continents due to the difference in pathogen population structures (*Leptosphaeria maculans* and *Leptosphaeria biglobosa*), climate and agricultural practices. *L. maculans* and *L. biglobosa* is a complex of two closely fungal species. *L. maculans* has colonized countries where *L. biglobosa* was prevalent such as Poland and central Canada (Fitt *et al.,* 2008). *L. maculans* isolates is responsible of basal stem damaging (crown canker), whereas *L. biglobosa* causes pale brown lesions with a dark margin on the upper stem (Dilmaghani *et al*., 2009). Discrimination between the two fungal species is based on phytopathology and molecular criteria.

Initial infections are mainly caused by ascospores dispersed from pseudothecia on infected debris (Smith *et al.,* 1988). The ascospores infect the plant through the stomata and symptoms can cause damping off on germinated seedlings or gray lesions and black pycnidia on the leaves. Once established in a crop, the pycnidia form conidia and the pathogen can be spread by rain-splash. These spores cause secondary infections which are usually less severe than primary infections with ascospores. Mild wet conditions favour the spread of the disease to epidemic proportions. The fungus is able to survive on crop debris in the soil for at least three years (Smith *et al.,* 1988).

The current reference method for the detection of *L. maculans* is the ISTA Rule 7-004 (ISTA, 2014). In this method 1,000 seeds are placed on blotter paper (Whatman No. 1 or equivalent) containing 2,4 Dichlorophenoxyacetic acid (2,4-D) to prevent seed germination, and incubated for 11 days at 20°C with alternating 12 h near ultra-violet light/12 h dark regime. As 2,4-D is toxic its use is not recommended for routine laboratories. In an alternative to using 2,4-D developed by ISHI-Veg (ISF, 2011) after incubation for 24 h at 20°C in the dark, seeds are placed at -20°C for 24 h to prevent germination.

A third method for the detection of the pathogen was developed in the GEVES-SNES laboratory and uses malt agar. This method does not entail preventing seeds from germinating; in contrast to the blotter paper method seeds adhere to the medium reducing the risk of cross contamination. Also fewer seeds can be tested in a Petri dish (10 instead of 25 in blotter). Pre-tests carried out in GEVES and Science and Advice for Scottish Agriculture (SASA) (https://www.sasa.gov.uk/) in the TESTA[1] project compared the different conditions between the three protocols and have shown that the method based on malt agar was equivalent to the blotter Whatman No 1 method (ISTA, 2014) and the deep freezing step resulted in a higher inhibition of seed germination compared with 2,4-D.

According to Toscano-Underwood *et al.* (2001) *L. maculans* is characterized as pale grey spots, often turning pale brown with abundant black pycnidia, while leaf lesions caused by *L. biglobosa* are smaller-with dark margins around a light brown centre and can contain pycnidia that form only on old leaf lesions. Despite these differences in symptom expression the two species *L. maculans* and *L. biglobosa* cannot be distinguished in any of the three methods. During the TESTA project GEVES tested different molecular and pathogenicity assays to confirm the identity of both species. Two PCR protocols (Liu *et al.,* 2006 and Plant Research International [PRI], Wageningen) were used to identify *L. maculans* and *L. biglobosa.* After comparing different methods of inoculation, aspersing the leaf, injecting the leaf vein and the stem at the two leaf stage and injecting the cotyledons according to Balesdent *et al*., (2006) was used for the pathogenicity assay.

This report describes the validation of the two blotter and malt agar protocols for detecting *P. lingam* (*L. maculans* and *L. biglobosa*) on untreated seeds. Based on the EPPO PM 7/98 (2) (EPPO, 2014) and using the

[1] TESTA: EU FP7 project *Seed health: development of seed treatment methods, evidence for seed transmission and assessment of seed health*. See http://archives.eppo.int/MEETINGS/2015_conferences/testa.htm.

results of a comparative test, the performance characteristics sensitivity, specificity, repeatability and reproducibility are determined.

Analytical sensitivity

Material and methods

One artificially infected cabbage seed (with many *Phoma* look-alikes or other saprophytes) was added to 999 healthy seeds to make a sample of 1,000 seeds. Analytical sensitivity was analyzed on six 1,000- seed samples at the established limit of detection of the three methods described in Appendix 1.

Seeds were artificially infected by placing healthy seeds on a Petri dish previously colonized by *P. lingam*. The media was composed of malt agar and mannitol. Mannitol was used to inhibit germination so that the seeds stayed in contact with the colony and the mycelium could penetrate (figure 1). After several pre-tests, a concentration of mannitol at 100 g/L and 48 h of seed exposure to the colony was selected. Seeds were tested to verify they were infected.

Fig. 1: Artificial infection of cabbage seeds on a Petri dish

To check if artificial infection was only on the surface of (superficial) or also inside the seeds, seeds were treated with 1% sodium hypochlorite. Results of artificially infecting pre-treated seeds and those without treatment are presented in figure 2. The very high percentage of infection after pre-treatment shows that infection is not superficial.

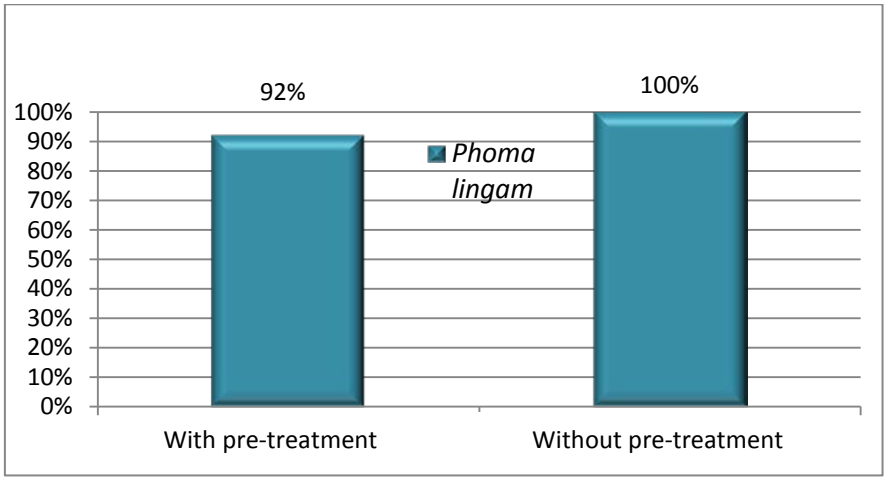


Fig. 2: Infection rate of seed artificially infected by *P. lingam*

Results

Six samples of 1,000 seeds each were tested using the malt agar, blotter deep freeze and blotter 2,4-D methods to evaluate the detection threshold of each method. The results are presented in table 1.

Table 1: Detection threshold of the three different protocols

Samples	Malt Agar (%)	Blotter deep freeze (%)	Blotter 2,4-D (%)
1	0.10	0.10	0.10
2	0.10	0.10	0.10
3	0.10	0.10	0.10
4	0.10	not applicable*	0.10
5	0.10	0.10	0.10
6	0.10	0.10	0.10
Average	0.10	0.10	0.10

* This sample developed a bacterial colony instead of *P. lingam*

All the samples tested on malt agar and 2,4-D methods were positive. On every occasion, the artificially infected seed added to 999 healthy seeds was detected. The malt agar test was easier to read when compared with the blotters due to a slower growth of fungal colonies.

The analytical sensitivity of the methods (level of detection) is 0.1%.

Analytical specificity

Material and methods

In the performance criteria for specificity a few of the most relevant targets (morphologically similar but different strains) and non-targets (seed lot infected with *L. maculans* and other saprophytes) organisms were selected. GEVES tested the three methods using three naturally infected seed lots with varying levels of infection [healthy (A), low (B) and medium(C)] for target and non-target organisms and recorded the number of seeds infected with other fungi. One sample each of 1,000 seeds was tested using the three methods.

Results

Results showed no difference between the methods in detecting target (*P. lingam*) and non-target (saprophytes) with similar standard deviations and confirmed that the analytical specificity of the three methods of detection were similar (figure 3).

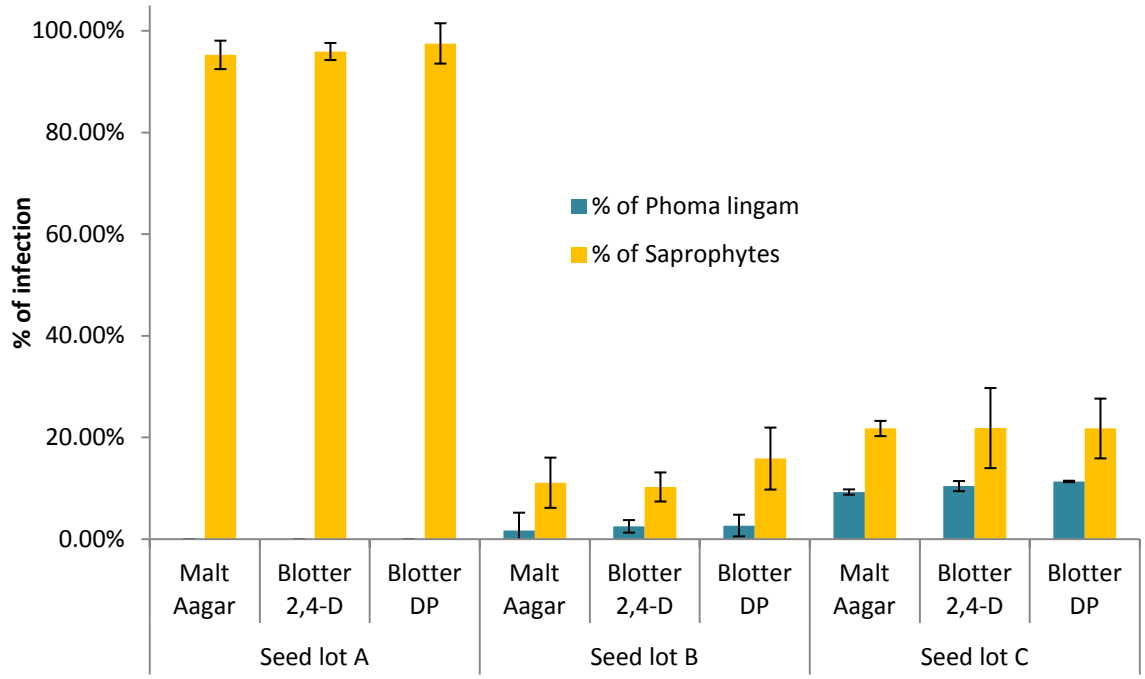


Fig. 3: Comparison of the analytical specificity of the three different method of detection

Repeatability and Reproducibility

Introduction

The objectives of this comparative test (CT) are to improve the ISTA method 7-004 by replacing 2,4-D to inhibit germination of seeds on the blotter by a deep freezing step and to compare the blotter and malt agar detection methods for the seed borne disease *Phoma* stem canker caused by *L. maculans*. This CT was also used to validate the deep-freeze blotter and malt agar methods to detect *L. maculans* on untreated seeds by comparing them to the 2,4-D blotter method. It was also used to evaluate the efficiency of PCR and the pathogenicity assay in identifying suspect *Leptosphaeria* detected on blotter or malt agar.

Materials and Methods

Seed lots and samples

Three untreated and naturally infected cabbage seed lots A (O 480437), B (P 19000) and C (P 19014) with variable levels of natural *P. lingam* infection and saprophytes were obtained from seed companies. They were characterized by the GEVES-SNES Laboratory in France prior to the comparative test based on the average number of *P. lingam* infected seeds. From each seed lot 3 samples of 1,000 seeds were tested. An average of 0, 3.7 and 11% of *P. lingam* infected seeds were observed in the lots A, B and C, respectively. Accordingly, the seed lots were characterized as being healthy, low and medium infected.

For the comparative test of the three detection methods the participating laboratories received three 1,000-seed samples from the three seed lots. The 9 samples for each method were randomly coded to ensure a blind comparative test. All seed samples were prepared by the sampling department of GEVES-SNES with the use of the rotary sample divider apparatus based on the thousand-seed weight of each seed lot. Not all methods were used by the participating laboratories due to a lack of time or a lack of experience with the different methods (see table 2).

Table 2: Laboratories participating in the comparative test

	Blotter deep freeze	Malt Agar	Blotter 2,4-D
Laboratory 1	x	x	x

Laboratory 2	x		x
Laboratory 3	x		x
Laboratory 4	x		x
Laboratory 5	x		x
Laboratory 6	x	x	x
Laboratory 7	x	x	x
Laboratory 8	x		x
Laboratory 9		x	
Laboratory 10	x	x	

Homogeneity and stability test of the seed lots

The three naturally infected seed lots were tested at GEVES-SNES with the malt agar method to check that the infection rates were homogeneous. Homogeneity was tested taking 10 samples of 400 seeds from the healthy, low and medium infected lots.

The stability test was also performed at GEVES-SNES at the end of the validation study to verify the infection levels of each lot. Each seed lot was tested on 10 samples of 400 seeds with the malt agar method after receiving the study results from the participating laboratories.

Detection methods

Table 3 below summarizes the key elements of the methods used in this test.

Table 3: Differences in the conditions of each detection method

Substrate	Inhibition of germination	Seeds per plate *	Temperature	Light condition	Notation	Sample Size
Malt agar	None	10	20°C	Darkness	7 and 11 days	1,000
Blotter	2,4-D	25	20°C	12 h NUV/12 h dark	11 and 14 days	1,000
Blotter	Deep Freezing	25	20°C	12 h NUV/12 h dark	11 and 14 days	1,000

* Number of seed per plate is for a 9 cm diameter Petri dish
Note: If a germination box is used, up to 100 seeds per box can be tested

It is important to note that in the malt agar test (see Appendix 1), the concentration of the malt obtained from a local bakery is 2%. The commercially available malt used in pre-tests by SASA did not give the same colony morphology (mycelium colours and pycnidia formation) when compared with the malt provided by GEVES to all the participating laboratories.

Strain collections

To confirm the identity of the *Leptosphaeria* strains, GEVES used a *P. lingam* collection and the suspect isolates from the comparative test.

During the TESTA project, a strain collection was established with target and non-target isolates. In total 20 target isolates identified as *P. lingam* (11 characterized as *L. maculans* and 9 as *L. biglobosa*) from the collections of INRA Versailles, SASA, GEVES and Bejo Zaden B.V. were tested. A collection of 16 isolates considered as non-target were collected from *Brassica* seed saprophytes (*Alternaria* sp., *Cladosporium* sp., *Rhizopus* sp., *Botrytis cinerea*, *Mucor* sp., *Trichoderma*., *Sclerotinia* sp., *Stysanus* sp., *Epicoccum* sp., *Sordaria* sp. and others), pathogens (*Alternaria brassicicola*, *Alternaria brassicae*, *Fusarium oxysporum)* and different *Phoma* subspecies (e.g. *Phoma exigua* and *Phoma valerianellae*).

Identification of *Leptosphaeria* strains using PCR

The Liu and PRI PCRs were used to differentiate *L. maculans* and *L. biglobosa*.

Collecting and grinding mycelium

Two methods were used for grinding mycelium. For testing isolate collections, fresh mycelium (grown on malt agar for 10 days) was sampled, placed in a filter plastic bag with 1 mL of sterilized water and crushed with a "ball mill". For the comparative test, after transferring suspect *P. lingam* colonies to a plate, fresh mycelium was sampled after 7 days of incubation, placed in sterilized water (50-100 µL) and crushed with silicone micro beads.

Phoma lingam DNA extraction

DNA was extracted by placing the suspension in a micro-tube twice at 100°C for 4 min and then at 4°C for 2 min.

PCR primers, positive and negative controls, reaction mix, PCR program and gel visualisation

Laboratories used DNA of their own reference *Leptosphaeria maculans* / *Leptosphaeria biglobosa* strains as PCR positive control and sterile water as PCR negative control. The primers sets developed by Liu *et al.* (2006) were used:

L. maculans	LmacR	5'-GCAAAATGTGCTGCGCTCCAGG-3'	with DNA amplification product size of 331 bp targeting *L. maculans*
	LmacF	5'-CTTGCCCACCAATTGGATCCCCTA-3'	
L. biglobosa	LmacR	5'-GCAAAATGTGCTGCGCTCCAGG-3'	with DNA amplification product size of 444 bp targeting *L. biglobosa*
	LbigF	5'- ATCAGGGGATTGGTGTCAGCAGTTGA -3'	

The PCR was carried out in a 25 µL reaction mix (see table 4a) containing 5 µL of DNA extract.

Table 4a: Mix used with Liu primers

Compound	Concentration	Final concentration	Volume in 25 µL
Sterile Milli Q			6.875
Buffer	10x	1x	2.5
$MgCl_2$	25 mM	2.5 mM	2.5
dNTPs	2 mM	0.2 mM	2.5
F-Primer	10 µM	1 µM	2.5
R-Primer	10 µM	1 µM	2.5
RedTaq polymerase	1U/µl	0.025 U/µl	0.625
DNA			5

The PCR profile was 2 min incubation at 95°C followed by 30 cycles of 15 sec at 95°C, 30 sec at 70°C and 1 min at 72°C, a final 10 min incubation at 72°C and infinity at 10°C.

Ten (10) µL of each PCR sample and the PCR negative control (sterile saline) were run on a 1.5% agarose gel in 1x TAE (Tris Acetate EDTA) buffer. A 100 bp DNA ladder was included. The gel was stained with ethidium bromide. The amplification products were analysed for a *L. maculans* or *L. biglobosa* specific product under UV-light. The PCR result of each tested sample was recorded next to the corresponding suspect *P. lingam* colony in the data record sheet.

Two qPCR assays were provided by P. Bonants from Plant Research International [PRI], Wageningen (http://www.wur.nl/) targeting *L. maculans* and *L. biglobosa*:

L. maculans	P.lin F	5' CGC GCA GGA AAC AGA TTT TT 3'
	P.lin R1	5' GAA GCT GGA ATT GAG TTA GCA TGT AC 3'
	P.lin Pr	5' FAM-CGT GCT TCT GCC GGC TCT AGC G-NFQ 3'
L. biglobosa	P.big F	5'-CGCATCGAAATGT**G**CATT-3' (Nucleotides in bold are locked nucleic acids (LNA))
	P.big R	5'-TCGCAGGCCACGTCAG-3'
	P.big Pr	5'-FAM-TAAC**T**C**T**GTT**C**CAGCTTCCATTG-NFQ-3' (Nucleotides in bold are LNAs)

The PCR was carried out in a 30 µL reaction mix (see table 4b) containing 1 µL of DNA extract.

Table 4b: Mix used with PRI primers

Compound	Concentration	Final concentration	Volume in 30 µL
Sterile Milli Q			10.4
Master mix *	2x	1x	15
Primer F	5 µM	0.25 µM	1.5
Primer R	5 µM	0.25 µM	1.5
Probe	5 µM	0.083 µM	0.6
DNA			1

*(PerfeCta® MultiPlex qPCR ToughMix, Quanta Biosciences)

Pathogenicity test

Only GEVES preformed this test. Suspect *P. lingam* colonies were transferred to a malt agar plate and isolates were incubated 10 days at 20°C in 24 h darkness until pycnidia formed. At the same time, a healthy susceptible cauliflower seed lot (variety Bill) was sown at a rate of one seed per pot in a substrate mix of soil and sand. Four pots (replicates) per suspect *P. lingam* isolate and four replicates each for the negative (inoculated with buffer only) and positive controls (inoculated with an isolate PAS 155 used in GEVES as a reference) were sown. All the plants were placed in controlled conditions at 23°C, 80 % relative humidity and 12 h light/12 h darkness until the cotyledon stage 7 days after sowing.

5 mL of buffer (water and 0.85% salt) was added to the malt agar plate and the mycelium with pycnidia was scraped with a glass slide at the surface of the agar to recover the pycnidia. The inocula containing pycnidia suspensions were placed in a BIOREBA plastic bag and ground with a pneumatic press to expulse pycnidiospores. Concentration of the inocula was calculated by a haemocytometer (Malassez cell) using a microscope and adjusted to 1 x10^5 spores per milliliter.

The cotyledons of each seedling were pierced with a needle. The inoculum was pipetted behind the filter in the BIOREBA plastic bag in order to eliminate any agar, mycelium and impurities and to keep the pycnidiospores suspension. The cotyledon, supported by a finger in a finger glove to facilitate penetration of the inoculum, was injected with the inoculum in the lesion area using a needleless syringe. The seedlings were placed in controlled conditions at 23°C, 80 % relative humidity and 12 h light/12 h darkness. The symptoms on seedlings were observed 5 and 10 days after inoculation. Symptom expression is characterized by black necrosis forming a macula on the cotyledon. After 8 to 10 days, pycnidia formation could be observed on the cotyledon with the naked eye.

Data analysis

All 10 participating laboratories submitted the results they had generated. For each (laboratory x protocol x contamination level x seed subsample) combination, the number of *P. lingam* suspect colonies was

recorded on the 7th day after plating for malt agar and 11th day for the blotters. Seed germination was recorded for the two blotters protocols.

The decision on whether a seed sample and the combinations (laboratory x protocol x contamination level x seed subsample) were *P. lingam* positive or negative was determined based on the malt agar and blotter protocol results. A seed subsample was considered *P. lingam* negative if there were no suspect *P. lingam* colonies observed. A seed subsample was considered *P. lingam* positive if at least one suspect *P. lingam* colony was identified with the naked eye or using a microscope.

The statistical analysis was performed on the final results (binary data; positive or negative) for the different protocols:

- For the malt agar method on the results of laboratories 1, 6, 7, 9 and 10. The other laboratories (2, 3, 4, 5, 8) did not use this protocol.
- For the blotter 2,4-D method on the results of laboratories 1-8. The laboratories 9 and 10 did not use this protocol.
- For the blotter deep freeze method on the results of laboratories 1-8 and 10. The laboratory 9 did not use this protocol.

For the homogeneity and stability tests the results were analyzed using Hampel's outlier test (Gregoire *et al.*, 2016). The method first determines the median result of the 10 laboratories. In a next step the absolute deviation (AD) of each result from the median results is calculated. Finally, the median absolute deviation (MAD) is determined and multiplied by 5.2 to obtain the maximum allowed absolute deviation. If all absolute deviations are smaller than the maximum allowed absolute deviation, the results are assumed to be homogeneous.

A qualitative analysis was performed on the expected and obtained positive and negative results. The ISO 16140 standard (AFNOR, 2012) was followed to evaluate the performance criteria - sensitivity, specificity and accuracy – of the three methods for the three infection levels. This evaluation was performed by comparing the expected results of all laboratories with those obtained. The results were in the form of positive and negative agreements and deviations. Accordance (repeatability of qualitative data) and concordance (reproducibility of qualitative data) of each method per infection level was evaluated using the method developed by Langton *et al.,* 2002.

The quantitative analysis of the percentage of infected seed in each seed lot (combined from all participants) was made using box plot to explore data. An ANOVA was used to compare the detection capacity of each method between laboratories.

The PCR and pathogenicity results on the different *Phoma* strains and saprophytes from the strain collection tested were analysed by qualitative analysis following the ISO 16140 standard mentioned above in order to determine specificity, sensitivity and accuracy of the PCR and pathogenicity tests.

Results and statistical analysis

Homogeneity and stability tests

The seed lots used to test for homogeneity and stability in the validation study were analyzed on 10 samples from each seed lot. Results are presented in figure 4.

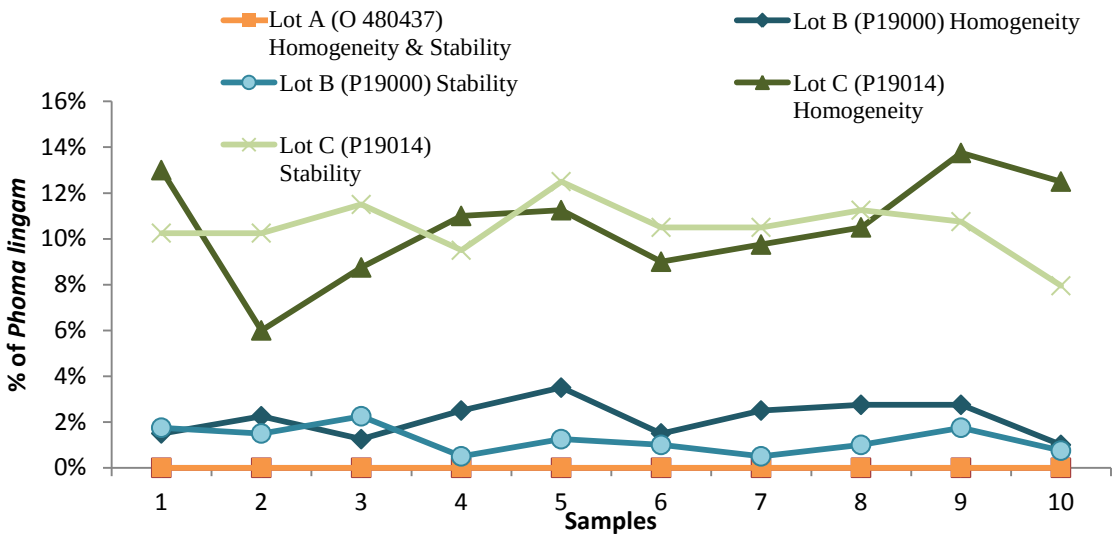


Fig. 4: Results of the homogeneity and stability tests on seed lots

Homogeneity and stability were confirmed for the three seed lots tested. The quantitative analysis (Hampel's method) showed that the seed lots had no outliers (see table 5).

Table 5: Quantitative analysis of results obtained after the homogeneity and stability test

	Seed lots	Median ($\tilde{x}$)	MAD	5.2 x MAD	Status of the x_i
Homogeneity test	A (healthy)	0.00	0.00	0.00	homogeneous
	B (low)	1.65	0.65	3.38	homogeneous
	C (medium)	10.75	1.75	9.1	homogeneous
Stability test	A (healthy)	0.00	0.00	0.00	Stable
	B (low)	1.25	0.50	2.60	Stable
	C (medium)	10.5	0.50	2.5	Stable

P. lingam distribution was homogeneous in two infected seed lots and the healthy seed lot was confirmed as being without the presence of *P. lingam* (table 6).

These results confirm the homogeneity of the infection level for the infected seed lots B and C and the healthy control A before the start (homogeneity test) and at the end (stability test) of the validation study. Moreover, the percentages of contamination obtained during homogeneity test and stability tests were comparable, showing that there were no changes in the infection level during the CT.

Table 6: Qualitative analysis of results obtained after the homogeneity and stability test

Seed lots	Positive samples in total	
	Homogeneity test	Stability test
A (healthy)	0	0
B (low)	10	10
C (medium)	10	10

CT results and analysis

Malt agar

All the five laboratories (laboratories 1, 6, 7 9 and 10) (see Appendix 2 for the results) recorded zero suspect *P. lingam* colony on the three samples from the healthy seed lot, and three positives samples from the low and medium infected seed lots. In total, 30 positives results were obtained on 30 expected results (5 laboratories x 2 infected seed lots x 3 samples = 30 expected results) and 15 negative results were obtained on the 15 expected results for all laboratories (5 laboratories x 1 healthy seed lots x 3 samples = 15 expected results).

The qualitative analysis was based on these results and gave a specificity, sensitivity and accuracy of 100% for the media protocol. Accordance (repeatability) and concordance (reproducibility) were calculated using Langton *et al*. (2002) to be 100% (table 7).

Table 7: Qualitative analysis of the malt agar results (%)

			ISO 16140			Langton *et al.,* (2002)	
	Results expected +	**Results expected -**	**Sensitivity**	**Specificity**	**Accuracy**	**Reproducibility**	**Repeatability**
Results obtained +	30	0	100	100	100	100	100
Results obtained -	0	15					

Blotter 2,4-D

Laboratory 1 recorded one suspect *P. lingam* colony in one subsample from the healthy seed lot (see Appendix 2). The others laboratories (2, 3, 4, 5, 6, 7 and 8) recorded zero suspect *P. lingam* colony on the three samples from healthy seed lot. All the laboratories recorded three positives samples from the low and medium infected seed lots. In total, 48 positives results were obtained on 48 expected results (8 laboratories x 2 infected seed lots x 3 samples = 48 expected results) and 23 negative results were obtained on 24 expected for all laboratories (8 laboratories x 1 healthy seed lots x 3 samples = 24 expected results). This means that 1 false positive result was obtained.

The qualitative analysis was based on these results and gave a specificity at 95.83 %, sensitivity at 100% and accuracy at 98.61% for the blotter 2,4-D protocol. Accordance (repeatability) was calculated to be 97.70% and concordance (reproducibility) was calculated to be 95.50% (table 8).

Blotter deep freeze

Laboratory 1 recorded suspect *P. lingam* colonies in the three samples from healthy seed lot whereas the laboratory 8 recorded one suspect *P. lingam* colony in one sample from the healthy seed lot (Appendix 2). The others laboratories (2, 3, 4, 5, 6, 7 and 10) recorded zero suspect *P. lingam* colony on the three samples from the healthy seed lot.

Table 8: Qualitative analysis of the blotter 2,4-D results (%)

			ISO 16140			Langton *et al.,* (2002)	
	Results expected +	**Results expected -**	**Sensitivity**	**Specificity**	**Accuracy**	**Reproducibility**	**Repeatability**
Results obtained +	48	0	100	95.83	98.61	95.5	97.7
Results obtained -	0	23					

All the laboratories recorded three positives samples from the low and medium infected seed lots. In total, 54 positives results were obtained on 54 expected results (9 laboratories x 2 infected seed lots x 3 samples = 54 expected results) and 23 negative results were obtained on 27 expected for all laboratories (9

laboratories x 1 healthy seed lots x 3 samples = 27 expected results). This means that 4 false positives results were obtained.

The qualitative analysis was based on these results and gave specificity at 85.19 %, sensitivity at 100% and accuracy at 95.06% for the blotter deep freeze method. Using the Langton method accordance (repeatability) was calculated to be 97.76% and concordance (reproducibility) was calculated to be 91.60% (table 9).

Table 9: Qualitative analysis of the Blotter deep freeze results (%)

			ISO 16140			Langton *et al.,* (2002)	
	Results expected +	**Results expected -**	**Sensitivity**	**Specificity**	**Accuracy**	**Reproducibility**	**Repeatability**
Results obtained +	54	4	100	85.19	95.06	91.60	97.76
Results obtained -	0	23					

Results of the quantitative analysis comparing the three protocols

Detection of Phoma lingam

The comparison of the detection of *P. lingam* by the three detection methods was done using ANOVA. All the samples from seed lots were analysed by Hampel's method and box plot to detect the outliers (Appendix 3). The data for each lab was combined for each seed lot and method. In case there were no expected results, the box plot identified the value as an outlier and notified it as a red point. The raw data for each participant are not included in this report.

For the healthy seed lot A, laboratory 1 recorded one positive subsample using the blotter 2,4-D method and three positives samples in blotter deep freeze method (Appendix 2). This result is the 0.03% observed in the box plot for blotter 2,4-D method and the 0.20% observed in blotter deep freeze method (Appendix 3). Laboratory 8 recorded one positive subsample in blotter deep freeze method corresponding to the 0.03% observed in the box plot.

For the low infection seed lot B, laboratory 9 reported samples with a lower level of infection compared to the expected value of the seed lot with 0.10% of *P. lingam* observed in malt agar method instead of 2.15% observed in the mean (Appendix 3). Laboratory 4 recorded a higher level of infection compared to the expected infection level of the seed lot (6.70% of *P. lingam* observed instead of 2.15% in the mean) in blotter 2,4-D method. Laboratory 5 recorded a higher level of infection compared to the expected infection level of the seed lot (7.80% instead of 2.15%) in both methods. These results are indicated by red point in the box plot of the seed lot B.

For the medium infection seed lot C, laboratory 9 recorded a lower level of infection compared to the expected infection level of the seed lot (0.30% of *P. lingam* observed in malt agar method instead of 10.55% observed in the mean in blotter deep freeze method. These results are also indicated as a red point in the box plot of the seed lot C.

All these values were considered as outliers and were not included in the ANOVA done using the Statistica software (STAT SOFT STATISTICA 12 ©DELL INC 1984-2015). After combining the results, the ANOVA showed no significant difference between the three methods of detection with a probability of 0.58 (Appendix 3).

The comparison of percentages of detection between the three methods (malt agar, Blotter + 2,4-D and Blotter deep freeze) shows that all three infected seed lots (healthy, low and medium) were detected at the expected infection level and that there are no significant differences between the three detection methods for the level of infection (Figure 5).

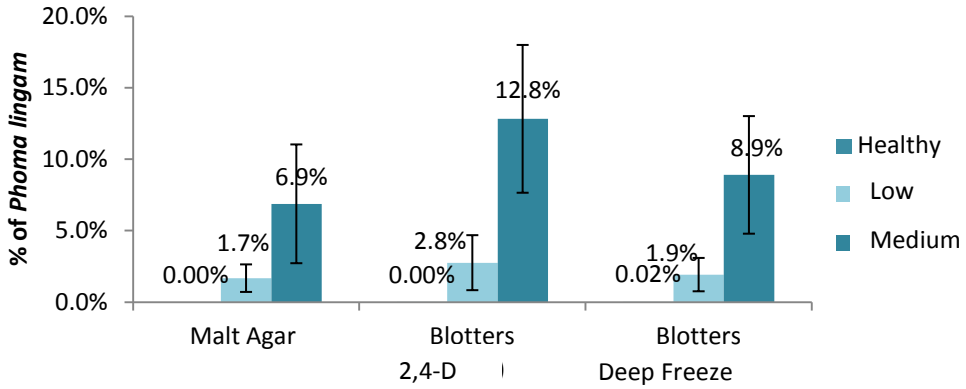


Fig. 5: Infection level (combined for all labs) of the seed lots detected with the three different methods

Inhibition of germination

A comparison of the percentage of germination of seeds in the blotter methods was done using an ANOVA. All the germination rates recorded by participants from all the samples tested (1 000 seeds evaluated per sample) were analyzed by Hampel's method in order to detect the outliers and BoxPlot. All the raw data for each lab are presented in Appendix 4 along with the outliers highlighted in yellow in the table. All these values considered as outliers were not included in the statistical analysis by ANOVA.

For the healthy seed lot (A), the results showed that laboratories 3 and 4 recorded outliers values for germination rates with respectively 98% and 2.5% instead of a mean of 29.5% for the 2,4-D method. Laboratories 2 and 3 recorded high germination rate with respectively 0.8% and 1.1 % instead of 0.0% mean for the deep freeze method.

For the low infected seed lot (B), laboratory 3 recorded a high germination rate (19.7% instead of 0.1% mean) for the 2,4-D protocol. Moreover, regarding the percentage of germination of the medium infected seed lot (C), laboratory 3 and 4 recorded outliers values with respectively 95.3% and 1.6% instead of 48.8% mean for the 2,4-D protocol while laboratories 2 and 3 recorded high germination rate (respectively 2.3% and 0.5 % instead of 0.0%) in deep freeze method.

After combining the results of all the participants without the outliers the healthy seed lot was shown to germinate at an average of 30.4% in 2,4-D and 0.03% in the blotter deep freeze while the low infected seed showed no difference between the two methods (0.1% germination) (see figure 6). The medium infected seed lot showed a 47.3% germination in 2,4-D protocol and 0% germination in the deep freeze protocol.

The ANOVA showed there was a significant difference in germination between the deep freeze and 2,4-D methods. The deep freeze method was more efficient in preventing germination of seeds during the test with a p value at 0.02 (Appendix 4). The box plot of the comparison of germination in the deep freeze and 2,4-D methods confirmed the results obtained in the ANOVA (Appendix 4).

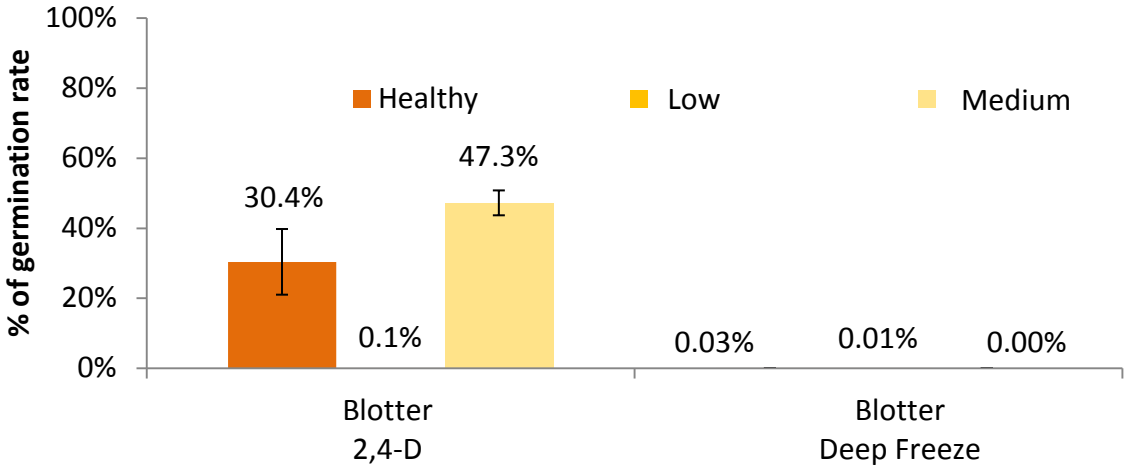


Fig 6: Comparison of germination in the deep freeze and 2,4-D methods (data combined for all participants)

Identification step

Identification by PCR of isolates in the collection

A collection of 36 fungal isolates were tested using the two PCRs. The Liu primers were able to identify the 11 *L. maculans* strains with the primers LmacR and LmacF and the 9 *L. biglobosa* strains with the primers LmacR and LbigF.

The PRI q-PCR was able to identify the 11 *L. maculans* strains. Concerning PRI primers on *L. biglobosa*, 3 strains were lost due to cross-contamination and 2 strains gave false negative results. Negative results were obtained on the 16 expected non-target strains.

A qualitative analysis of these results gave 100% of accuracy (100% sensitivity and specificity) for the Liu primers (*L. maculans* and *L. biglobosa*) and the PRI qPCR for *L. maculans* (table 10). The accuracy for the PRI qPCR for *L. biglobosa* was calculated at 94.44%.

For the *L. biglobosa* PCR with Liu primers the repeatability was not good and the tests had to be done several times on these strains to obtain a result. Depending of the repetition the same colony could not be amplified and could be attributed to lack of homogeneity in extracting DNA.

Identification of the isolates using the PCR

Four isolates from the CT (from the low and medium lots and the two blotter methods) were analysed by GEVES using the two PCRs. Both identified three strains as belonging to the *L. biglobosa* species. Only one strain of lot B gave a negative result for both *Leptosphaeria* species whereas the pathogenicity test gave a positive result (in table 10). These unexplained results need further tests to investigate the problem. For the other three strains (two of lot C and one of lot B), positive results for *L. biglobosa* obtained were confirmed by positive pathogenicity tests.

Table 10: Qualitative results obtained with Liu and PRI primers

			Strains (No.)	Accuracy (%)
Liu primers	*L. maculans*	Target	11	100
		Non Target	25	
	L. biglobosa	Target	9	100
		Non Target	27	
PRI primers	*L. maculans*	Target	11	100
		Non Target	25	
	L. biglobosa	Target	4	94.44

		Non Target	32	

Pathogenicity tests on isolates from the collection

Different inoculation methods showed different symptoms expression on plants. Inoculation by spraying did not give any symptoms. In the case of inoculation on leaf veins, macula and pycnidia were observed at D+14 (figure 7a) whereas inoculation on the stem lead to the development of a stem canker at D+14 (figure 7b). Aspersing the leaf, injecting the leaf vein and the stem at the 2 leaf stage and injecting the cotyledons according to Balesdent *et al*., (2006) showed an easier formation on the macula and pycnidia at D+5 (figure 7c). This method was selected for inoculation.

Figure 7. Symptom on plant after on (a.) leaf, (b.) stem, and (c.) cotyledon

All the collection (in table 11) was screened by pathogenicity tests on cotyledon stage plants. The results are also presented in table 11.

Table 11: Result of the pathogenicity test of the *Phoma* collection

n° entry	Anamorph	Genus	Species	original code	Pathogenicity
1559	*Phoma lingam*	*Phoma*	*lingam*	P19014	+
1558	*Phoma lingam*	*Phoma*	*lingam*	P19009	+
1674	*Phoma lingam*	*Phoma*	*lingam*	P19007	+
1673	*Phoma lingam*	*Phoma*	*lingam*	P19000	+
1737	*Phoma lingam*	*Phoma*	*lingam*	444	+
1738	*Phoma lingam*	*Phoma*	*lingam*	445	+
1739	*Phoma lingam*	*Phoma*	*lingam*	446	+
1740	*Phoma lingam*	*Phoma*	*lingam*	447	+
1741	*Phoma lingam*	*Phoma*	*lingam*	448	+
1742	*Phoma lingam*	*Phoma*	*lingam*	449	+
1904	*Phoma lingam*	*Leptospharia*	*maculans*	UK1	+
1905	*Phoma lingam*	*Leptospharia*	*biglobosa*	UK21	+
1906	*Phoma lingam*	*Leptospharia*	*maculans*	GER2	+
1907	*Phoma lingam*	*Leptospharia*	*biglobosa*	GER14	+
1908	*Phoma lingam*	*Leptospharia*	*biglobosa*	GER16	+
1909	*Phoma lingam*	*Leptospharia*	*biglobosa*	POL1	+
1910	*Phoma lingam*	*Leptospharia*	*maculans*	POL2	+
1911	*Phoma lingam*	*Leptospharia*	*maculans*	SWE1	+

1912	*Phoma lingam*	*Leptospharia*	*biglobosa*	SWE2	+
1913	*Phoma lingam*	*Leptospharia*	*maculans*	SWI1	+
1983	*Phoma exigua*	*Phoma*	*exigua*	482679	-
218	*Phoma valerianellae*	*Phoma*	*valerianellae*	C0635877	-
1675	*Alternaria sp.*	*Alternaria*	*sp*	P19012	-
	Stysanus sp.				-
	Epicoccum sp.				-
	Aspergillus sp.				-
	Alternaria brassicae				-
	Botrytis cinerea				-
	Fusarium oxysporum				-
	Cladosporium				-
	Mucor				-
	Rhizopus				-
	Trichoderma				-
	Sondaria sp				-
	Sclero				-
	M. brassisicola				-

It is important to note that for *L. biglobosa* colonies, the symptoms were more difficult to observe than the symptoms of *L. maculans*. This pathogen is less aggressive than *L. maculans* (Liu *et al*., 2006). The qualitative results are presented in table 12.

Table 12: Qualitative analysis of results obtained after the pathogenicity test

	Results expected - positive	Results expected - negative	Sensitivity	Specificity	Accuracy
Results expected - positive	20	0	100	100	100
Results expected - negative	0	16			

In total, on the 20 target isolates expected positives, 20 positives results were obtained and 16 negative results were obtained on 16 expected. Qualitative analysis was based on these results and gave 100% of specificity, sensitivity and accuracy.

Pathogenicity test for confirmation on isolates from the CT

During the comparative test, four isolates from the CT (two lots, two blotter methods) were tested. The pathogenicity test gave positive results with 4 positives isolates out of the 4 expected positives. These results confirm the qualitative analysis with 100% of specificity, sensitivity and accuracy.

Discussion and conclusions

Results show that level of infection in the healthy, medium and highly infected seed lots was constant during the comparative test. Nevertheless, some laboratories experienced some difficulty in identifying *P. lingam* colonies on malt agar compared to the blotter test (2,4-D and deep freeze) but it had no impact on their results. Some false positive results were also obtained for the blotter methods (2,4-D and deep freeze).

According to Champion (1997) some fungi can be confused with *P. lingam* based on their visual characteristics; mycelium shape and colours, growing brownish silver mycelium and pycnidial primordia of *P. lingam* on the seed and substrate and pycnidia formation with purple (amethyst) exudates (ISTA, 2014). Champion (1997) is also of the view that whatever the method chosen, it is easy to confuse *P. lingam* with several saprophytes. A saprophyte like *Stemphylium* sp. is easily confused with *P. lingam* on blotter as only conidia which look like *P. lingam* pycnidia are present. The identification using malt agar is easier due to presence of two fructifications stages (sexual with perithecia and asexual with conidia). This could explain the difficulties encountered by laboratories 1 and 8 with saprophytes using the blotter method and the variable infection levels observed by different laboratories resulting in some outliers.

There was no significant difference in detecting *P. lingam* using the malt agar, blotter + 2,4-D and blotter deep freeze methods for the low and medium infected lots. The qualitative analysis showed a good sensitivity, specificity, accuracy, repeatability and reproducibility of each method with each performance criteria higher than 95% and better performance in the malt agar method.

Earlier research work by PRI, NAKT and Bejo (Margreet Asma, pers comm.) concluded that the deep freeze step could replace 2,4-D in the blotter method. The results of this study support this assertion and show that the deep freeze step inhibits the germination of seeds even better than the use of 2,4-D in the blotter method. Therefore it can be concluded that deep freeze blotter method is a good replacement for the use of 2,4-D to detect *P. lingam*. However, because the CT showed false positive results with the two blotter methods, it is strongly recommended to add a confirmation step when suspect colonies of *P. lingam* are detected and/or when the *Leptosphaeria* species need to be confirmed.

A confirmation method is currently available to identify *P. lingam* isolates. Inoculation of seedlings at the cotyledon stage produced expression of brown necrosis with pycnidia formation. Distinction between *L. biglobosa* and *L. maculans* can be done on symptom expression (Toscano-Underwood *et al.,* 2001) but this method is time consuming and was less efficient than molecular confirmation.

Due to the lack of specificity of primers, repeatability issues on *L. biglobosa* strains with Liu primers and a PCR false negative isolate observed during the CT with PRI primers, a PCR result cannot be used as final confirmation test. Therefore, a work flow (Appendix 5) is proposed to confirm the presence of *Leptosphaeria* sp. If, in case of a suspect colony the PCR result is negative, a pathogenicity test on cotyledons must be carried out as a confirmatory step.

Combination of all these results show that the blotter deep freeze and malt agar methods detect *P. lingam* on cabbage seeds. The toxic herbicide 2,4-D can be replaced by a deep freeze step to inhibit seeds from germinating in the blotter method.

As the identification of the fungus is based on the way they grow on seeds and on the morphological characters of fruiting bodies, these methods can also be used on a variety of *Brassica* and related crops (Mathur and Kongsdal, 2003).

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Appendix 1 - Methods for the detection of *Phoma lingam* in *Brassica oleracea* seeds

Malt Agar:

1. Media: Pour 20 mL of malt agar with 50 ppm of Streptomycin in each Petri dish.
2. Plating: Aseptically place 10 seeds per plates.
3. Incubation: Incubate for 7 days at 20 °C with 24 h darkness.
4. Examination: After 7 days (D+7) examine at ×25 magnifications for loose growing silver brownish white mycelium of *Phoma lingam* (teleomorph, *L. maculans)* on substrate (Fig.1). Examination for pycnidia on infected seeds and on media. Pycnidia are relatively large, about 250 µm, with papilla, sometimes developed as a neck. The ubiquitous saprophyte *Phoma herbarum* Westend occurs also on *Brassica* seed, but has smaller pycnidia formed superficially on the seed coat, not papillate (Fig 2c.), with white yellow or pink but not purple (amethyst) exudate. Seeds on which typical mycelium with or without pycnidia of *P. lingam* have developed are recorded as infected. Examination can be prolonged until 11 days if detection of *P. lingam* cannot be concluded only by the presence of typical mycelium.

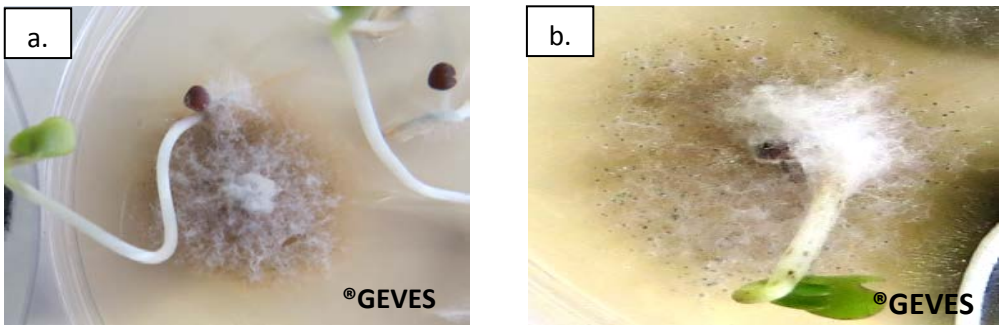


Fig 1: Aspect of *Phoma lingam* colony (a.) and pycnidia (b.) at D+7 on media

Blotter with 2,4-D:

1. Blotter: Place three pieces of filter paper (Whatman No. 1 or equivalent) in each Petri dish, in a fume cupboard wearing protective gloves, add 5 mL of a 0.2% solution of the sodium salt of 2,4 dichlorophenoxyacetic acid (2,4-D). Pour off the excess 2,4-D solution and place 25 seeds in each dish (up to 100 seeds if a germination box is used).
2. Incubation: Incubate for 14 days at 20 ºC (±2ºC) with alternating cycles of 12 h NUV light and 12 h darkness.
3. Examination: After 11 days, note the percentage of germination in order to compare with the deep freezing method. Then, examine at ×25 magnifications for loose growing silver white mycelium and pycnidial primordia of *P. lingam* on the seed and substrate. After 14 days, make a second examination for pycnidia on infected seeds and on the filter paper near infected seeds. Seeds from which pycnidia of *P. lingam* have developed are recorded as infected. Pycnidia are relatively large, about 250 µm, with papilla, sometimes developed as a neck (Figs. 2) and often have purple (amethyst) exudate. The ubiquitous saprophyte *P. herbarum* Westend occurred also on *Brassica* seed, but has smaller pycnidia formed superficially on the seed coat, not papillate, with white yellow or pink but not purple (amethyst) exudate.

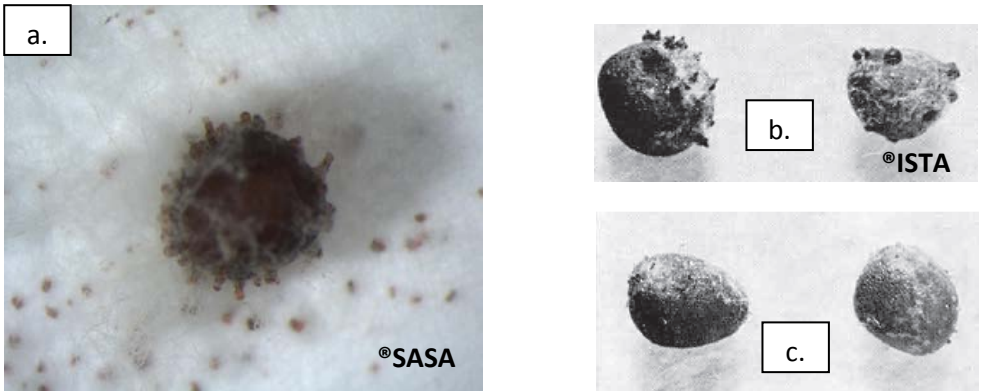


Fig 2: a) and b) Pycnidia of *Phoma lingam* on *Brassica oleracea* seeds at D+11 on blotter - amethyst exudate from pycnidia/ c) *Phoma herbarum* on *Brassica* seeds (ISTA, 2014)

Blotter with deep freeze:

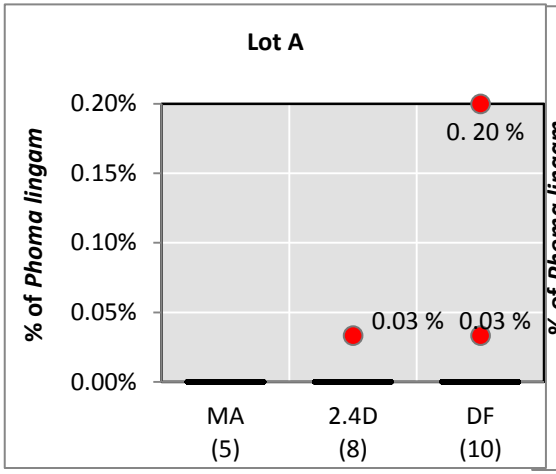
1. Blotter: Place three pieces of filter paper (Whatman No. 1 or equivalent) in each Petri dish with distilled water and place 25 seeds in each dish.
2. Incubation: Incubate for 24 h at 20 ± 2˚C in the dark. Carefully so not to disturb seeds, transfer dishes to freezer and maintain at -20˚C± 2˚C for 24 h.
3. After freezing, incubate for 14 days at 20˚C± 2˚C with alternating 12 h periods of darkness and NUV light.
4. Examination: After 11 days, note the percentage of germination in order to compare with the 2,4D method and examine pycnidia on infected seeds and on the filter paper near infected seeds in order to compare with protocol 2 and make equal comparisons. Make a second examination at 14 days in order to follow the protocol. Seeds from which pycnidia of *P. lingam* have developed are recorded as infected.

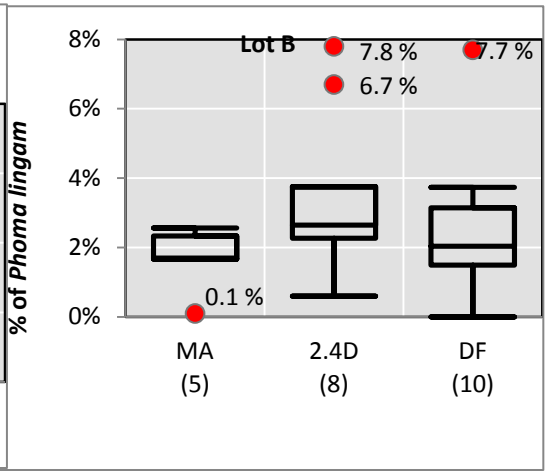
Appendix 2 - Results of the comparative test provided by the participating laboratories

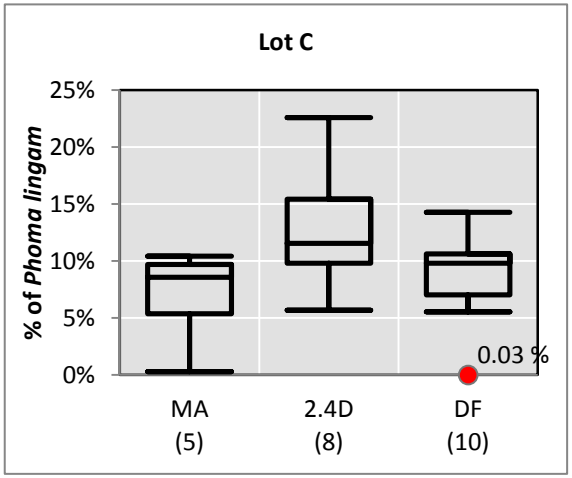
				No. of positive seed samples obtained									
				Laboratories									
	Seed Lot	Infection Level	No. of expected positive seed samples	1	2	3	4	5	6	7	8	9	10
Malt agar	A	Healthy	0	0					0	0		0	0
Malt agar	B	Medium	3	3					3	3		3	3
Malt agar	C	High	3	3					3	3		3	3
Blotter 2,4-D	A	Healthy	0	1	0	0	0	0	0	0	0		
Blotter 2,4-D	B	Low	3	3	3	3	3	3	3	3	3		
Blotter 2,4-D	C	Medium	3	3	3	3	3	3	3	3	3		
Blotter Deep Freeze	A	Healthy	0	3	0	0	0	0	0	0	1	0	0
Blotter Deep Freeze	B	Medium	3	3	3	3	3	3	3	3	3	3	3
Blotter Deep Freeze	C	High	3	3	3	3	3	3	3	3	3	3	3

Appendix 3 - Quantitative analysis of the contamination rate provided by participants analyzed by box plot

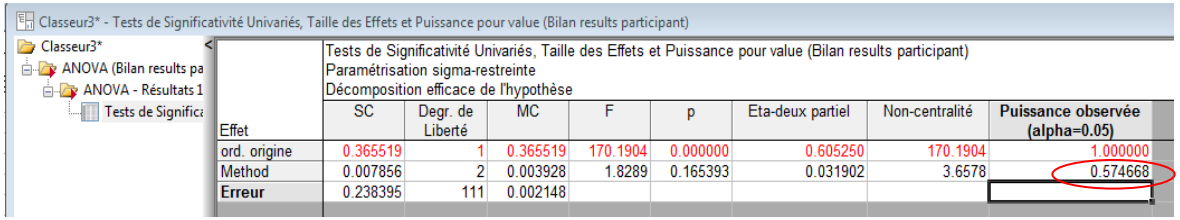
		% *Phoma lingam*		
Infection Level	Lab	Malt Agar	2,4-D	Deep Freeze
Seed lot A Healthy	1	0.00%	0.03%	0.20%
	2		0.00%	0.00%
	3		0.00%	0.00%
	4		0.00%	0.00%
	5		0.00%	0.00%
	6	0.00%	0.00%	0.00%
	7	0.00%	0.00%	0.00%
	8		0.00%	0.03%
	9	0.00%		0.00%
	10	0.00%		0.00%
Seed lot B Low	1	1.70%	0.60%	0.90%
	2		1.37%	2.03%
	3		2.57%	3.50%
	4		6.70%	3.73%
	5		7.80%	7.70%
	6	2.33%	2.77%	2.07%
	7	2.57%	2.67%	1.57%
	8		2.63%	2.03%
	9	0.10%		0.00%
	10	1.67%		1.47%
Seed lot C Medium	1	5.37%	5.67%	5.53%
	2		9.77%	9.40%
	3		15.00%	14.27%
	4		22.57%	10.23%
	5		16.63%	13.33%
	6	9.70%	9.83%	10.27%
	7	10.43%	11.63%	10.73%
	8		11.43%	8.73%
	9	0.30%		0.03%
	10	8.57%		6.47%







Percentage of infection from all participants combined and used to calculate ANOVA by STAT SOFT STATISTICA 12 ©DELL INC 1984-2015



Classeur3* - Tests de Significativité Univariés, Taille des Effets et Puissance pour value (Bilan results participant)

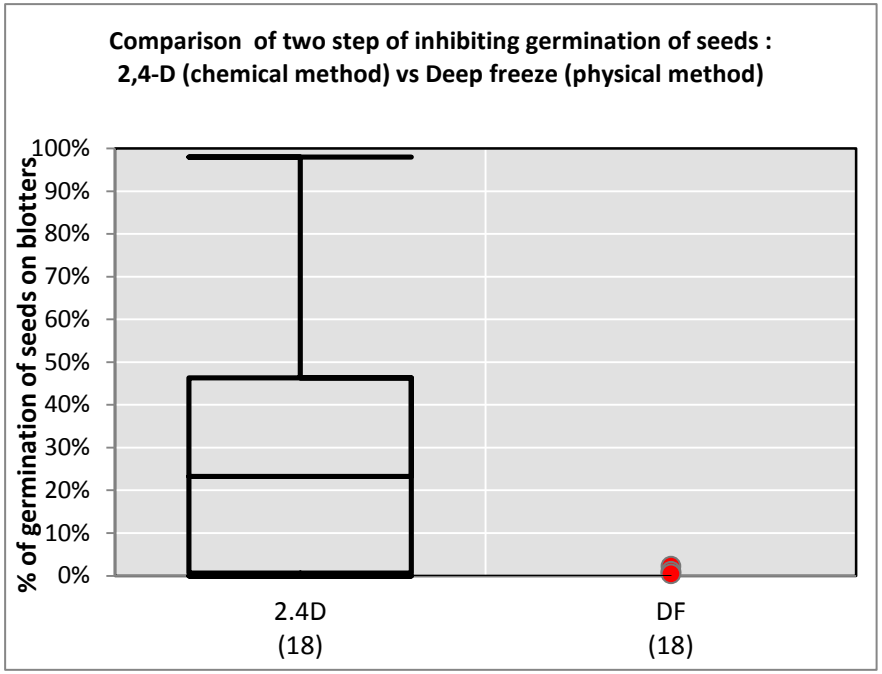
Classeur3*
ANOVA (Bilan results pa
ANOVA - Résultats 1
Tests de Significa

Tests de Significativité Univariés, Taille des Effets et Puissance pour value (Bilan results participant)
Paramétrisation sigma-restreinte
Décomposition efficace de l'hypothèse

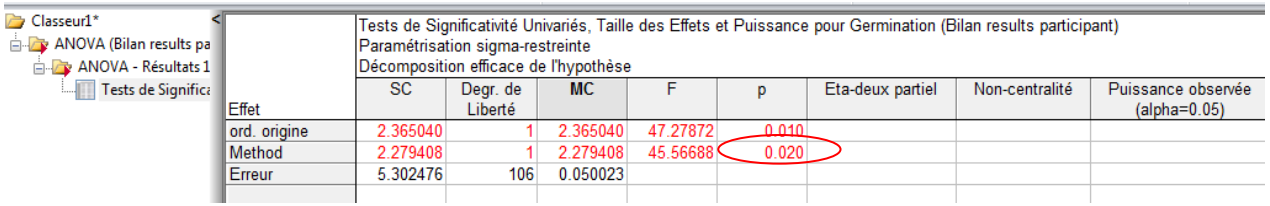
Effet	SC	Degr. de Liberté	MC	F	p	Eta-deux partiel	Non-centralité	Puissance observée (alpha=0.05)
ord. origine	0.365519	1	0.365519	170.1904	0.000000	0.605250	170.1904	1.000000
Method	0.007856	2	0.003928	1.8289	0.165393	0.031902	3.6578	0.574668
Erreur	0.238395	111	0.002148					

Appendix 4 - Comparison between two techniques to inhibit germination of seeds: 2,4-D and deep freezing

		Blotters 2,4-D		Blotters Deep Freeze	
		Germination rate	Standard deviation	Germination rate	Standard deviation
Seed Lot A (Healthy)	Lab 1	20.4%	12.0%	0.1%	0.10%
	Lab 2	42.2%	0.3%	0.8%	0.00%
	Lab 3	98.0%	36.9%	1.1%	0.00%
	Lab 4	2.5%	8.2%	0.0%	0.70%
	Lab 5	33.0%	0.2%	0.0%	0.06%
	Lab 7	26.0%	4.5%	0.0%	1.97%
Seed Lot B (Low)	Lab 1	0.2%	1.4%	0.0%	0.90%
	Lab 2	0.3%	1.3%	0.0%	0.00%
	Lab 3	19.7%	0.9%	0.0%	0.42%
	Lab 4	0.0%	4.3%	0.0%	0.00%
	Lab 5	0.0%	0.0%	0.0%	0.00%
	Lab 7	0.0%	2.7%	0.0%	0.00%
Seed Lot C (Medium)	Lab 1	47.6%	8.5%	0.0%	0.00%
	Lab 2	42.4%	0.0%	2.3%	0.00%
	Lab 3	95.3%	4.0%	0.5%	0.00%
	Lab 4	1.6%	5.6%	0.0%	0.06%
	Lab 5	48.0%	0.3%	0.0%	0.00%
	Lab 7	51.0%	5.5%	0.0%	0.10%



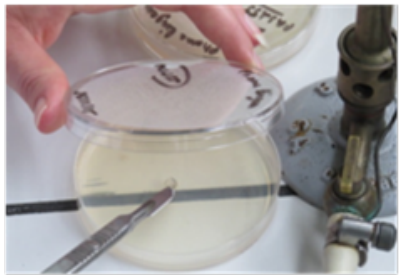
Germination data from all participants combined and used to calculate ANOVA by STAT SOFT STATISTICA 12 ©DELL INC 1984-2015



Tests de Significativité Univariés, Taille des Effets et Puissance pour Germination (Bilan results participant)
Paramétrisation sigma-restreinte
Décomposition efficace de l'hypothèse

Effet	SC	Degr. de Liberté	MC	F	p	Eta-deux partiel	Non-centralité	Puissance observée (alpha=0.05)
ord. origine	2.365040	1	2.365040	47.27872	0.010			
Method	2.279408	1	2.279408	45.56688	0.020			
Erreur	5.302476	106	0.050023					

Appendix 5 - Work flow to identify *L. maculans* and *L. bigobosa*



✓ Transfer suspect colony in MA and incubate at 20°C darkness

Validation study for filtration methods for the detection of *Ditylenchus dipsaci* and *D. gigas* on alfalfa and faba bean seeds to support C.7.2

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Abstract
The method for the detection of the seed-transmitted pathogens *Ditylenchus dipsaci* (Dd) and *Ditylenchus gigas* (Dg) on alfalafa and faba bean seed was validated in an international comparative test organized, between nine laboratories, by GEVES during the TESTA project. The method is a filtration protocol using different sieving steps in order to collect the nematodes present in a sample. After observation with a stereo microscope, *Ditylenchus* sp. was identified by morphological criteria under microscope (higher magnification) followed by a PCR confirmation to distinguish Dd and Dg. One healthy seed lot, and two (low and medium infestation) naturally contaminated seed lots of alfalfa and faba bean were tested. Each seed lot was tested in five blind subsamples of 100 g of alfalfa and 500 g of faba bean. The detection methods showed high values of accordance (repeatability), concordance (reproducibility), diagnostic sensitivity, diagnostic specificity and accuracy for all three infestation levels. Therefore, the filtration method using sievingsteps is considered reliable to detect Dd and Dg on alfalfa and faba bean seed lots and is highly recommended in routine seed health testing.

Introduction
Among the 60 species currently described in the genus *Ditylenchus*, *Ditylenchus dipsaci*, the stem and bulb nematode, is considered as the most important and severe plant pathogen worldwide. Responsible for many crops losses, it is present mainly in temperate zones and in the Mediterranean region (Volvas *et al.*, 2011). About 30 different biological races have been identified in many different hosts. Although they are morphologically similar they are host specific (Esquibet *et al*, 2003). A race, distinguished from *D. dipsaci*, owing to a greater body size was named “giant race” in the past (Sturhan and Brzeski, 1991; Kerkoud *et al.*, 2007) and was later described in 2011 by Volvas *et al*, as a new species named *D. gigas*. According to Kerkoud *et al*., presence of *D. dipsaci sensu stricto* was associated with alfalfa whereas Esquibet *et al* described presence of *D. dipsaci* and *D. gigas* (called giant race in the publication) in Faba bean. No studies have shown the presence of *D. gigas* in alfalfa. The absence of *D. dipsaci* in alfalfa seed lots is a phytosanitary requirement in Europe (import, export and sale of seed lots to farmers). One of the two options to fulfil this requirement is testing. Few studies have reported the difference in morphological characters between *D. dipsaci* and *D. gigas*. Two protocols used at GEVES and NIAB, based on filtration (MOA13 part A, 2013) and decantation (NIAB 013 STNEM beans v5) have been compared. For the decantation, the main limit was the presence of nematodes in the supernatant that can lead to a difference in the estimation of the infestation rate, particularly on samples with a low infestation level (Appendix 1, III.a).

Analytical sensitivity of both protocols was studied before this comparative test (according to the EPPO Standard PM 7/98,2014). Five repetitions of 3 samples artificially infested at very low infestation level with 10, 5 and 1 nematode per sample were tested. Results showed a detection threshold at 10 nematodes/sample for the filtration method and higher than 10 for the decantation method (Appendix 1, III.b). Based on the results of this comparison, the filtration method was chosen for validation. Confirmation was performed with a PCR method.

Molecular methods have been recently developed to confirm the *Ditylenchus* subsp. (Esquibet *et al*, 2003; Kerkoud *et al*, 2007; Volvas *et al,* 2011). Different PCR confirmations were compared

(Kerkoud; Esquibet; Wood and Clear Detection) before the comparative test (Appendix I, III.c-results of PCR) on the nematode collection at GEVES containing 38 Dd, 25 Dg and 19 non target nematodes. Based on the ISO Standard 16140 (AFNOR, 2012) the performance criteria of each method were evaluated. The Kerkoud method seemed best suited to the identification of the species *D. dipsaci* and *D. gigas* compared to Wood method. However, the repeatability defects observed for Kerkoud primers involved the implementation of a suitable number of repetitions compared to Wood primers. It was decided to focus the test on Kerkoud and Wood methods.

An inter-laboratory test was organized to validate the detection of *D. dispaci* and *D. gigas* by the filtration method and study performance criteria of the method.

Aim and objective of the comparative test

To validate the filtration method for detection of *D. dipsaci* and *D. gigas* and propose it for addition to the ISTA and EPPO protocols.

Laboratories from different countries (GEVES and ANSES from France, LfL and JKI from Germany, NIAB and SASA from United Kingdom, KIS from Slovenia, UKZUZ from Czech Republic) participated in this comparative test.

Materials and Methods

Seed lots and subsamples

Three untreated alfalfa (lots A B and C) and faba bean seed lots (lots D E and F) with variable levels of natural Dd and Dg infestation and saprophytes were selected by the GEVES-SNES Laboratory in France. The seed lots were characterized prior to the comparative test based on the number of positive samples contaminated by Dd and Dg. From each seed lot five subsamples of 100 g of alfalfa and 500g of faba bean seeds were tested. In the alfalfa lots an estimate of 0, 1-<50, 50-500 *Ditylenchus* sp. were observed respectively in the 3 lots whereas 0, 1-<50, 50-500 *Ditylenchus* sp. were observed in faba bean lots. Thus, infestation in the seed lots was considered as healthy (non-infested by *Ditylenchus* sp.), low and medium, respectively (lot A to C and D to F).

For the comparative test , each participating laboratory received five subsamples of 100 g of alfalfa and 500 g of faba bean subsamples from each of the three seed lots. These 30 subsamples were randomly coded to ensure a blind test . All seed subsamples were prepared by the sampling department of GEVES-SNES with the use of the rotary sample divider apparatus based on the thousand-seed weight of each seed lot.

Homogeneity was tested after sampling procedure using divider, on 10 subsamples of alfalfa and faba bean seed lots for the healthy, low and medium infestation-level.

A stability test was performed by organizer for faba bean seed lots by testing 10 subsamples of each lot after reception of results by participants. A stability test was not carried out on the alfalfa infested seed lots due to a very good knowledge of the infestation stability over time. Both these seed lots (low and medium infested) were previously used in Thermoluz project in 2012, 2013 and 2014, and in the pre-tests of TESTA project defining the performance criteria of the decantation and filtration method in 2014 and 2015. In all alfalfa subsamples tested, none of them became negative over time.

Detection method

Filtration protocol on alfalfa and faba seed lot

Seeds were immersed in water to facilitate nematodes migration from the host tissues according to the Baermann method. The extraction was performed in 1000 mL of water (Fig. 1) for each sample. The sample and water were placed on a sieve (250 µm) covered with soft filter paper, in 1000mL tap water and nematodes were allowed to migrate during 24 hours at least (Fig. 1- a). After migration, the sieve containing the seeds and the filter paper is removed and the water containing nematodes is filtered through a 20 µm mesh sieve (Fig. 1- b to d).

All materials collected on the 20 µm mesh sieve were transferred to a glass Petri dish. To ensure that all nematodes are recuperated, a minimum quantity of water is added to create a suspension (Fig. 1- e and f). Glass Petri dish were examined under binocular microscope and microscope.

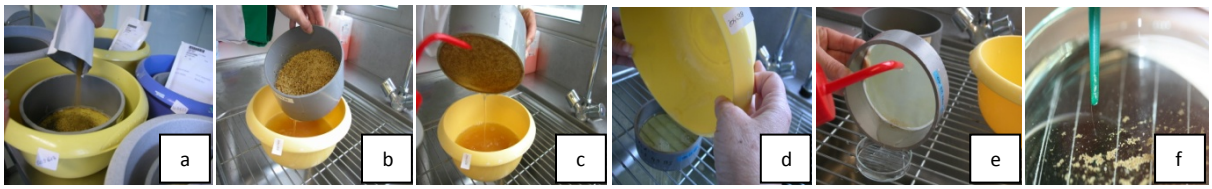

Fig. 1: Nematodes' extraction by filtration method.

The identification of *D. dipsaci* and *D. gigas* was based on morphological characters observed under a stereomicroscope at low magnification: aspect of the body (long, thin); swimming style of the nematode (undulating); shape of head (round to slightly flattened and not swollen compared to the body); shape of the tail (pointed conidial and short); head and tail slightly transparent and middle of the body is more dense.. Suspect nematodes are harvested and placed individually between slide and cover slides for identification of the *Ditylenchus* genus and Dd or Dg suspect species. Examination of the specific morphological characters was done at high magnification (x100) and nematodes needed to be immobile. To ensure immobility, nematodes are heated (to approximately 60°C for about 10 to 30 seconds) until they became immobile (about 10 to 30 seconds) and observed with a microscope. The following observations are made under a microscope : the size of the stylet (10-12µm); body size (1000-1300 µm for Dd and 1373-1950 µm for Dg); number of lateral lines (4 total) and the shape of tail (should be conical and pointed). Morphological characters are described in PM 7/87 (EPPO 2008) and Volvas *et al.* (2011) (Table 1).

Table 1: Morphological characters for *Ditylenchus dipsaci* and *D.gigas* identification on microsocope

	According to Bulletin OEPP bulletin 38, 2008		According to Volvas *et al*, 2011	
Characteristics	***Ditylenchus dipsaci* female**	***Ditylenchus dipsaci* male**	***Ditylenchus gigas* female**	***Ditylenchus gigas* male**
Body length (µm)	1000-1300µm	1000-1300µm	1780µm (1561-1932)	1557µm (1373-1716)
Stylet length (µm)	10-12µm	10-12µm	12µm (11.5-13)	11.6µm (11-12.5)
Body width	36-40µm	37-41µm	48.9µm (43.0-56.4)	56.7µm (34.3-63.0)
Number of lateral lines	4	4	4	4
Vulva position (%)	82% (79-82)	/	81.5% (80-83)	/
vulva-anus distance (µm)	1 3/4–2 1/4 tail length	/	228 (208-266)	/
Pharengial length	6.5-7.1µm	6.5-7.3µm	8.5µm (7.3-9.3)	8.3µm (6.7-10.7)
Tail length	14-18µm	11-15µm	20.µm (16.8-27.6)	17.9µm (15.7-20.0)

To confirm species identification, a PCR was used.

PCR confirmation
Individual nematodes were isolated and placed in a microtube for further extraction. DNA extraction was performed using a commercial kit (Macherey Nagel NucleoSpin Tissue, protocol “animal tissue”) according to the instructions provided.

WOOD protocol
Nematode is collected, and transferred for lysis and DNA extraction by Macherey Nagel kit. Tom Wood from the NIAB has developed species-specific primers for *D. gigas* Dg.F (5’-TGCGTTGAAGAGAATCGACAG-3’), Dg.R (5’-AAAAGCACCCGCTAGTTTTG-3’) and for *D. dipsaci* Dd.F (5’-GCGTTGAAGAGAACTGGCAC-3’) and Dd.R (5’-AAGCAC CCAACCAGTACCG-3’). Two PCR are run at the same time, one specific for *D. gigas* and the other specific for *D. dipsaci.* Ten (10) µL of each PCR-sample containing loading buffer were run on a 1.5% agarose gel in 1x TAE buffer. A 100 bp DNA ladder was included. The gel was stained with ethidium bromide. The amplification products were visualized under UV-light.

Each PCR gives a band at 330 bp for *D. gigas* and *D. dipsaci*. The amplicon size is similar and therefore the PCR are run as simplex. The mix (using Taq Platinium) and program for Wood protocol is described in table 2.

Table 2: PCR mix and program for Wood protocol

Mix PCR	Unit	Initial concentration	final concentration	Volume 1 tube
H2O				12.2
Buffer 10X		10	1	2
MgCl2	mM	50	1.5	0.6
dNTP	mM	2	0.2	2
F-primer	µM	5	0.25	1
R-primer	µM	5	0.25	1
Platinum Taq	U/µL	5	0.05	0.2
DNA	µL	5		1
Total volume	µL			20

Program		
96°C	5 min	
96°C	15sec	
60°C	30sec	**30** cycles
72°C	30sec	
72°C	7 min	
10°C		

KERKOUD protocol

Kerkoud method is based on two forward primers: (DdpS1 and DdpS2); one reverse (rDNA2 5'-TTTCACTCGCCGTTACTAAGG-3'). DdpS1 (5'-TGGCTGCGTTGAAGAGAACT-3') is specific to *D. dipsaci*, while DdpS2 (5'-CGATCAACCAAAACACTAGGAATT-3') anneal to both *D. dipsaci* and *D. gigas*. Ten (10) µL of each PCR-sample containing loading buffer were run on a 1.5% agarose gel in 1x TAE buffer. A 100bp DNA ladder was included.. The gel was stained with ethidium bromide. The amplification products were visualized under UV-light.

An electrophoresis profile showing both a 517 bp (DdpS2 / rDNA2) and a 707 bp band (DdpS2 / rDNA2) identifies *D. dipsaci*. If only the 707 bp band is observed, the tested individual is identified as *D. gigas.* The mix (using Taq Platinum) and program for Kerkoud PCR is described in table 3:

Table 3: PCR mix and program for Kerkood protocol

Mix PCR	Unit	Initial concentration	final concentration	Volume 1 tube
H2O				6.20
Buffer 10x		10	1	2
MgCl2	mM	50	1.5	0.6
dNTP	mM	2	0.2	2
DdpS1	µM	10	0.5	1
DdpS2	µM	10	1	2
rDNA2	µM	10	1	2
Platinum Taq	U/µL	5	0.05	0.2
DNA	µL			4
Total volume	µL			20

Program		
94°C	1 min	
94°C	30 sec	
55°C	30 sec	**40** cycles
72°C	45 sec	
72°C	5 min	
10°C		

Data analysis

Homogeneity of the seed lots used was analyzed by qualitative analysis (positives or negatives samples obtained out of total samples tested). In case of heterogeneous infection of the seed lot, the seedcalc8 software was used to determine the percentage of infested seeds per sample and the Prob pos sample software was used to determine the number of positive samples expected by participant.

All 9 participating laboratories submitted the results they had generated. For each (laboratory x seed species x infestation level x seed subsample) combination, the number of Dd and Dg suspect were recorded after 24h of migration. Number of suspect Dd and Dg and PCR positives nematodes identified by Kerkoud and Wood primers on the total tested were additionally recorded.

The decision on whether a seed sample and the combinations (laboratory x protocol x infestation level x seed subsample) were Dd or Dg positive or negative was determined based on morphological identification. A seed subsample was considered Dd or Dg negative if there were no suspect Dd or Dg observed. A seed subsample was considered Dd or Dg positive if at least one suspect Dd or Dg was observed and identified by morphological characters under the microscope.

The statistical analysis was performed on the final results (binary data; positive or negative) obtained by all participants. Laboratory 9 was not able to complete the comparative test on time and requested not to participate in October.

The ISO Standard 16140 (AFNOR, 2012) was followed to evaluate the performance criteria – diagnostic sensitivity, diagnostic specificity and accuracy - for protocols and infestation levels. This evaluation was performed by comparing the expected results with those obtained. The results were in the form of positive and negative agreements and deviations.

Table 4: Performance assessment criteria on diagnostic sensitivity, diagnostic specificity and accuracy calculated

	Expected result + (contaminated sample)	Expected result – (healthy sample)
Obtained result +	positive agreement +/+ (PA)	positive deviation -/+ (PD)
Obtained result -	negative deviation +/- (ND)	negative agreement -/- (NA)

- ⇨ Diagnostic sensitivity: Percentage of samples correctly identified as positives. ΣPA/(ΣPA+ΣND)x100.
- ⇨ Diagnostic specificity: Percentage of samples correctly identified as being negative. ΣNA/(ΣNA+ΣPD)× 100.
- ⇨ Accuracy: (ΣNA+ΣPA)/ (ΣPA+ΣNA+ΣPD+ΣND) x100.

PA = positive agreement / ND = negative deviation / NA = negative agreement / PD = positive deviation / N = total number of possible agreement

Conformity of results:

Performance criteria	Level to obtain
Diagnostic sensitivity	Results conform if sensitivity diagnostic is 100% : all contaminated samples are positive which means no false negative result has been obtained
Diagnostic specificity	Results are conform if specificity diagnostic is 100% : all healthy samples are negative which means no false positive result has been obtained
Accuracy	Synthesis of the two performance criteria. So no false positive or negative results have been obtained

The method of Langton et al. (2002) was used to evaluate the accordance (repeatability of qualitative data) and concordance (reproducibility of qualitative data) of each method per infection level.

Results

Homogeneity test

The healthy seed lot of alfalfa (Lot A) and faba bean (Lot D) were confirmed as healthy with no *D. dipsaci* and *D. gigas* detected in 10 subsamples. The two alfalfa seed lots (Lot B and C) were homogeneous with 10 samples positives out of 10 tested. The two faba bean infested seed lots (Lot E and F) were not homogeneous with 8 samples positive out of 10 tested for Lot E (low level) and 9 samples positives out of 10 tested for Lot F (medium level).

Table 5: Qualitative analysis of results obtained after the homogeneity test and stability test

	Homogeneity test	Stability test
Seed lots	No. positive samples/total	No. positive samples/total
Seed lot A	0/10	0/10
Seed lot B	10/10	Already tested
Seed lot C	10/10	Already tested
Seed lot D	0/10	0/10
Seed lot E	8/10	9/10
Seed lot F	9/10	10/10

As it has been difficult to obtain infested faba bean samples and no other ones were available, it was decided to use these seed lots for the comparative test , taking their heterogeneity into account for the statistical analysis. Based on homogeneity test results, the % of infestation of the low and medium seed lots was calculated with Seedcalc8 software. The upper bound of true percentage of impurity (95% confidence) is 0.37% for the lot E and 0.56% for the lot F. The computations of the probability of observing a given number of positive samples are based on a seed number per pool: evaluated to 880 seeds for the lot E and 947 seeds for the lot F. With a thousand seed weight around 520 g per seed lot, the probability tool of ISTA (Prob pos sample software) allowed to calculate the number of expected positive subsamples out of the 5 tested at a 95% confidence. The expected number of positive subsamples was estimated to be between 2 to 5 for low infested seed lot and between 2 to 5 for medium infested seed lot, when 5 subsamples were tested (Appendix II). Moreover, the percentages of infestation obtained during homogeneity test and stability tests were comparable, showing that there were no modifications of the infestation level during the comparative test.

Alfalfa

All the laboratories recorded zero suspect Dd out of the five subsamples from healthy seed lot and five positives subsamples out of five tested from the medium infested seed lot. Laboratory 8 recorded one negative subsample out of the five tested from low infested seed lot whereas the others laboratories (1 to 7) recorded five positives subsamples out of 5 from the low infested seed lot. In total, 79 positive results were obtained out of 80 expected positive results (8 laboratories x 2 infested seed lots x 5 subsamples = 80 results) and 40 negative results were obtained out of 40 expected negative results for all laboratories (8 laboratories x 1 healthy seed lots x 5 subsamples = 40 expected results).

Table 6: Qualitative analysis of results obtained by participants on alfalfa

	expected result + (contaminated sample)	expected result - (healthy sample)	Diagnostic sensitivity	Diagnostic specificity	Accuracy
Obtained result +	79	0	98.8	100.0	99.2
Obtained result -	1	40			

Qualitative analysis of the expected filtration protocol was based on these results and gave 100% of diagnostic specificity, 98.8% of diagnostic sensitivity and 99.2% of accuracy. According to Langton, accordance (repeatability) and concordance (reproducibility) were at 98% respectively.

Faba bean

A remark was made by participants for the Faba bean seed sample size analyzed by sieving method. During the comparative test, 500 g of seed per subsample was tested according to NIAB protocol sample size (NIAB 013 STNEM beans v5). But the capacity of imbibitions of the seed caused problems in the sieve. The containers (sieve and plastic basin) can contain usually a maximum of 1L of water. This volume is enough for alfalfa sample to completely cover seed during soaking. But in case of 500g of faba bean seeds analyzed, the seeds were not completely covered with 1L of water. In this case, the step of migration of nematodes was not suitable. Samples had to be divided.

Laboratory 7 recorded one suspect Dd out of five subsamples from healthy seed lot whereas the others laboratories recorded zero positive subsample out of the five subsamples tested from healthy seed lot. In total, 39 negative results were obtained on 40 expected for all laboratories (8 laboratories x 1 healthy seed lots x 5 subsamples = 40 expected results).

Table 7: Qualitative analysis of results obtained by participants on faba bean

	Expected results (healthy sample)	Specificity
Obtained result +	1	97.5
Obtained result -	39	

Qualitative analysis of the filtration protocol was based on these results and gave 97.5% of specificity.

Laboratory 3 and 8 recorded one negative subsample out of the five tested from the low infection level. The other six laboratories recorded five positives subsamples out of five tested from the low infested seed lot. Laboratory 6 recorded one negative subsample out of the five tested from medium infested seed lot whereas the others laboratories recorded five positives subsamples from the medium infested seed lot. With 2 positive subsamples expected, all laboratories obtained the expected results: at least 2/5 positives samples obtained. Based on these results, the diagnostic sensitivity was at 100% and accuracy was at 97.9%.

Detection of Ditylenchus spp. by filtration method

Criteria of performance of the filtration method were:

- Diagnostic specificity: 98.8%.
- Diagnostic sensitivity: 98.8 % (alfalfa).
- Accuracy: 99.2 % (alfalfa).
- Accordance (repeatability): 98% (alfalfa).
- Concordance (reproducibility): 99%

PCR confirmation

Kerkoud and Wood methods were tested in the comparative test. Due to a lack of equipment, or a lack of experience, or a lack of time, only 5 different laboratories were involved out of the 8 participants (laboratory 1, 2, 3, 7 and 8). After identification of nematode species by morphological criteria, each analyzed 20 individuals (belonging to *Ditylenchus* sp.).

Unfortunately, laboratory 7 had difficulties with Kerkoud primers because they did not distinguish *D. dipsaci* from *D. gigas* due to the appearance of multiple non-specific bands. Results were given as *Ditylenchus* sp. After discussion, the participant analyzed 3 nematodes pooled instead of analyzed 3 replicates of individual nematode. This confusion was due to a lack of understanding and an improper explanation of the organizer during emails exchange. But for the Wood primers, the results were correct and as expected. The Kerkoud results are considered as undetermined and not included in the comparison between Wood and Kerkoud primers. Laboratory 3 found alfalfa samples positive as Dd and Dg with Wood primers. After discussion, the participant analyzed nematodes pooled instead of analyzed replicates of individual nematode. . Nevertheless, due to no presence of Dg in alfalfa, the Dg results were considered as a false positive. Only the result of Dd was analyzed.

Kerkoud method

All the laboratories recorded 10 positives Dd out of 10 subsamples tested from the two infested alfalfa seed lots.

Concerning the Dg from the faba bean infested seed lots, laboratory 1 recorded one negative Dg out of 10 subsamples whereas the others laboratories recorded 10 Dg out of the 10 subsamples tested.

In total, 79 positives results were obtained out of 80 expected positive results (4 laboratories x 2 infested seed lots x 10 subsamples = 80 expected results).

Wood method

Laboratories 1 and 8 had difficulties with the Wood primers and found 5 positives Dd out of 10 tested whereas Laboratories 2, 3 and 7 confirmed 10 Dd out of 10 tested.

Concerning the Dg tested from the faba bean infested seed lot, laboratories 1 recorded one negative Dg out of 10 subsamples. This negative result was also obtained with the Kerkoud primers. Morphological identification clearly showed a *Ditylenchus* spp., so in the absence of internal amplification control, we can suspect an extraction problem. Laboratory 8 detected 7 Dg out of 10 tested whereas the other laboratories (2, 3 and 7) recorded 10 Dg out of the 10 tested.

In total, 86 positives results were obtained on 98 expected results.

Qualitative analysis of the expected confirmation protocol was based on these results and performance criteria of each method were summarized in Table 8.

Table 8: PCR results of the comparative test

		Sensitivity	Accuracy	Reproducibility	Repeatability
Kerkoud	*D. dipsaci*	100	100	100	100
	D. gigas	96.7	96.7	93.3	93.3
	D. dipsaci & D. gigas	98.3	98.3	97	97
Wood	*D. dipsaci*	81.6	81.6	65.0	77.8
	D. gigas	93.8	93.8	84.6	86.7
	D. dipsaci & D. gigas	87.8	87.8	74.4	81.2

When analyzing *D. dipsaci*, the Kerkoud method provided extremely reliable results with 100% accuracy, reproducibility and repeatability. These results are slightly lower when analyzing *D. gigas*. The overall results remain high with 98 % accuracy, 97% reproducibility and repeatability.

Considering the method proposed by Wood, the calculated accuracy on all the tested individuals is of 87.8%. The evaluated criteria of reproducibility and repeatability are also lower with respectively 74.4% and 81.2%.

As the results obtained with the Wood protocol did not match to morphological identification, laboratory 1 decided to add more DNA in the PCR (4µL instead of 1µL). With a higher DNA quantity, better results were obtained with 8 samples positive as *D.gigas* instead of 7, and 10 Dd instead of 5. One sample gave a false negative answer with both PCR. A universal nematode PCR was ran in parallel as a control and no DNA was amplified. Probably the nematode remained stuck to the eppendorf tube or something went wrong in the DNA extraction.

Discussion and conclusions

Combination of the results on 30 samples tested in eight participating laboratories showed a very good ability of the filtration method to detect Dd and Dg in alfalfa and faba bean infested seed lot with a very good sensitivity diagnostic and specificity diagnostic, accuracy, concordance and accordance on alfalfa and faba bean seed lots with each performance criteria higher than 95%.

According to the EPPO Standard PM 7/98 (EPPO 2014), the analytical specificity of the filtration method is not applicable and was therefore not studied. Extraction of the target organism from a sample is per definition non-specific due to the capacity to collect all nematodes presents.

The sample size tested for alfalfa was convenient for routine testing while the one for faba bean was not. The sampling of the seed lot was studied in WP2 of the TESTA project and a project named DITYLUZ in French will study the sample size during the next years. Regarding the lack of information, the sample size proposed in the method will refer to MOA 13 part A.

PCR confirmation was very important to distinguish Dd and Dg. The lack of morphological difference resulted in difficulties to identify both species. According to Volvas *et al.*, the body size was the only morphological criteria (1000-1300 µm for Dd and 1379-1950 µm for Dg) able to distinguish Dd and Dg at the L4 stage. But if a juvenile stage L3 of Dg is observed, the body size is similar to the Dd at the L4 stage. In this case, identification between both *Ditylenchus* sp. can be very problematic. The easiest way is still the PCR confirmation.

Among the different tested molecular detection protocols, the method published by Kerkoud provided the best results during in-house evaluation and the test performance studyinvolving four different laboratories showing >95% accuracy. This protocol is recommended for the identification of nematodes belonging to *D. dipsaci* and *D. gigas* species. However, an important limitation of this protocol has to be highlighted. Since one band is expected for both species and the second one for *D. dipsaci*, this method is only suitable to identify isolated individuals for example for confirmation of suspect nematodes after identification of the *Ditylenchus* genus by morphology in the filtration method. The Wood primers with <95% accuracy are less recommended for *Ditylenchus* identification.

Combination of all these results proved that a detection method using filtration (sieve at 20µm) in order to concentrate population of nematodes present in alfalfa and faba bean seed lot was validated. The morphobiometric and PCR identification were correlated showing that identification of the Dd and Dg can be done by either methods. So a work flow (Appendix 5) can be proposed to identify the genus *Ditylenchus* sp. by morphological criteria (shape of head and tail, number of line, presence of stylet) without morphobiometric measurement (size of body and stylet, number of lines) and use PCR to confirm the results and identify the species (Dd and Dg) or use morphobiometry.

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Appendix 1: TESTA WP5: Validate detection methods of *Ditylenchus dipsaci* and *D.gigas* on seeds by filtration/decantation (GEVES report Nov 2012-March 2014)

Aim: To harmonize and validate a European detection method of *Ditylenchus dipsaci* and *D. gigas*.

Objectives: To compare performance of the biological and molecular protocols currently used in Europe in order to validate a method that enable the detection of the two pathogens *Ditylenchus dipsaci* and *D.gigas* and propose it as an official ISTA and EPPO protocol.

Partners:
- GEVES (Valérie GRIMAULT, Geoffrey ORGEUR, Mathieu ROLLAND and Céline ANDRO)
- NIAB (Jane THOMAS and Tom WOOD)

I. Introduction

Among 60 species presently in the genus *Ditylenchus,* the stem and bulb nematode, *Ditylenchus dipsaci*, is the most important and severe plant pathogen worldwide. Responsible of many crops losses, it is present mainly in temperate zones, including the Mediterranean region (Volvas *et al*, 2011). According to their host, about 30 biological races of *D. dipsaci* are described and represent a species complex with morphological similarity (Esquibet *et al,* 2003). A race, distinguished from *D. dipsaci*, owing to a greater body size was named “giant races” in the past (Sturhan and Brzeski, 1991; Kerkoud *et al, 2007*) and then according to Volvas *et al*, (2011), described as a new species named *D. gigas.* The detection and identification of *D. dipsaci* and *D. gigas* in seed lots is an obligatory part of the sanitary control and regulation in Europe (the import, export and sale of seed lots to farmers) on alfalfa seeds. Few studies have reported the difference on morphological characters between the two pathogens. However molecular methods have been recently developed to confirm the *Ditylenchus* subsp. (Esquibet *et al,* 2003; Kerkoud *et al,* 2007; Volvas *et al,* 2011). Two protocols have been collected (from GEVES and NIAB) and compared in order to choose the best ones to be validated. An inter-laboratory test will be organized to detect *Ditylenchus dispaci* and *D. gigas*. It will enable identification based on morphological characters as well as the quantification of nematodes which is needed to provide infestation rates of the seeds. Confirmation will be allowed through a robust PCR method.

II. Material and Methods:

a. Availability of infested seed lots

An exchange of seed samples (Alfalfa and Faba bean) with different levels of infestation (low, medium and high) was organized between NIAB and GEVES laboratories.

Three Faba bean seed lots (4, 5 and 6) infested by *Ditylenchus gigas* have been provided by NIAB and GEVES sent three Alfalfa seed lots (1, 3 and 2) infested by *Ditylenchus dipsaci* to NIAB for analysis. Each seed lot was sampled in five samples (Table 1).

Table 1: Total of Faba bean and Alfalfa seed lots samples tested by NIAB and GEVES protocol's

Host	Sample	Variety
Alfalfa	Sample 1	1 1-0-1
	Sample 2	1 1-0-2
	Sample 3	1 1-0-3
	Sample 4	1 1-0-4
	Sample 5	1 1-0-5
	Sample 6	3 2-0-1
	Sample 7	3 2-0-2
	Sample 8	3 2-0-3
	Sample 9	3 2-0-4
	Sample 10	3 2-0-5
	Sample 11	2 3-0-1
	Sample 12	2 3-0-2
	Sample 13	2 3-0-3
	Sample 14	2 3-0-4
	Sample 15	2 3-0-5
Faba Bean	Sample 16	4 1-0-1
	Sample 17	4 1-0-2
	Sample 18	4 1-0-3
	Sample 19	4 1-0-4
	Sample 20	4 1-0-5
	Sample 21	5 2-0-1
	Sample 22	5 2-0-2
	Sample 23	5 2-0-3
	Sample 24	5 2-0-4
	Sample 25	5 2-0-5
	Sample 26	6 3-0-1
	Sample 27	6 3-0-2
	Sample 28	6 3-0-3
	Sample 29	6 3-0-4
	Sample 30	6 3-0-5

b. Detection and identification by morphological characteristics

Two protocols (Annex 1 and 2), filtration (used in GEVES) and decantation (used in NIAB), are currently used to detect nematodes after soaking of seeds (to enable extraction and migration of nematode). The filtration protocol uses a sieve to collect nematodes whereas the other protocol uses decantation to recover nematodes in the pellet (Table 2). Both are compared in the first pre-test.

Table 2: Comparison of protocols availables for detection of *Ditylenchus* sp.

	Decantation method	Sieving method
Name method	NIAB : 013 STNEM beans v5	ANSES : MOA13 part A
Quantity of seeds Faba bean	500g	200g
Quantity of Aalfalfa seeds	Not tested	70g
Seeds in a sieve	No, using decantation	Yes (250µm)
Mousseline	Yes dirty samples	No using a paper
Soaking	Overnight (minimum of 17 hours)	Overnight (minimum of 24 hours)
Sieving	No	20µm sieve, nematodes on the sieve, wash sieve : nematodes concentrated
Examination	leave 4h water standing, pour off the top liquid, keep 100ml in Petri dish, x25	on the remaining liquid in Petri dish x60
Identification	Morphological. In case of doubt →PCR on a pool of nematodes to confirm *D.gigas*. Sequences from Tom wood	Morphological. Not in MOA13 part 1: In case of doubt →PCR on individual nematodes to confirm *Ditylenchus* sp. but no difference D.*dipsaci, D.gigas*. Sequences from Kerkoud *et al,* 2007
Counting	semi quantitative: light (1-15), medium (15-50), heavy (50-500), very heavy infestation (>500), but do not like using	No

The identification of *D. dipsaci* and *D. gigas* was carried out on morphological characters (Table 3). Nematodes need to be immobile. Different criteria can be observed to distinguish both nematodes. Examination is done with a microscope and has to focus on stylet, body size and a conical pointed tail. Morphological characters of *D. dipsaci* are described in EPPO bulletin 38 in 2008 when Volvas *et al*, in 2011 listed morphological characters to identify *D. gigas.* For precise identification, examination of the specific morphological characters at high magnification is necessary.

Table 3: Morphological characters for *Ditylenchus dipsaci* and *D.gigas* identification

	According to Bulletin OEPP bulletin 38, 2008		According to Volvas *et al*, 2011	
Characteristics	***Ditylenchus dipsaci* female**	***Ditylenchus dipsaci* male**	***Ditylenchus gigas* female**	***Ditylenchus gigas* male**
Body length (mm)	1000-1300µm	1000-1300µm	1780µm (1561-1932)	1557µm (1373-1716)
Stylet length (µm)	10-12µm	10-12µm	12µm (11.5-13)	11.6µm (11-12.5)
Body width	36-40µm	37-41µm	48.9µm (43.0-56.4)	56.7µm (34.3-63.0)
Number of lateral lines	4	4	4	4
Vulva position (%)	82% (79-82)	/	81.5% (80-83)	/
vulva-anus distance	1 3/4–2 1/4 tail length	/	228 (208-266)	/
Pharengial length	6.5-7.1µm	6.5-7.3µm	8.5µm (7.3-9.3)	8.3µm (6.7-10.7)
Tail length	14-18µm	11-15µm	20.µm (16.8-27.6)	17.9µm (15.7-20.0)

The giant race shares the same morphological criteria but the body size (length and width) is bigger.

c. Threshold detection of the two methods

After the first pre-tests, the threshold detection of the two methods (filtration and decantation) was tested. In order to evaluate both methods, an artificial infestation of the seed samples by a precise number of *Ditylenchus dipsaci* was done.

Three infection levels at 1, 5 and 10 *Ditylenchus dipsaci* were prepared. For each laboratory and each method tested, five samples were infested. A healthy seed lot of Alfalfa was found by GEVES and sampled in 60 samples of 100g (3 levels of infection x 5 samples per level of infection x 2 methods x 2 labs = 60 samples) to test the two protocols in the two laboratories on 15 samples. The precise numbers of nematodes were added in each sample. Half of the conditions were sent to NIAB for testing.

d. PCR confirmation

Three molecular methods suitable for the identification of *D. dipsaci* and *D. gigas* have been identified. One protocol was collected by GEVES in a peer reviewed publication (Esquibet *et al*, 2003). A second was developed and published by GEVES (Kerkoud *et al* 2007). The third was developed by Tom Wood (NIAB). Kerkoud method (Kerkoud *et al* 2007) is based on two forward primers (DdpS1 and DdpS2) and one reverse (rDNA2). DdpS1 (5'-TGGCTGCGTTGAAGAGAACT-3') is specific to *D. dipsaci*, while DdpS2 (5'-CGATCAACCAAAACACTAGGAATT-3') and rDNA2 (5-TTTCACTCGCCGTTACTAAGG-3') anneal to both *D. dipsaci* and *D. gigas*. An electrophoresis profile showing both a 517bp (DdpS2 / rDNA2) and a 707 bp band (DdpS2 / rDNA2) identifies *D. dipsaci*. If only the 707bp band is observed, the tested individual is identified as *D. gigas.* Esquibet method (Esquibet *et al* 2003) uses two pairs of primers each specific to one species. D09 (5'CAAAGTGTTTGATCGACTGGA-3') and D10 (5'CATCCCAAAACAAAGAAAGG-3') provide a 200bp band specific to *D. gigas*. H05 (5'-TCAAGGTAATCTTTTTCCCCACT-3') and H06 (5'-CAACTGCTAATGVGTGCTCT-3') provide a 250bp band specific to *D. dipsaci.* Both primers

pairs can be used in multiplex. Tom Wood from the NIAB has developed species-specific primers for *Ditylenchus gigas* Dg.F (5'-TGCGTTGAAGAGAATCGACAG-3'), Dg.R (5'-AAAAGCACCCGCTAGTTTTG-3') and for *Ditylenchus dipsaci* Dd F (5'-GCGTTGAAGAGAACTGGCAC-3') and Dd.R (5'-AAGCACCCAACCAGTACCG-3').

These three PCR protocols (Annex 4) have been compared according to their trueness and repeatability. The reproducibility of the methods has not been evaluated yet.

e. Collection of nematodes:

Different nematodes populations of *D. dipsaci* and *D. gigas* species from different hosts were collected for the evaluation of the PCR tests by NIAB and GEVES in the framework of their routine analysis (Annex 3):

- 37 nematodes *D. dipsaci* from alfalfa
- 25 nematodes *D. gigas* from *Vicia* faba
- 20 non target nematodes (*D. destructor, Aphelencoïdes*…)
-

III. Results:

a. First pre-tests : comparison of the two methods

1. Alfalfa seed lots

Sieving protocol was compared with decantation protocol for detect *D. dipsaci*. The supernatant, normally thrown out in decantation protocol, was kept and filtrated in order to check if nematodes can be present.

The comparison between both protocols showed some difference between samples (Table 4).

Table 4: Comparison between decantation and sieving protocols tested in GEVES and NIAB

Seed lots		Tested at GEVES				Tested at NIAB	
		Sieving protocol		Decantation protocol		Decantation protocol	
		Nb positive/ nb samples	Counting	Nb positive/ nb samples	Estimation	Nb positive/ nb samples	Estimation
1	Sample 1	5/5	185	5/5	1 to 15	4/5	1 to 15
	Sample 2		281		>500		50 to 500
	Sample 3		38		50 to 500		50 to 500
	Sample 4		272		1 to 15		1 to 15
	Sample 5		67		15 to 50		0
2	Sample 6	5/5	76	5/5	>500	5/5	50 to 500
	Sample 7		175		>500		50 to 500
	Sample 8		360		>500		50 to 500
	Sample 9		244		50 to 500		50 to 500
	Sample 10		349		50 to 500		50 to 500
3	Sample 11	5/5	318	5/5	>500	5/5	>500
	Sample 12		424		>500		>500
	Sample 13		270		>500		>500
	Sample 14		315		50 to 500		>500
	Sample 15		77		50 to 500		>500

Overall, all the samples tested at GEVES were found positive for each seed lot (1, 2 and 3) with the two protocols (decantation and sieving).

Regarding the samples tested at NIAB, one on five coming from the seed lot 1 was found negative.

The results showed some differences between the number of nematodes counting (sieving protocol) and the estimation of nematodes presents (decantation protocol). The estimation of nematodes by decantation protocol was not in the same proportion of nematodes counted in the sieving protocol. Estimations of *Ditylenchus dipsaci* were also different between the two labs for the seed lot 3. This observation can be explained by heterogeneity of the infection between samples from a same seed lot.

Nevertheless, a limit was observed for the decantation protocol. Normally in decantation protocol, the supernatant was poured back after soaking. Here it was kept and filtered through a 20µm sieve to check if nematodes were present. The results (Table 5) showed that in most of the case many *Ditylenchus dipsaci* were found and sometimes even more nematodes were detected in supernatant than in bottom part. For instance, in the sample 4 of the first seed lot (1), 1 to 15 *Ditylenchus dipsaci* were estimated present but in the supernatant, 15 to 50 have been found.

Same remark for the sample 5 of the same seed lot, 15 to 50 *Ditylenchus dipsaci* were estimated present but in the supernatant 50 to 500 have been found.

For a high contaminated seed lot, it was not a problem because the results are given by qualitative analysis (presence or absence of the pathogen). But for a low infested seed lots it could be a problem and lead to a false negative result.

Table 5: NIAB protocol tested on Alfafa seed lots in GEVES

		Tested at GEVES			
		Decantation protocol (Bottom)		Decantation protocol (Supernatant)	
Seed lots		Nb positive/ nb samples	Estimation	Nb positive/ nb samples	Estimation
1	Sample 1	4/5	1 to 15	4/5	0
	Sample 2		>500		1 to 15
	Sample 3		50 to 500		1 to 15
	Sample 4		1 to 15		15 to 50
	Sample 5		15 to 50		50 to 500
2	Sample 6	5/5	>500	5/5	50 to 500
	Sample 7		>500		50 to 500
	Sample 8		>500		50 to 500
	Sample 9		50 to 500		15 to 50
	Sample 10		50 to 500		50 to 500
3	Sample 11	5/5	>500	5/5	50 to 500
	Sample 12		>500		50 to 500
	Sample 13		>500		50 to 500
	Sample 14		50 to 500		50 to 500
	Sample 15		50 to 500		15 to 50

The presence of *Ditylenchus dipsaci* in the supernatant can also be an explanation of the different estimation between the labs. Nematodes could have been lost during step of “throw out the supernatant”.

To overcome this problem, the step of filtration of all the water (used in sieving protocol) can resolve the loss of nematode. With this step, all the nematodes present in the sample are recovered and can be observed.

2. Faba bean seed lots

The comparison between both protocols (decantation and sieving) showed less difference between the infection levels of the Faba bean samples compared to the previous tests on the Alfalfa seed lots (Table 6).

The counting (realized with sieving protocol) was correlated with the estimation of *Ditylenchus gigas* (realized with decantation protocol) for most of the samples.

Table 6: Comparison between NIAB and GEVES protocol tested in GEVES

		Tested at GEVES			
		Sieving protocol		Decantation protocol	
Seed lots		Nb positive/ nb samples	Couting	Nb positive/ nb samples	Estimation
4	Sample 16	5/5	5000	5/5	> 500
	Sample 17		490		> 500
	Sample 18		1755		> 500
	Sample 19		1180		> 500
	Sample 20		1765		> 500
5	Sample 21	4/5	30	4/5	50 to 500
	Sample 22		0		15 to 50
	Sample 23		5		0
	Sample 24		19		15 to 50
	Sample 25		400		50 to 500
6	Sample 26	5/5	38	4/5	50 to 500
	Sample 27		4		15 to 50
	Sample 28		20		15 to 50
	Sample 29		15		0
	Sample 30		47		50 to 500

For the second seed lot (5), two samples were found negative, one with the sieving protocol (sample 22) and one with the decantation protocol (sample 23). These results can be explained by the low infection level of the seed lot 5 coupled with the heterogeneity of the infection. It can show that for medium/low infested seed lots, the sample taken for the analysis can lead to false negative result due to heterogeneous repartition of nematodes.

Regarding the last seed lot (6) one sample out of five was found negative with the decantation protocol whereas five of five were positive with the sieving protocol.

Like previously, the supernatant of all the samples were kept and filtered through a 20µm sieve to check if nematodes were present. Result is presented in table 7.

Table 7: NIAB protocol tested on Faba bean seed lots in GEVES

Seed lots		Tested at GEVES			
		Decantation protocol (Bottom)		Decantation protocol (Supernatant)	
		Nb positive/ nb samples	Estimation	Nb positive/ nb samples	Estimation
4	Sample 16	5/5	> 500	5/5	> 500
	Sample 17		> 500		> 500
	Sample 18		> 500		> 500
	Sample 19		> 500		> 500
	Sample 20		> 500		> 500
5	Sample 21	4/5	50 to 500	4/5	15 to 50
	Sample 22		15 to 50		15 to 50
	Sample 23		0		0
	Sample 24		15 to 50		1 to 15
	Sample 25		50 to 500		15 to 50
6	Sample 26	4/5	50 to 500	5/5	50 to 500
	Sample 27		15 to 50		15 to 50
	Sample 28		15 to 50		15 to 50
	Sample 29		0		1 to 15
	Sample 30		50 to 500		50 to 500

The most important observation concerned the last seed lot 6. One sample was found negative by decantation protocol but 1 to 15 *Ditylenchus dipsaci* were found in supernatant. This example shows the risk to have false negative sample due to the limit of the decantation method on the low infested seed lot. Nevertheless, the infection level of two seed lots 4 and 5 varied between the samples from the same seed lot. For the 4 seed lot, the infection level varied between 5000 (sample 16) and 490 (sample 17) *Ditylenchus dipsaci*. For the 5 seed lot the infection level various between 0 (sample 22) and 400 (sample 25) *Ditylenchus dipsaci*. These observations confirm that the infection level have many variations between samples from a same seed lots, also in the Faba bean seed lot. Most of the time, the quantity of nematodes detected in highly infested Faba bean seed lots was more important than in Alfalfa seed lots. The Petri dish is completely full of nematodes (figure 3). In this case, it was easier to do estimation instead of a counting.

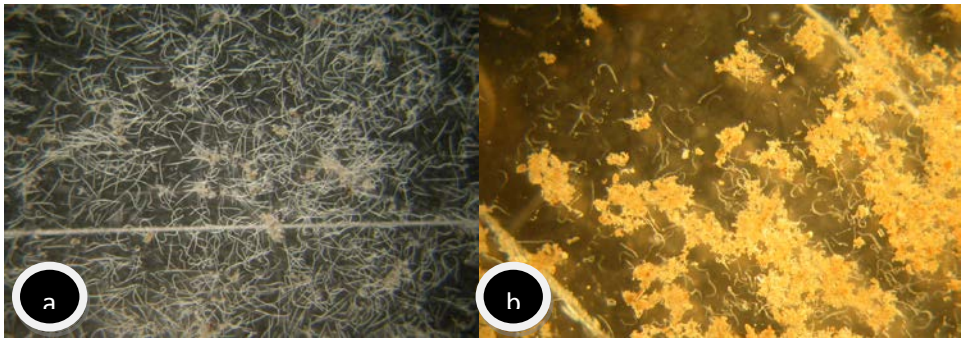

Figure 3: Difference between Faba bean (a.) and Alfalfa (b.) high infested seed lot

Conclusion*:*

The comparison showed the limit of the decantation and sieving methods. For the first one, the main limit was the presence of nematodes in the supernatant that can lead to a difference in the estimation of the infestation rate, particularly on low infested samples. This could be resolved by the step of filtration of the water by a sieve for catching and observing all the nematodes present in a sample. For the second one (sieving protocol), the main limit was the counting of *Ditylenchus dipsaci* specimens recovered. This step takes too much time and is not necessary for a quarantine pest because there is no tolerance of presence. A qualitative analysis with presence or absence is enough for *Ditylenchus dipsaci*. This could be resolved by an estimation of the presence of the pathogen similar to that described in the NIAB protocol.

b. Threshold detection of the two methods

To determine their level of detection, the two methods were compared on very low infested seed lots artificially infested. This comparison was done in GEVES only on the Alfalfa seeds lot (Table 8). The entire subsamples were coded and analyzes were done in blind test.

Table 8: Recovering of the NIAB and GEVES protocol tested on artificial infested seed lots

	Tested at GEVES			
	Sieving protocol (Sieving)		Decantation protocol (Decantation)	
Number of nematodes in samples	Nb positive/ nb samples	Recovery	Nb positive/ nb samples	Recovery Estimation
10	5/5	60%	3/5	8%
5	4/5	48%	3/5	24%
1	2/5	40%	1/5	20%

The results showed a better percentage of recovery of *D. dipsaci* with the sieving protocol compared with the decantation protocol. Regarding the sub samples artificially infested by 10 *D. dipsaci*, 5/5 were positive with the sieving protocol when only 3/5 were positive with the decantation protocol. Moreover, for this condition (10 nematodes) the subsample positives with decantation protocol have low percentages of recovery of *D. dipsaci*.

About the conditions with five *D. dipsaci*, one subsample was negative with the sieving protocol and two with the decantation protocol. Overall, the percentage of recovery of *D. dipsaci* is better with the sieving protocol (48%).

The last condition (one nematode *D. dipsaci*) showed the limit of the two methods. On five subsamples tested, two were positives with the sieving protocol and one with the decantation protocol.

The limit of detection (table 9) of the sieving and the decantation method was based on guide line LNPV (LNPV/Guide 01 Version 09, 2010, p10).

Table 9: Limit of detection between the sieving and the decantation protocol.

	Repeat 1		Repeat 2		Repeat 3		Repeat 4		Repeat 5	
	Sieving	Decantation	Sieving	Decantation	Sieving	Decantation	Sieving	Decantation	Sieving	Decantation
Condition 1: 10 nematodes	+	-	+	+	+	+	+	-	+	-
Condition 2: 5 nematodes	-	+	+	-	+	-	+	+	+	+
Condition 3: 1 nematodes	-	-	+	-	-	+	+	-	-	-

Regarding the table, limit of detection for the sieving method is at 10 nematodes. This method allowed to detect all the five samples positives for the condition 1 (10 nematodes) whereas the decantation protocol detected only 2 samples. For the condition 2 (5 nematodes), only 4 repeat on 5 were detected positive.

The table 9 shown that the decantation protocol has a limit of detection up to 10 nematodes.

Conclusion:

This study on alfafa showed the high capacity of sieving protocol to detect nematode in low infested seed lots compared to the decantation protocol. Decantation protocol showed a higher detection limit and could lead to more false negatives than the filtration method.

Perspectives:

- Test the detection threshold on Alfalfa seed lot artificially infested at 1, 5 and 10 *Ditylenchus dipsaci* in NIAB laboratory.
- Test the detection threshold on Faba bean seed lot artificially infested at 1, 5 and 10 *Ditylenchus gigas* in GEVES and NIAB laboratory
- Test plan to validate one or two methods with respect of the EOPP norms (Annex 5).
-

c. Results of PCR tests

1. BioGEVES

An evaluation of the three methods (Kerkoud, Esquibet and Wood/NIAB) has been performed by BioGEVES on single nematodes previously identified as *D.dipsaci, D. gigas* and on single nematodes belonging to other species (Annex 3). Nematodes collection contained 38 Dd, 25 Dg and 19 non target nematodes. The identifications obtained using the different tests are presented in table 10, according to the initial identification.

Table 10: Molecular methods evaluated for the identification of *D.dipsaci* and *D.gigas*

			Morphological Identification Expected results		
			D. dipsaci	D. gigas	Other
Assay (Obtained results)	Kerkoud	D. dipsaci	35	0	2
		D. gigas	0	23	0
		Other	1	2	18
	Esquibet	D. dipsaci	28	1	1
		D. gigas	1	14	0
		Other	7	10	19
	NIAB	D. dipsaci	31	0	0
		D. gigas	0	21	0
		Other	5	4	20
	Clear Detection	D. dipsaci	30	0	0
		Other	6	23	15

Definitions of the concepts of sensitivity diagnostic and specificity diagnostic are provided in the EPPO document present in Annex 5. The accuracy of the methods has been defined as the ability of the assays to provide the correct identification (% of matching identification among the total number of reactions). For each method, several samples have been run several times. These data (more than 30 possible matching pairs of results) have been used in order to calculate an approximate repeatability (% of matching pairs of results obtained from a common sample). The results obtained for the three methods are presented in table 11.

Table 11: Accuracy and repeatability calculated for the three evaluated methods

Method	Results for a specific target				overall results	
	D. dipsaci		*D. gigas*			
	Sensitivity	Specificity	Sensitivity	Specificity	Accuracy	Repeatability
Kerkoud	97%	96%	92%	100%	94%	84%
Esquibet	78%	96%	56%	98%	75%	35%
NIAB	86%	100%	84%	100%	89%	100%
Clear Detection	83%	100%			92% *	

* Value only based on the ability to specifically detect *D. dipsaci*

The method published by Kerkoud et al. provided the best sensitivity for both species. Among the 81 individuals tested, 5 bad assignments are to be deplored, 3 negative and 2 false positives. A repeatability calculation was made on the basis of results obtained on 49 duplicates, 3 samples were subjected to a bad assignment among 3 repetitions. This penalizes the method with a level of repeatability estimated at 84%.

The method Esquibet *et al.* provided accuracy results close to 89%. The evaluation of this method is however characterized by a 35% repeatability level (84 double results in agreement on a total of 241).

The method provided by the NIAB has not detected 9 target extracts, 5 of *D. dipsaci* and 4 of *D. gigas*. Apart from that the results are optimal with 100% analytical specificity (no false positives). Repeatability is estimated at 100% of a total of 30 duplicate results.

Clear Detection method could be assessed only on 74 extracts. This method does not detect individuals of *D. gigas*, the calculation accuracy is therefore only based on its ability to detect *D. dipsaci*. The method does not meet the expectations; the estimate of the repeatability has not been completed.

Regarding the PCR tests realized by NIAB (on a pool of nematode), no differences between Kerkoud and Wood primers were observed (Table 12).

Table 12: Three pairs of primers tested in NIAB for detection of *D.dipsaci sensu stricto* and *D.gigas*

Date	Host	Name	Kerkoud Duplex	Esquibet Duplex	NIAB simplex
30/07/2013	Alfalfa	Fravier 2-0-1	Ddi	No data	Ddi
		Fravier 2-0-2	Ddi		Ddi
		Fravier 2-0-3	Ddi		Ddi
		Fravier 2-0-4	Ddi		Ddi
		Fravier 2-0-5	Ddi		Ddi
		2 3-0-1	Ddi		Ddi
		2 3-0-2	Ddi		Ddi
		2 3-0-3	Ddi		Ddi
		2 3-0-4	Ddi		Ddi
		2 3-0-5	Ddi		Ddi
30/07/2013	Faba bean	5	D. gigas	No data	D. gigas
		4	D. gigas		D. gigas
		6 - Lincoln	D. gigas		D. gigas
		D. dipsaci sensu-stricto?	D. gigas		D. gigas
		D. dipsaci sensu-stricto?	D. gigas		D. gigas
		D. dipsaci sensu-stricto?	D. gigas		D. gigas
		D. gigas?	D. gigas		D. gigas
		D. gigas?	D. gigas		D. gigas
		D. gigas?	D. gigas		D. gigas

Ddi = *Ditylenchus dipsaci sensu stricto*

The Esquibet primers were not tested but a comparison between the Kerkoud and NIAB primers was performed. For faba bean, all the samples tested positive for *D. gigas* with the Kerkoud protocol. This was in agreement with the NIAB protocol that also identified only *D. gigas* in the same samples. It is important to note that Kerkoud protocol was improved in BioGEVES in parallel. This first result with Kerkoud primers need to be retested with the improved Kerkoud protocol. In conclusion for NIAB, although the Esquibet methods were not used to screen samples, the Kerkoud method proved to be the most resource efficient (only requiring a single test per sample), whereas, the NIAB primers proved an easier test.

Prospects:

- Evaluate the repeatability and robustness of Kerkoud and NIAB methods according to EPPO standards
-

Conclusion:

Based on the evaluation of the accuracy of the results provided by the methods, two protocols might be suitable for routine analysis and one provides unsatisfactory results. The two remaining methods are able to identify the two species of interest on isolated nematodes. However, only one method (NIAB) could be used on more complex samples susceptible to contain both species.

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Appendix 2: Heterogeneity test with seedcalc8 and Prob pos sample

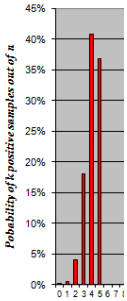
Impurity Estimation & Confidence Intervals (Assay measures impurity characteristic)
(Number of seed sampled should not exceed 10% of total number in population)

# of Seed Pools	10	Computed % in sample	0.18 %
# of Seeds per Pool	880		
Total Seeds Tested	8800	*Measured property on seed pools*	
# Deviants Pools	8		
		Desired Confidence Level	95 %
Upper Bound of True % Impurity		0.37	
		(95% confident that the lot impurity is below 0.37%.)	
2-sided CI for True % Impurity		*0.07* to *0.42*	
Lower Bound of True % Purity		99.63	
		(95% confident that the lot purity is above 99.63%.)	
2-sided CI for True % Purity		*99.58* to *99.93*	

Lot E

# of seeds per sample (m)	947
True contamination rate (π)	0.1800%
Total # of samples (n)	5

k	Prob (%) of k positive out of n
0	0.0197%
1	0.4447%
2	4.0093%
3	18.0726%
4	40.7323%
5	36.7213%
6	#NOMBRE!
7	#NOMBRE!
8	#NOMBRE!
9	#NOMBRE!
10	#NOMBRE!
11	#NOMBRE!
12	#NOMBRE!
13	#NOMBRE!
14	#NOMBRE!
15	#NOMBRE!



Impurity Estimation & Confidence Intervals (Assay measures impurity characteristic)
(Number of seed sampled should not exceed 10% of total number in population)

# of Seed Pools	10	Computed % in sample	0.24 %
# of Seeds per Pool	947		
Total Seeds Tested	9470	*Measured property on seed pools*	
# Deviants Pools	9		
		Desired Confidence Level	95 %
Upper Bound of True % Impurity		0.56	
		(95% confident that the lot impurity is below 0.56%.)	
2-sided CI for True % Impurity		*0.09* to *0.63*	
Lower Bound of True % Purity		99.44	
		(95% confident that the lot purity is above 99.44%.)	
2-sided CI for True % Purity		*99.37* to *99.91*	

Lot F

# of seeds per sample (m)	947
True contamination rate (π)	0.2400%
Total # of samples (n)	5

k	Prob (%) of k positive out of n
0	0.0011%
1	0.0500%
2	0.8731%
3	7.6252%
4	33.2957%
5	58.1549%
6	#NOMBRE!
7	#NOMBRE!
8	#NOMBRE!
9	#NOMBRE!
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11	#NOMBRE!
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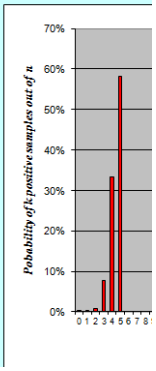


Appendix 3: Results of the participants

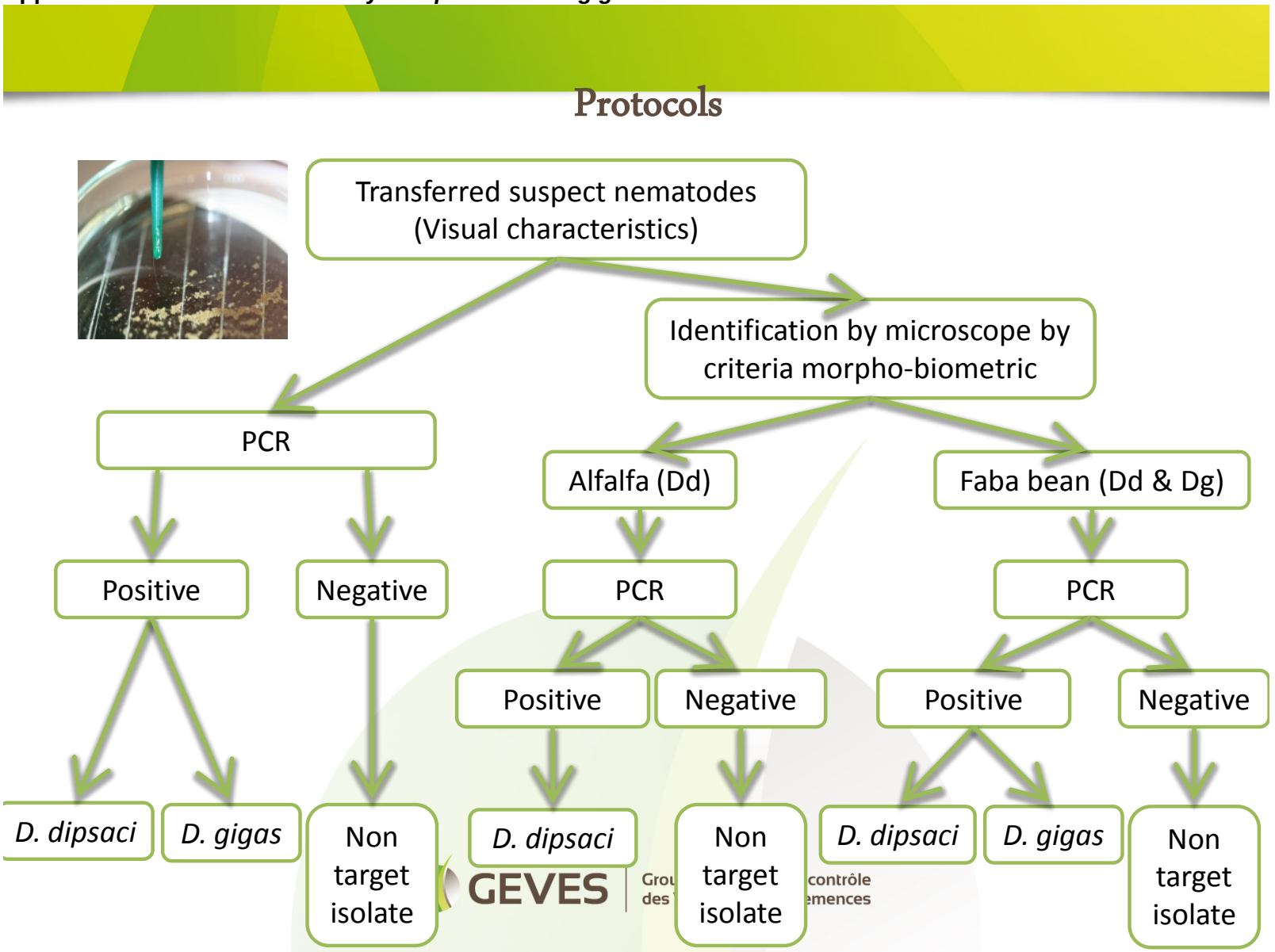
				No. of obtained positive seed subsamples							
				Laboratories							
	Seed Lot	Infestation Level	No. of expected positive seed subsamples	1	2	3	4	5	6	7	8
Alfalfa	Lot A	Healthy	0	0	0	0	0	0	0	0	0
Alfalfa	Lot B	Low	5	5	5	5	5	5	5	5	4
Alfalfa	Lot C	Medium	5	5	5	5	5	5	5	5	5
Faba bean	Lot D	Healthy	0	0	0	0	0	0	0	1	0
Faba bean	Lot E	Low	5	5	5	4	5	5	5	5	4
Faba bean	Lot F	Medium	5	5	5	5	5	5	4	5	5

Appendix 4: PCR Results of the participants

		No. of positive seed subsamples obtained							
		Laboratories							
		1	2	3	4	5	6	7	8

PCR Method	Seed lot species	Suspect nematode	No. of expected positive seed subsamples	1	2	3	7	8
Kerkoud	Alfalfa & Faba bean	Healthy	0	0	0	0	0	0
Kerkoud	Alfalfa	*D. dipsaci*	10	10	10	10	Undetermined	10
Kerkoud	Faba bean	*D. gigas*	10	9	10	10	Undetermined	10
Wood	Alfalfa & Faba bean	Healthy	0	0	0	0	0	0
Wood	Alfalfa	*D. dipsaci*	10	5	10	10	10	5
Wood	Faba bean	*D. gigas*	10	7	10	10	10	9

Appendix 5: Work Flow to identify *D. dipsaci* and *D. gigas*



Validation study for blotter paper method for the detection of *Verticillium dahliae* on fungicide-treated spinach seed to support C.7.3

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Summary

Two comparative tests were organized by ISHI-Veg in the last four years to evaluate the efficacy of NP-10 agar medium and blotter paper methods for the detection of *Verticillium dahliae* on non-treated and fungicide-treated spinach seed. Results supported the use of the NP-10 agar method for non-treated seeds and the blotter paper method for testing both non-treated and fungicide-treated seed. In this comparative test seven laboratories used the blotter method to quantitatively detect *Verticillium* on treated and non-treated spinach seed and comparing the results of carrying out the seed assay for 5, 9, 14 and 21 days. The results show that fungicide treatment can delay the development of internal infection of *Verticillium dahliae* in the seed. Prolongation of the incubation period to 21 days is therefore necessary.

Introduction

Verticillium dahliae is a seed borne and seed transmitted pathogen of spinach (du Toit *et al*., 2005). In 2011, ISHI-Veg collaborated with L. J. du Toit (Washington State University, Mount Vernon, U.S.A.) on evaluating the efficacy of NP-10 agar and blotter paper methods for the detection of *Verticillium dahliae* and other potential seed-borne pathogens of spinach, particularly *Stemphylium*, on fungicide-treated and non-treated spinach seed. Results supported the efficacy of the blotter paper method in detecting the *Verticillium dahliae* on both types of seed (du Toit, 2011a; du Toit 2011b). The difference in incidence of *Stemphylium* detected on treated vs. non-treated seeds was, however, far greater with the freeze blotter assay than with the NP-10 assay. This suggested that fungicides on the treated seed might dissipate into the agar medium, reducing efficacy of the fungicide treatment against any *Stemphylium* present on the seed. It also suggested that the freeze blotter assay may be more appropriate than the NP-10 assay for testing fungicide-treated seed lots (du Toit, 2011a; du Toit 2011b).

The increase in the detection of these fungi for the reading taken on day 21 compared to day 14 using the freeze blotter test was, however, not conclusive due to differences in results among labs. This led to a repeat of the comparative test of just the blotter paper method with

fungicide-treated seed and non-treated seed in which seven laboratories from The Netherlands, U.S.A. and Japan participated.

Scope and objective of the comparative test

The scope of this comparative test is to evaluate the performance of the blotter paper test method for detection of *Verticillium dahliae* on fungicide-treated spinach seed. The objective is to determine the importance of the day 14 and day 21 readings of fungicide-treated seeds in the blotter paper test under the hypothesis that any internal seed infection may be delayed by the fungicide treatment and, therefore, will only become visible after a longer duration of incubating the seed. The influence of other seed treatments such as biological or physical seed treatments on the test method was not studied.

Materials and Methods

Seed lots and seed subsamples

Three (3) spinach (*Spinacia oleracea*) seed lots with different levels of natural infection with *Verticillium dahliae* and saprophytes were used in this comparative test. Seed lots were selected by the laboratory of Rijk Zwaan in De Lier, The Netherlands based on their infection levels of untreated seed batches: a highly infected seed lot **A** with >50% *Verticillium* infection, a medium infected lot **B** with 10 - 25% infected seeds, and a low infected lot **C** with 0 - 5% infected seeds.

All seed lots were then treated as in the previous comparative test (du Toit, 2011a; du Toit, 2011b). Subsamples of each lot were treated with Thiram (thiram) + Apron (metalaxyl) at the rates typically recommended for planting baby leaf spinach crops in California, USA (du Toit *et al*., 2010).

Each laboratory received 4 subsamples of 250 seeds from each treated seed lot. However, each laboratory only tested 100 seeds of each of the 12 subsamples using the blotter paper assay. Subsamples were coded randomly, and their correspondence to seed lots was known only to the test organiser.

In addition, each laboratory received 1,000 seeds from the highly infected seed lot before they were treated with fungicides in order to serve as the positive control of the non-treated seed lots. The code of this non-fungicide treated sample was known to laboratories. Each

laboratory tested 4 subsamples of 100 seeds. Distribution of the extra 150 seeds per subsample was done to allow repetition of the test, if necessary.

Pre-treatment of seeds

At each of the participating laboratories, surface sterilization of the non-treated control seed subsamples was done in order to reduce the level of saprophytic fungi that inhibit development and detection of *V. dahliae* on spinach seed. Surface-sterilization was performed as follows: Seeds were placed in a tea strainer and immersed in a 1.2% NaOCl solution for 60 seconds while being constantly agitated. The tea strainer with seeds was then placed in sterilized de-ionized or distilled water for 30 seconds while being constantly agitated to rinse the NaOCl from the seeds. The seeds were rinsed two more times using a new batch of sterilised de-ionised or distilled water each time. Surface-sterilised seeds of each subsample were then spread onto a dry, sterilized paper towel in a laminar flow hood or biological safety cabinet for 60 minutes or more.to dry thoroughly. No surface-sterilisation was applied to the fungicide-treated seeds before placing them on blotter paper.

Seed plating and incubation

In each participating laboratory acrylic boxes and lids (e.g. DBP plastics, 20 cm x 14 cm) were sterilised by spraying with 70% isopropyl alcohol or equivalent in a biological safety cabinet or laminar flow hood. Boxes and lids were then air-dried. Likewise, blotter papers (e.g. Steel blue germination blotter paper, Anchor Paper Co., D1.360.560, All Paper) were sterilised by autoclaving or soaking in 70% isopropyl alcohol or equivalent, and dried in sterile conditions.

One blotter paper was placed in each sterilized acrylic box, soaked with sterilized water, and any excess water drained off the blotter. For each seed subsample, a maximum of 100 seeds was placed on the blotter paper in each box (e.g. up to 34 seeds per 10 cm x 10 cm box). Seeds were incubated at 20-24°C for 24-25 h in the dark, and then at -18 to -22°C for 24-25 h to kill the embryos. Incubation continued at 20-24°C under a day/night cycle of 12 h light (near ultraviolet (NUV) and cool white fluorescent light)/12 h dark.

Verticillium dahliae identification

Laboratories used a dissecting microscope (8-100x magnification) to examine the seeds for development of *Verticillium dahliae*. The non-fungicide treated positive seeds were examined on

days 5, 9 and 14 after plating and fungicide treated seeds were read on days 5, 9, 14 and 21 after plating. To facilitate microscopic examination of seeds, the lid of each box was removed during the reading.

Typical structures of *Verticillium dahliae* are:

i) microsclerotia: black survival structures ranging in size from 10 µm to 230 µm and
ii) conidiophores and conidia: tree-like structures (conidiophores) with phialides borne in verticillate whorls on the conidiophores, and clumps of hyaline, single-celled conidia borne at the end of each phialide.

It is possible to confuse *Verticillium dahliae* with *Acremonium* spp. However, the mycelium of *Acremonium* spp. tends to form in rope-like 'strands' from which individual conidiophores branch off at right angles, and which do not normally form phialides in distinct whorls. *Acremonium* spp. also do not form microsclerotia.

Results and Discussion

Laboratories recorded the number of seeds per box with *Verticillium dahliae* microsclerotia and/or conidiophores that were observed on reading days 5, 9, and 14 for the non-fungicide treated positive seed lot A and on days 5, 9, 14 and 21 for the fungicide-treated seed lots. The total number of seeds with both microsclerotia and conidiophores for each subsample was used in the statistical analysis. Results were subjected to a one-way analysis of variance (ANOVA) with companies as the factor and the infection% they measured on each reading date as the variable and the four reps as blocks. This was followed by Tukey's multiple range test to compare the seven participating companies. Data were analysed with GENSTAT statistical software (16th Edition) and visualized using a Tukey boxplot in which infection% of each batch on each date is shown (Fig. 1).

The average percentage of seed on which *Verticillium dahliae* was observed by all laboratories for all tested seed lots at the four readings is presented in Table 1. The results show that all laboratories scored similar results for the non-fungicide treated seed lot and the three fungicide-treated seed lots (Tables 2 - 5). A 21-day reading of the non-fungicide treated seed lot was not performed by the laboratories because experience has shown no further increase in *Verticillium dahliae* incidence is detected between 14 and 21 days for non-treated

spinach seed (Tables 1 and 2). Also, development of saprophytes on the non-fungicide treated seeds can be excessive by 21 days, making it difficult to get reliable assessment at this reading.

For the non-fungicide treated seed lot, the statistical difference among the laboratories at the 5 day reading (Table 2) might be a result of different varying conditions of seed incubation, e.g. humidity, lighting (NUV cool and white fluorescent light, distance between the lights and the boxes or plates, etc.), temperature (freezing temperature, or incubation at 20 vs. 24^{o}C), that could influence initiation of development of the fungus on seed. However, by 9 days differences among laboratories in terms of the incidence of seed on which *V. dahliae* was detected had disappeared. *Verticillium dahliae* development was present at an almost 50% incidence at the 5 day reading, increased by the 9-day reading and increased again only slightly by the 14-day reading. However, no significant differences were detected among the laboratories for the readings on days 9 and 14.

The correlation between the incidence of seed with microsclerotia and the incidence of seed with conidiophores of *Verticillum dahliae* was calculated for the non-fungicide treated seed lot and was 0.87 resp. 0.85 at 5 days and 14 days after plating (Fig. 2, 3). This shows that both fungal structures were able to form normally confirming the presence of *Verticillium dahliae*.

The fungicide-treated seed lot A, clearly showed the importance of a 21 day final reading for fungicide-treated seeds, as hardly any or a very limited incidence of *Verticillium dahliae* infected seed was observed on days 5 and 9 and with a significant increase in incidence by 14 days (Table 3). This result is in agreement with the results of the previous ISHI-Veg comparative test (du Toit, 2011a; du Toit, 2011b). The incidence of seeds with *Verticillium dahliae* more than doubled at the 21 day reading (Table 3). No significant differences were shown among laboratories for the 14 and 21 reading days (Table 3).

Fungicide-treated seed of lots B and C also showed a limited infection by *Verticillium dahliae* at the 21 day reading (Tables 4 and 5). There was no significant difference among laboratories at any of the four reading days for seed lot B (Table 4), and no significant difference for seed lot C (Table 5). Although the untreated batch B was selected because of its higher seed infection compared to batch C no difference was found between the fungicide treated batches. Apparently the fungicide treatment reduces the infection on batch B to a high extent.

This comparative test showed that the blotter paper method can be used reliably for the detection of *Verticillium dahliae* on spinach seeds, both for fungicide-treated seeds and non-treated seeds. For fungicide-treated seeds it is important to prolong the incubation until the 21st

day to detect all viable *Verticillium dahliae* seed infections, as some infected seed may not become visible until after 9 or 14 days, as some fungicides slow down the development of *Verticillium dahliae* in and on spinach seed.

Acknowledgements

The input and participation of laboratories from The Netherlands, U.S.A. and Japan in the comparative test is greatly acknowledged. Special thanks to the Pathology laboratory of Rijk Zwaan B.V. in the Netherlands for providing the testing material.

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Fig. 1. Tukey boxplot describing the *Verticillium dahliae* infection % of spinach seeds at the different days of assessment for the participating labs on the four seed batches used.

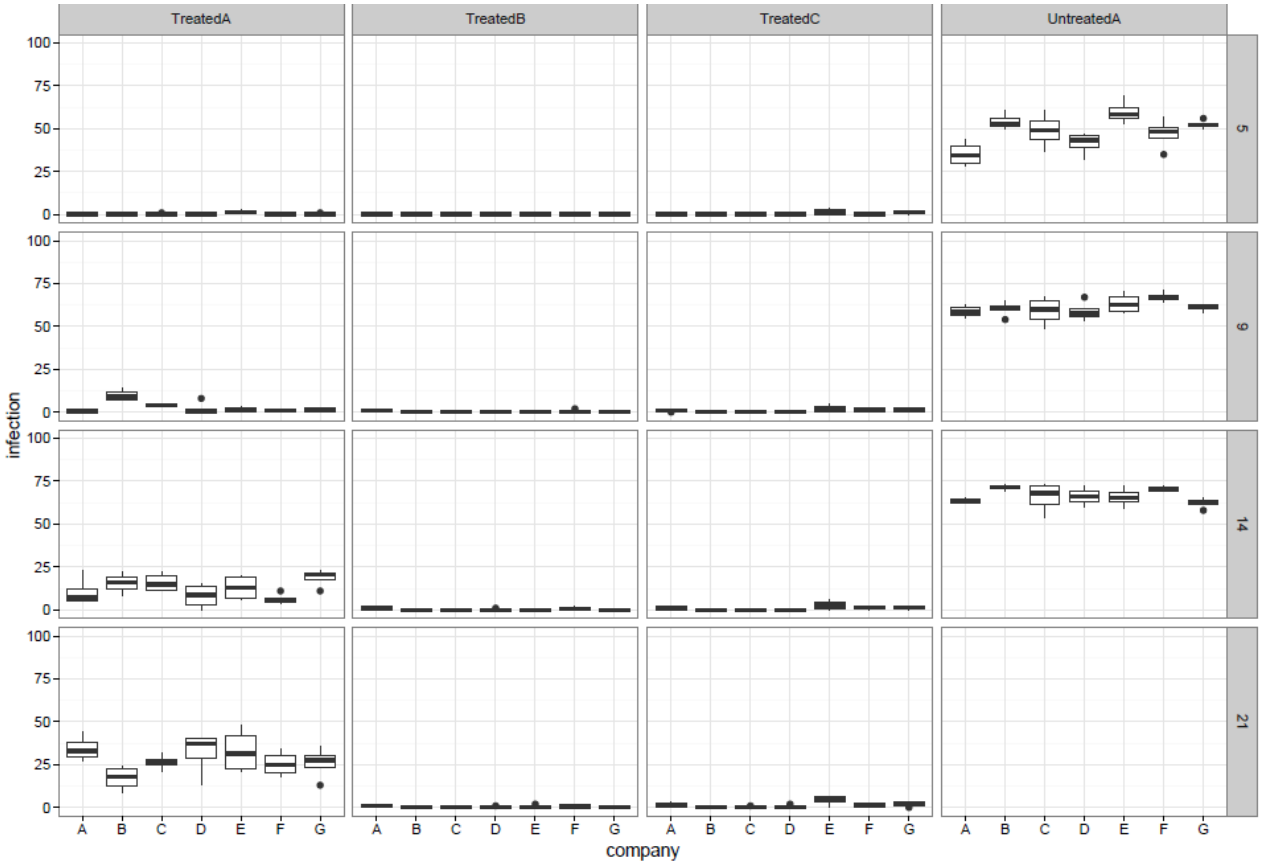

Fig. 2. Correlation between percentage of seeds with conidiophores versus microsclerotia of *Verticillium dahliae* that were observed on non-fungicide treated seeds 5 days after plating (n=28, 7 labs with 4 subsamples each).

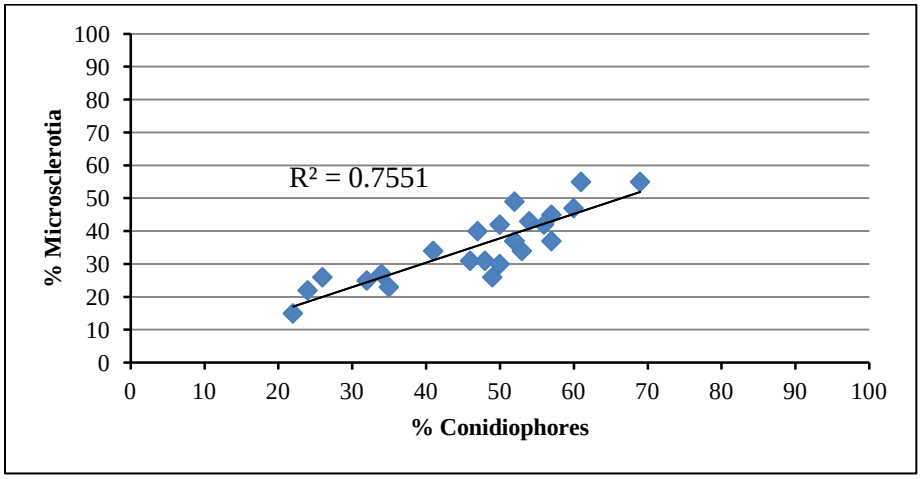

Fig. 3. Correlation between percentage of seeds with conidiophores versus microsclerotia of *Verticillium dahliae* that were observed on non-fungicide treated seeds 14 days after plating (n=28, 7 labs with 4 subsamples each).

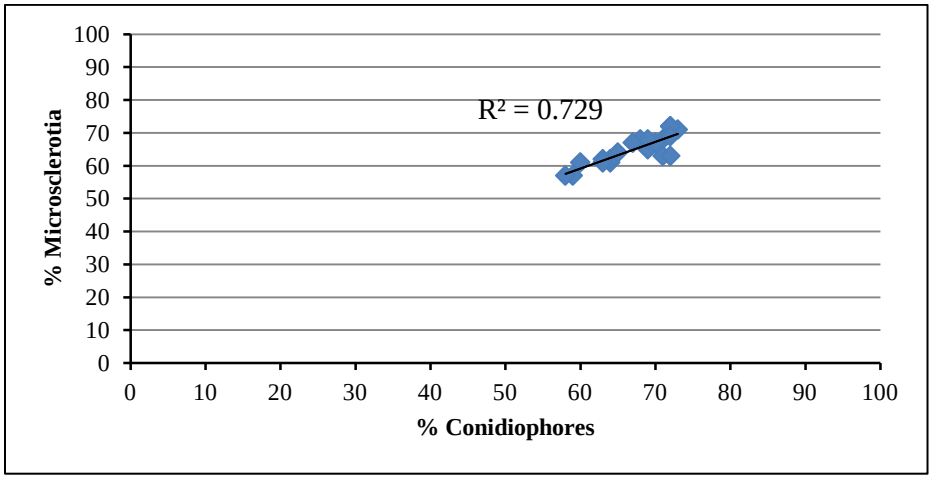

Table 1. Average ± standard error of the percentage (%) of *Verticillium dahliae* infected seed for the tested seed lots and all laboratories at 5, 9, 14 and 21 day readings.

Seed lot	5 day	9 day	14 day	21 day
Non-fungicide treated (A)	48.5 ± 5.1	61.1 ± 3.6	66.6 ± 3.3	Not read
A	0.3 ± 0.3	3.0 ± 1.5	12.5 ± 4.5	26.5 ± 7.2
B	0	0.2 ± 0.3	0.3 ± 0.4	0.4 ± 0.5
C	0.4 ± 0.6	0.8 ± 0.8	0.9 ± 0.8	1.4 ± 1.0

Table 2. Average percentage (%) of *Verticillium dahliae* infected seed of the non- fungicide treated seed lot A at 5, 9 and 14 days after plating on blotter paper (average of 4 sub-samples of 100 seeds).

Laboratory	5 day*		9 day (NS)	14 day (NS)
A	35	a	59	64
B	54	bc	60	71
C	49	abc	59	66
D	42	ab	59	66
E	60	c	63	66
F	47	abc	67	71
G	53	bc	61	62

Statistical differences after ANOVA (α=5% level) followed by Tukey's multiple range test among laboratories at different days of assessment.

NS= no significant difference among laboratories

*= significant difference among laboratories

Table 3. Average percentage (%) of *Verticillium dahliae* infected seed of fungicide-treated seed lot A at 5, 9, 14 and 21 days after plating on blotter (average. of 4 sub-samples of 100 seeds).

Laboratory	5 day*		9 day*		14 day (NS)	21 day (NS)
A	0.0	a	0.8	a	10.5	34.3
B	0.0	a	9.8	b	15.5	17.3
C	0.3	a	4.0	a	15.8	26.5
D	0.0	a	2.0	a	8.0	31.8
E	1.8	b	1.8	a	13.0	33.0
F	0.0	a	1.0	a	6.3	25.5
G	0.3	a	1.5	a	18.8	26.0

Statistical differences after ANOVA (α =5% level) followed by Tukey's multiple range test among laboratories at different days of assessment.

NS = no significant difference

*= significant difference among laboratories

Table 4. Average percentage (%) of *Verticillium dahliae* infected seed of fungicide-treated seed lot B at 5, 9, 14 and 21 days after plating on blotter (Average of 4 sub-samples of 100 seeds).

Laboratory	5 day (NS)	9 day (NS)	14 day (NS)	21 day (NS)
A	0	1	1	1
B	0	0	0	0
C	0	0	0	0
D	0	0	0.3	0
E	0	0	0	0.5
F	0	0.5	0.8	0.5
G	0	0	0	0

Statistical differences after ANOVA (α =5% level) followed by Tukey's multiple range test among laboratories at different days of assessment.

NS= no significant difference among laboratories

Table 5. Percentage (%) of *Verticillium dahliae* infected seed of fungicide-treated seed lot C at 5, 9, 14 and 21 days after plating on blotter (Average of 4 sub-samples of 100 seeds).

Laboratory	5 day (NS)	9 day (NS)	14 day (NS)	21 day*	
A	0	0.8	1	1.8	ab
B	0	0	0	0	a
C	0	0	0	0.3	a
D	0	0	0	0.5	a
E	1.5	2	2.8	4	b
F	0	1.3	1.3	1.3	ab
G	1.3	1.3	1.3	1.8	ab

Statistical differences after ANOVA (α =5% level) followed Tukey's multiple range test among laboratories at different days of assessment.

NS= no significant difference

*= significant difference among laboratories

Validation study for a new DNA based method for variety verification in *Zea mays*

Validation report authors: Ana Laura Vicario, INASE, Argentina and Daniel Perry, Canadian Grain Commission, Canada.

Abstract

To facilitate the process for the incorporation of DNA-based markers into the International Rules for Seed Testing (ISTA Rules) the ISTA Variety Technical Committee (TCOM) organized a series of comparative tests (CTs). As part of the ISTA Variety TCOM's DNA methods working group eight laboratories participated in the CTs for *Zea mays* (maize). Results from three CTs are complete and have been summarized.

Data from the CTs resulted in the selection of eight markers for DNA based varietal verification in *Zea mays*. Marker selection was based on plots per marker and per allele and only markers that had "substantial agreement" or "almost perfect agreement" are included in the ISTA Rules proposal.

The ISTA Rules proposal includes information on the markers, sample size and DNA test methodology. If the proposal is accepted into the ISTA Rules, as a minimum, the eight prescribed microsatellite markers must be used to issue an ISTA International Certificate for varietal verification using a DNA based method.

Background

Traditionally, ISTA's standardized procedures for the determination of varietal identity have been based on the examination of seeds, seedlings or plants in a laboratory, glasshouse, growth chamber or field plot, to assess morphology (grow-out tests), specific substances (biochemical methods) or protein characteristics (protein-based methods).

In 2014 a new chapter (Chapter 19: Testing seeds of genetically modified organisms) was added, which, for the first time in the context of ISTA rules, described the use of DNA-based methods for testing specified traits. However, DNA-based approaches are very useful not only for the determination of presence of traits introduced through modern biotechnology, but also for variety identification and assessment of purity. In comparison to traditional variety identification methods, DNA-based techniques may have higher throughput, may reveal more polymorphism thus allowing greater resolution among varieties, are independent of environmental conditions or developmental stages and normally require less space and time per data point collected.

To initiate the process for the incorporation of the DNA-based markers into the Rules, over a period of four to five years ISTA organized three or four comparative tests (CTs) for each of four selected crops (maize, wheat, rice and soybeans) with participation of laboratories from several countries from around the world. The results of the CTs carried out for maize are summarized here. The CTs were run with the following aims:

- The aim of the 1st CT was to evaluate the ability to reproduce the SSR analysis results between laboratories.
- The aim of the 2nd CT was to compare results among participant laboratories and evaluate if it is possible to obtain the same band pattern and same allele sizes even when using different reactants, equipment and working protocols. Varieties and SSR markers were the same for all participant laboratories. Some varieties and SSR were the same as in the previous CT and others were new.
- The aim of the 3rd CT was to compare results among participant laboratories and evaluate if it is possible to obtain the same band pattern and same allele sizes even when using different reactants, equipment and working protocols, and also to extend further the range of varieties tested for identification by the SSR markers validated during the 1st and 2nd CTs:

Materials and methods

Seed samples

For the 1st CT

The participating laboratories analyzed 8 maize inbred lines using 12 SSRs, under the analysis conditions of each participating laboratory.

Participating laboratories were given the following:

- primer sequences and all information available on the 12 SSRs selected
- one gram/variety of powders derived from ground seeds (30-50 kernels)
- Protocols used in BioGEVES for information.

For the 2nd CT

Participating laboratories received samples of new lines and also used some of the lines from the 1st CT. Some SSR were replaced by new ones and others were run again on the new and old set of lines.

For the 3rd CT

Laboratories received 24 lines previously tested and a list of SSRs composed of SSRs selected from those run in previous CTs and new SSRs to be tested for the first time in the 3rd CT.

The number of varieties and SSRs changed among CTs. New varieties were added with the aim to find more alleles for the markers tested. Markers that showed most divergent results, problems for scoring and heterogeneity for varieties were marked as observed. Markers that clearly did not perform well in CT1 or CT2 were discharged and replaced with new ones.

Equipment chemicals and procedures

This was as stated by each laboratory, as the strategy for inclusion on DNA-based techniques into the ISTA Rules is semi-performance based, although guidance was given for all CTs.

Evaluation and reporting of results

The evaluation of the data to select SSR useful for next runs and possible SSR to keep for the ISTA Rules proposals, were based on pattern similarity observed in the summarised data. Data analysis was performed by the ISTA Statistics TCOM.

Final comments and conclusions

For the statistical analysis, overall percentage agreements (p_a) and Cohen's kappas have been computed for all the possible laboratory pairs and considering as units either the marker alleles or the varieties.

The results indicate that agreement for scoring varieties across laboratories is quite good (Fleiss's kappa above 81% except for variety 16). Some marker alleles have very low Fleiss's kappa (below 40%). Laboratory 5 and sometimes laboratory 3 appear not to be in agreement with the others.

For marker selection the following criteria was applied:

$\hat{\kappa}_C$	**Interpretation**
< 0	No agreement
0.0 — 0.20	Slight agreement
0.21 — 0.40	Fair agreement
0.41 — 0.60	Moderate agreement
0.61 — 0.80	Substantial agreement
0.81 — 1.00	Almost perfect agreement

Landis and Koch table for the interpretation of $\hat{\kappa}_C$.

For maker selection the data was quantified based on plots per marker and per allele and only selected markers that had “substantial agreement” or “almost perfect agreement” were selected for inclusion in the ISTA Rules method proposal.

The list of markers selected is:

Marker	Approximate allele size range (bp)
umc1545	66-81
umc1448	165-180
umc1117	140-168
umc1061	99-108
phi109275	123-138
phi102228	123-129
phi083	123-135
phi015	81-102

Acknowledgements

Thanks to the staff at the following laboratories for participating in the study: CFIA Ottawa Plant Laboratory, Canada; COODETEC, Brazil; ENSE, Italy; INASE, Argentina; GEVES-Biogeves, France; Pioneer, USA; SGS, USA; and Syngenta, USA. Also thanks to the people responsible for the work, especially to Emanuela Casarini who was the former DNA working group leader.

Also a special thanks to the technical assessors: Verena Peterseil from AGES Austria and Anne Bernole from GEVES France for the revision of the results and Kirk Remund for the statistical revision.

In particular thanks to Ana Laura Vicario as Chair of the ISTA Variety (TCOM) for coordinating the activity, David Zhang as test leader, Nadine Ettel for her support during the method validation process and Daniel Perry for preparing the final version of the validation report. Special thanks to Kirk Remund as ISTA Statistics TCOM member and Jean-Louis Laffont as ISTA Statistics TCOM Chair for their ongoing support of the ISTA validation process and the statistics involved in this validation analysis report in particular.

Appendix 1:

Validation of a new method for "Microsatellite marker analysis for maize variety verification": statistical analysis of the CT results

Jean-Louis Laffont, ISTA Statistics Committee

1. Materials and methods

Allele results (0 or 1) from a Comparative Test (CT) for evaluating the performance of several microsatellite markers are available for 8 laboratories and 24 varieties. Figure 1 summarizes the structure of the data.

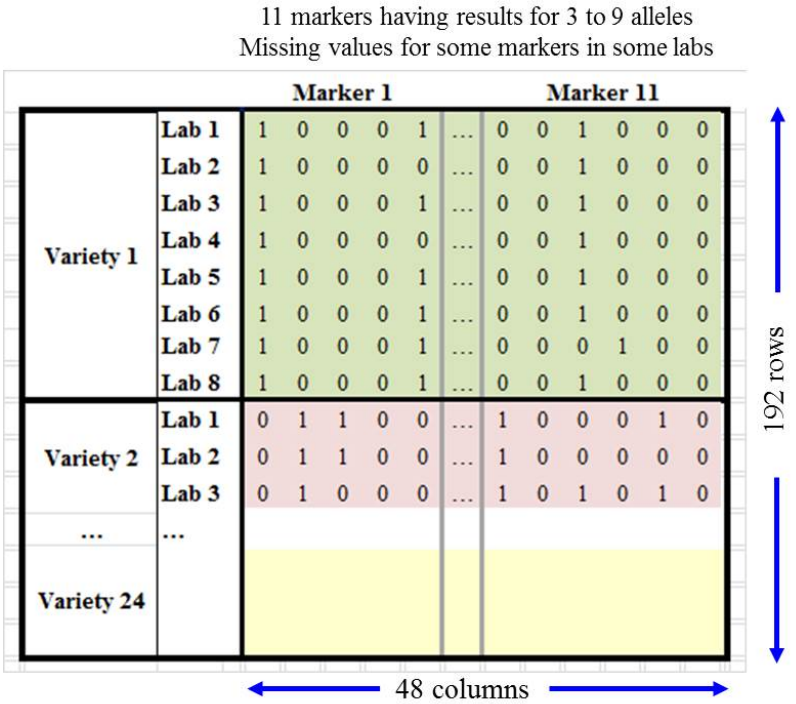

Figure 1: Structure of the data analyzed.

Consider two laboratories and the allele results (0 or 1) for one variety and for the different markers. We elaborate first a coincidence matrix giving the number of 0's and 1's observed in both laboratories and the number of 0's and 1's observed only in one of the two laboratories. Table 1 is an example of such a table. There is a total of 41 alleles and the two laboratories provide same results on 30 + 6 = 36 alleles leading to an overall percent agreement of 36 / 41 = 87.8%. However, this percent agreement is overestimated as agreement between the two laboratories can be due by chance only. That is the reason why many reliability measures taking into account the possible chance agreement have been developed. The most popular one for two laboratories is the Cohen's kappa coefficient (Cohen, 1960). It is computed as:

$$\hat{\kappa}_C = \frac{p_a - p_e}{1 - p_e}$$

in which:

. p_a is the overall percent agreement: $p_a = \frac{n_{00} + n_{11}}{n}$ using the notations in Table 2.

. p_e is the chance agreement probability computed by Cohen (1960) as

$p_e = \frac{n_{0+}}{n} \times \frac{n_{+0}}{n} + \frac{n_{1+}}{n} \times \frac{n_{+1}}{n}$ (other authors have proposed some other ways to compute p_e; see Krippendorff, 2004, for a review of the different ways of computing p_e). In the example, this gives $p_e = \frac{32}{41} \times \frac{33}{41} + \frac{9}{41} \times \frac{8}{41} = 0.671$ and therefore $\hat{\kappa}_C = \frac{0.878 - 0.671}{1 - 0.671} = 0.629$.

The range of possible values of $\hat{\kappa}_C$ is from -1 to 1. A value of 1 represents perfect agreement, 0 indicates agreement no better than that expected by chance, and a negative value indicates an agreement worse than that expected by chance (Sim and Wright, 2005). Although there is no universally accepted magnitude guidelines on the value of $\hat{\kappa}_C$ for characterizing agreement, we can use the ones established by Landis and Koch (1977) which are summarized in Table 3.

When the number of laboratories is greater than two, a popular measure of the reliability of agreement between the laboratories is the Fleiss' kappa (1971).

2. Results

Overall percentage agreements (p_a) and Cohen's kappas have been computed for all the possible laboratory pairs and considering as units either the marker alleles or the varieties. The computations have been performed with the R *irr* package (Gamer *et al*., 2012) which includes functions for computing various coefficients of reliability of agreement.

Figures 2 - 5 visualize with dot plots the overall percentages agreements; Figures 6 - 9 visualize Cohen's kappas. Table 4 provides the Fleiss's kappas; Tables 5 and 6 provide the percentages of varieties and the percentage of alleles respectively having a $\hat{\kappa}_C$ value falling in one of the categories defined by Landis and Koch (1977).

Agreement for scoring varieties across laboratories is quite good (Fleiss's kappa above 81% except for variety 16). Some marker alleles have very low Fleiss's kappa (below 40%). Laboratory 5 appears not to be in agreement with the others.

		Lab 2		
		0	**1**	**Total**
Lab 1	**0**	30	2	32
	1	3	6	9
	Total	33	8	41

Table 1: Coincidence matrix example.

		Lab 2		
		0	**1**	**Total**
Lab 1	**0**	n_{00}	n_{01}	n_{0+}
	1	n_{10}	n_{11}	n_{1+}
	Total	n_{+0}	n_{+1}	n

Table 2: Abstract coincidence matrix.

$\hat{\kappa}_C$	**Interpretation**
< 0	No agreement
0.0 — 0.20	Slight agreement
0.21 — 0.40	Fair agreement
0.41 — 0.60	Moderate agreement
0.61 — 0.80	Substantial agreement
0.81 — 1.00	Almost perfect agreement

Table 3: Landis and Koch table for the interpretation of $\hat{\kappa}_C$.

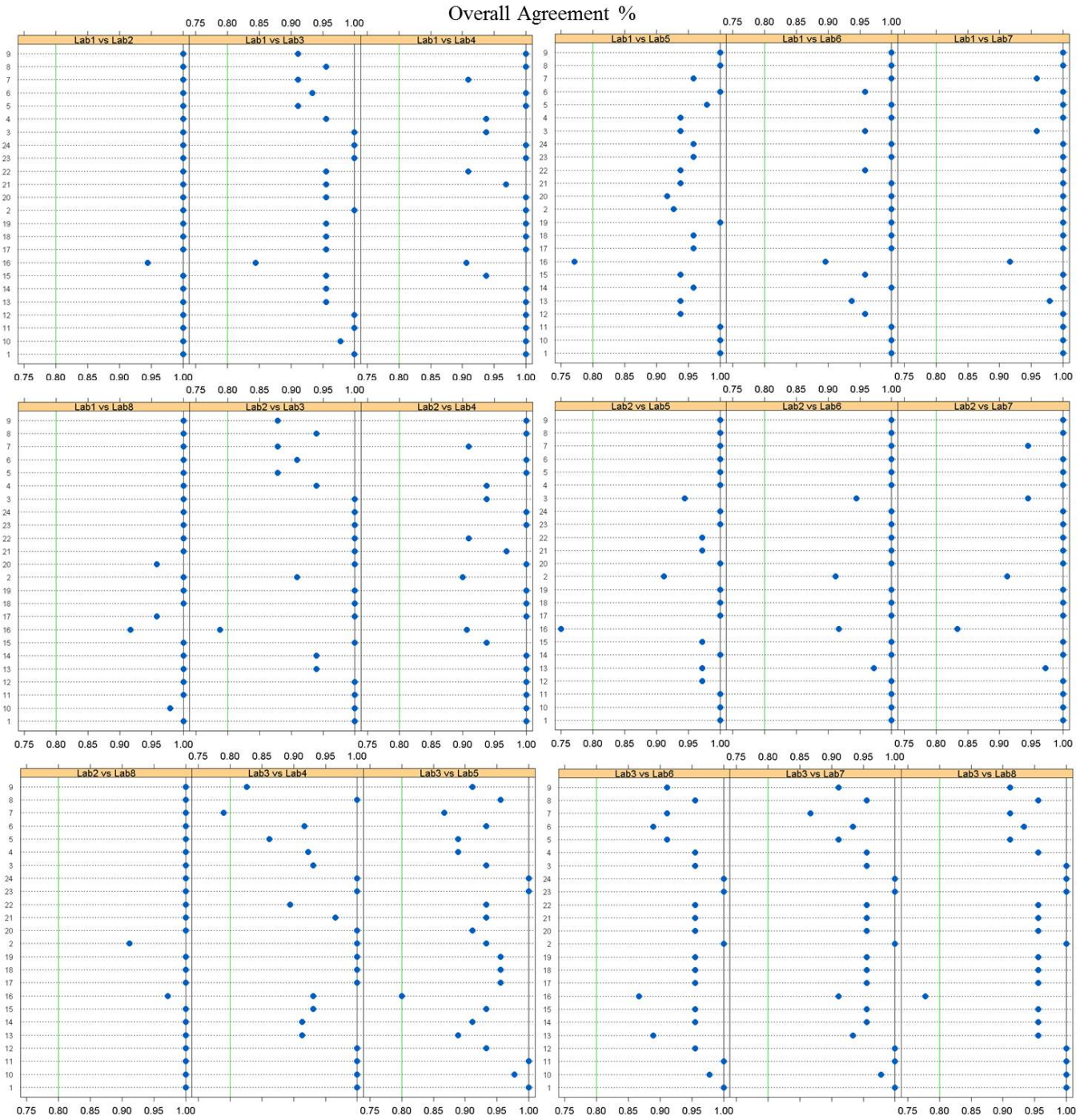

Figure 2: Overall percentage agreements considering allele results agreement for a given variety.

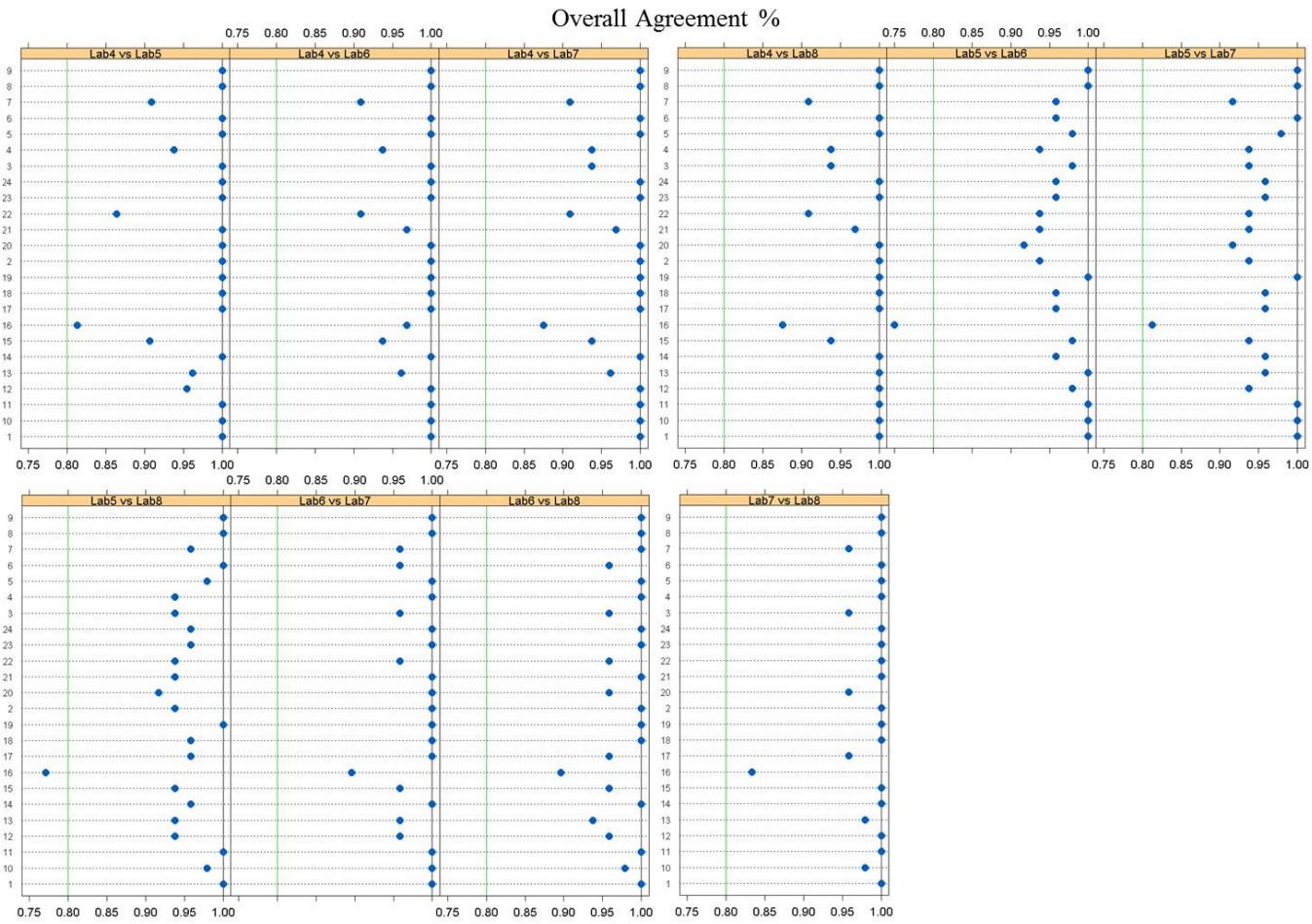

Figure 3: Overall percentage agreements considering allele results agreement for a given variety.

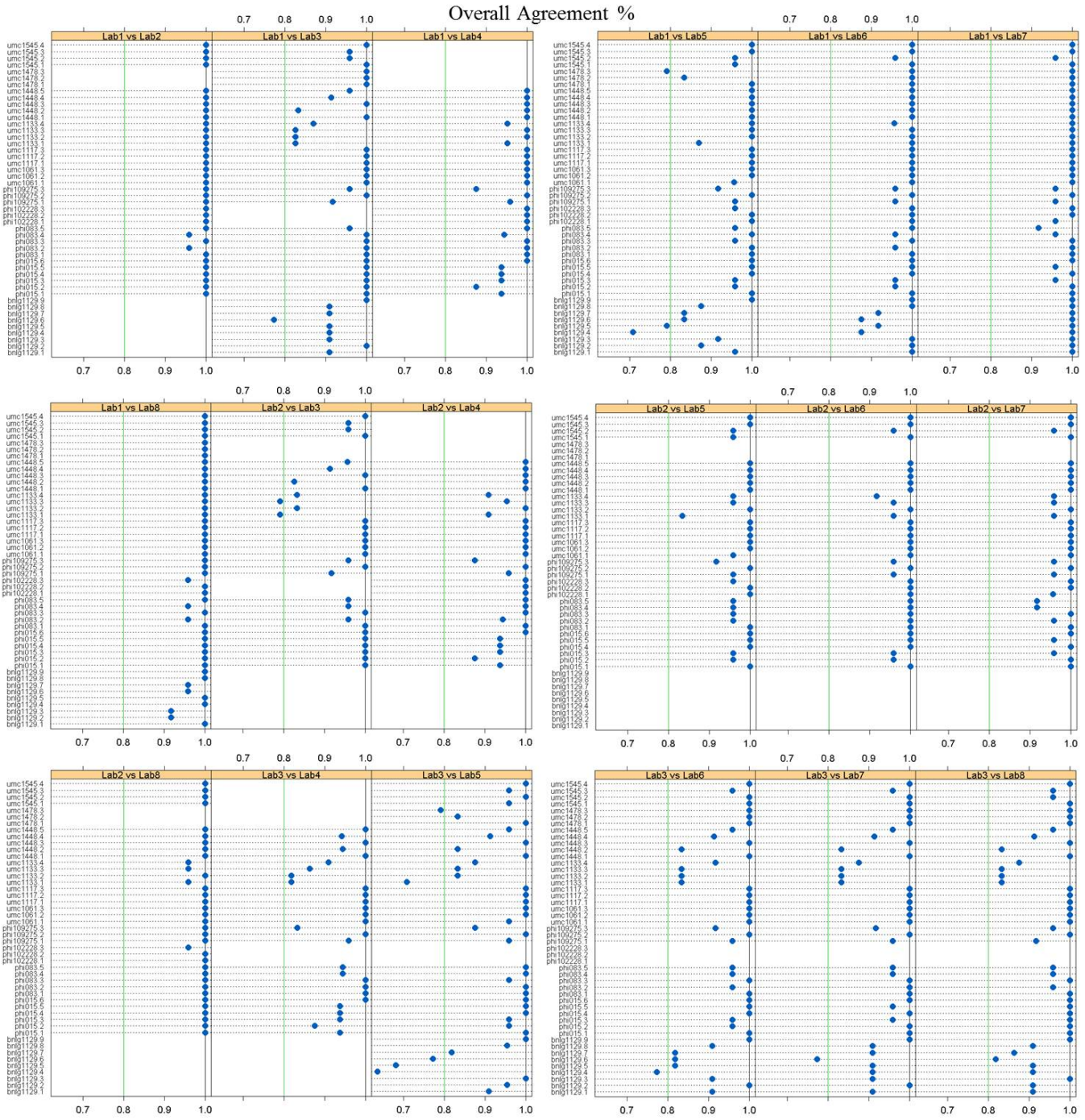

Figure 4: Overall percentage agreements considering allele results agreement across varieties for a given allele.

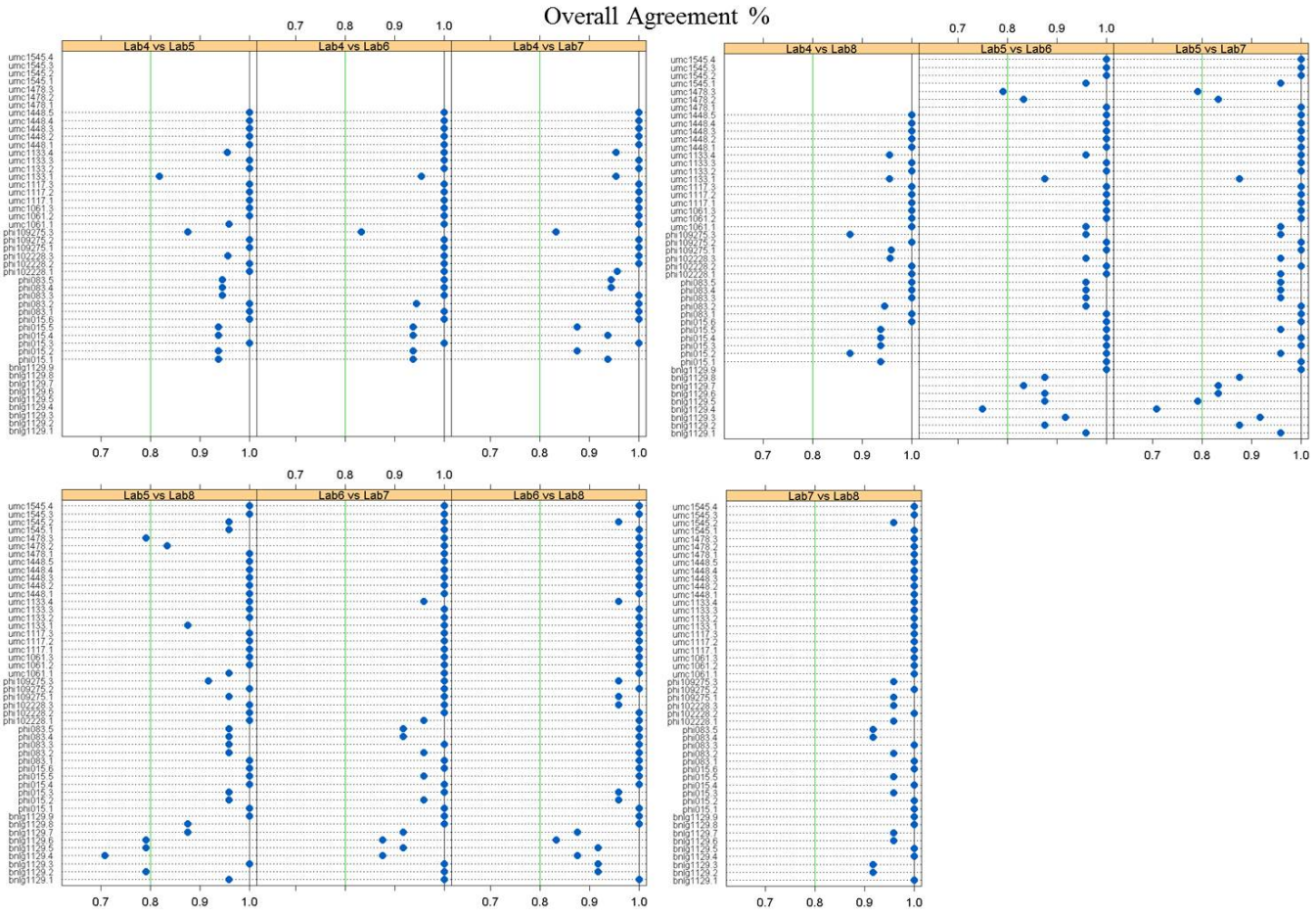

Figure 5: Overall percentage agreements considering allele results agreement across varieties for a given allele.

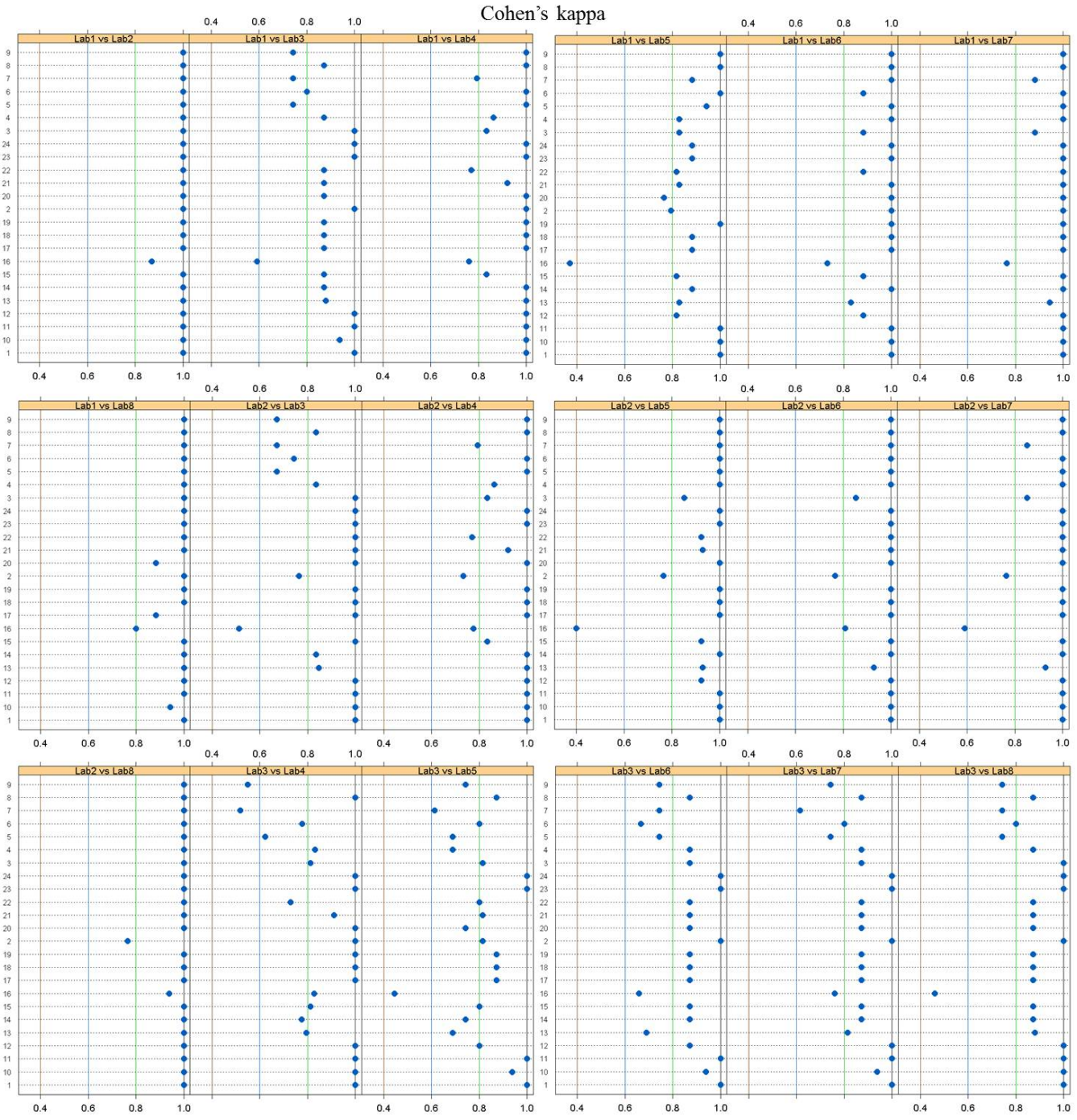

Figure 6: Cohen's kappas considering allele results agreement for a given variety.

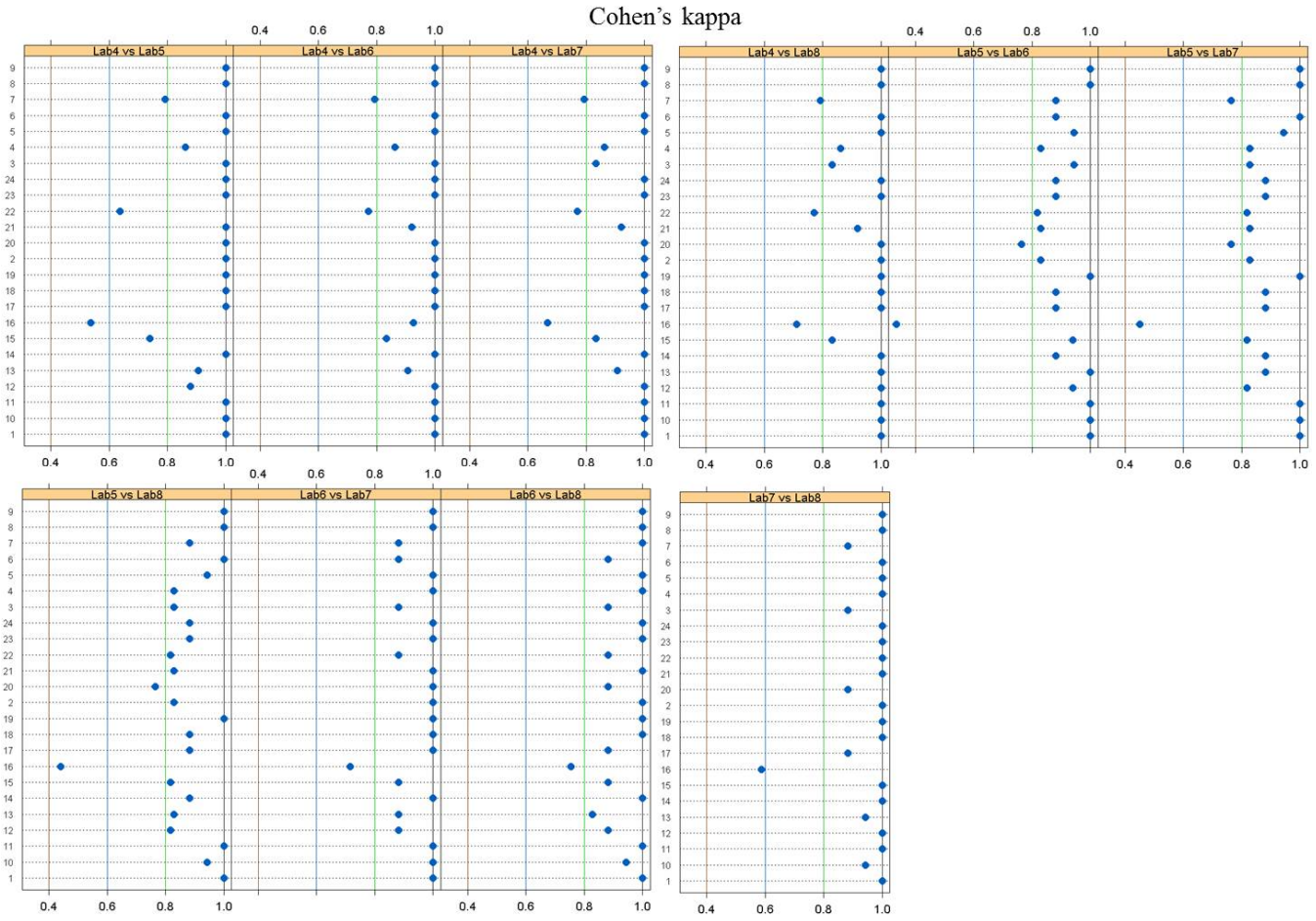

Figure 7: Cohen's kappas considering allele results agreement for a given variety.

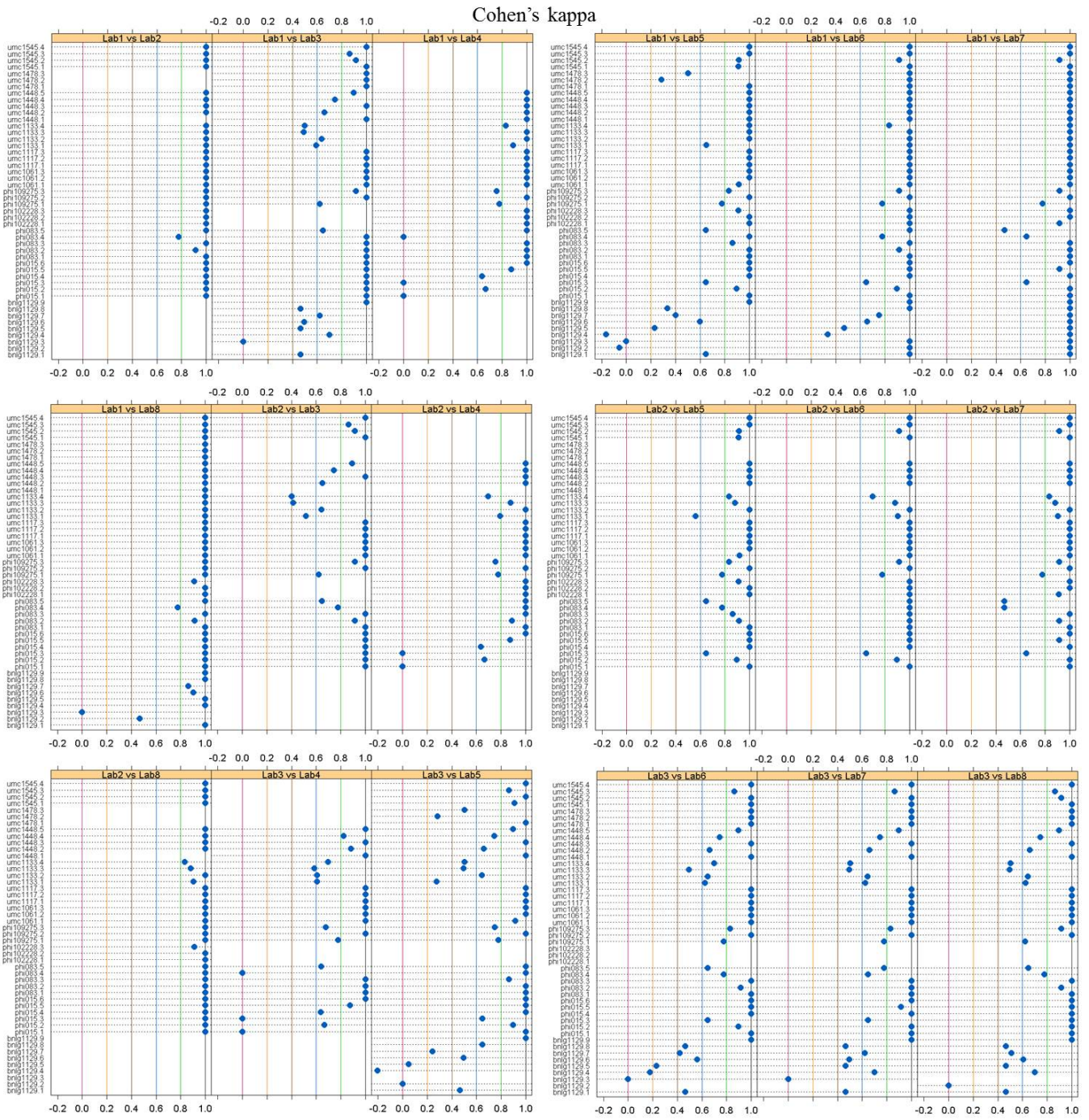

Figure 8: Cohen's kappas considering allele results agreement across varieties for a given allele.

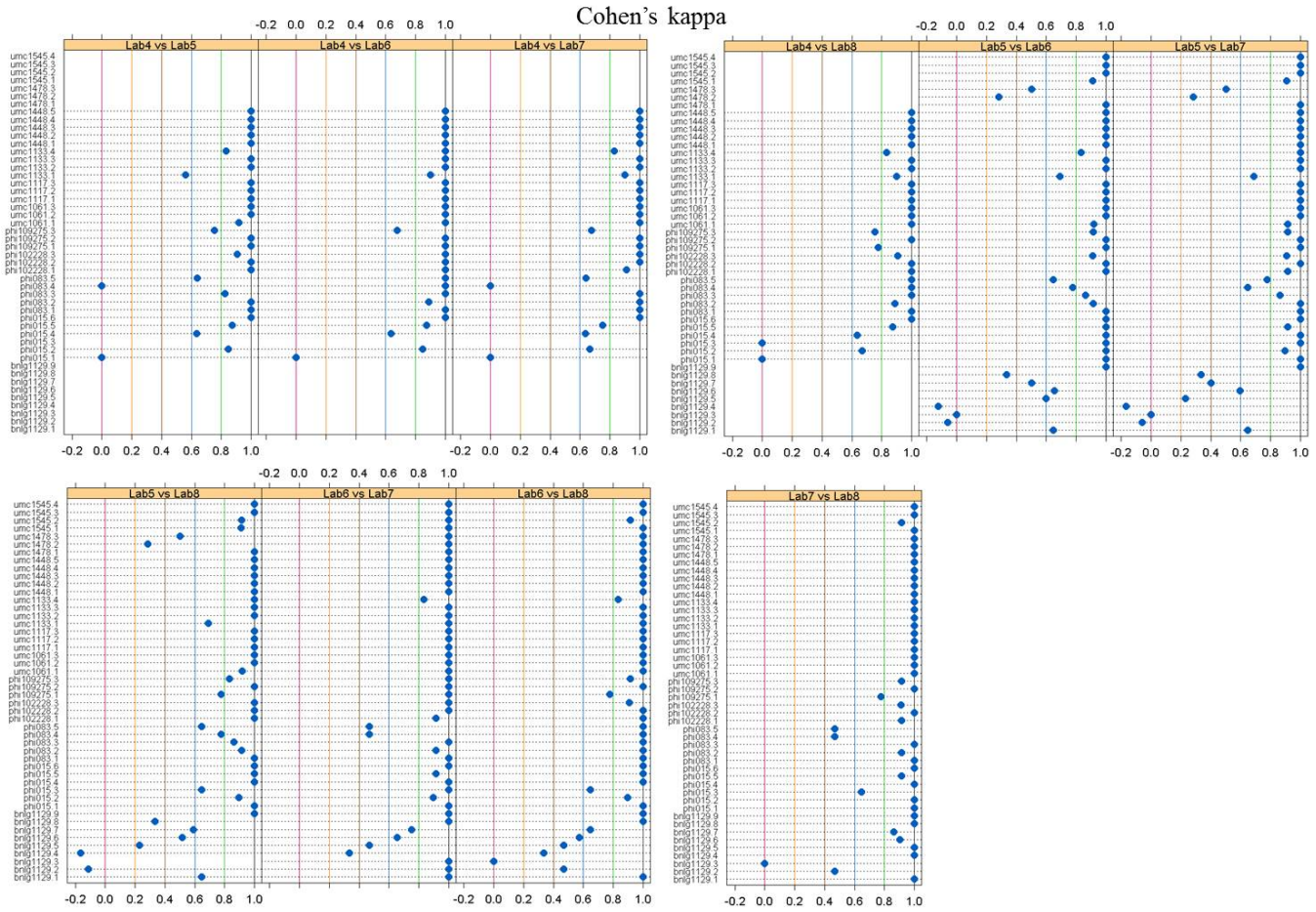

Figure 9: Cohen's kappas considering allele results agreement across varieties for a given allele.

Variety	Fleiss's kappa
Var1	100.0%
Var10	100.0%
Var11	100.0%
Var12	96.6%
Var13	89.0%
Var14	94.4%
Var15	92.9%
Var16	72.3%
Var17	100.0%
Var18	100.0%
Var19	100.0%
Var2	100.0%
Var20	100.0%
Var21	96.1%
Var22	89.7%
Var23	100.0%
Var24	100.0%
Var3	87.2%
Var4	95.6%
Var5	90.6%
Var6	94.4%
Var7	86.8%
Var8	100.0%
Var9	88.7%

Marker allele	Fleiss's kappa
phi109275.1	82.5%
phi109275.2	100.0%
phi109275.3	85.6%
phi102228.1	97.4%
phi102228.2	100.0%
phi102228.3	95.5%
phi083.1	100.0%
phi083.2	94.0%
phi083.3	95.2%
phi083.4	41.2%
phi083.5	78.9%
umc1448.1	-
umc1448.2	96.8%
umc1448.3	100.0%
umc1448.4	95.8%
umc1448.5	100.0%
umc1117.1	100.0%
umc1117.2	100.0%
umc1117.3	100.0%
umc1545.1	97.4%
umc1545.2	95.2%
umc1545.3	95.8%
umc1545.4	100.0%

Marker allele	Fleiss's kappa
phi015.1	-0.8%
phi015.2	87.4%
phi015.3	41.0%
phi015.4	88.0%
phi015.5	93.7%
phi015.6	100.0%
bnlg1129.1	73.4%
bnlg1129.2	-2.3%
bnlg1129.3	37.1%
bnlg1129.4	37.3%
bnlg1129.5	38.3%
bnlg1129.6	63.9%
bnlg1129.7	61.5%
bnlg1129.8	68.4%
bnlg1129.9	100.0%
umc1478.1	100.0%
umc1478.2	81.2%
umc1478.3	78.4%
umc1061.1	97.8%
umc1061.2	100.0%
umc1061.3	100.0%
umc1133.1	79.3%
umc1133.2	89.9%
umc1133.3	90.0%
umc1133.4	81.7%

Table 4: Fleiss’s kappa for variety’s scoring and allele’s scoring.

% of varieties for which $\hat{\kappa}_C$ is	< 0	0 – 0.2	0.21 – 0.40	0.41 – 0.60	0.61 – 0.80	0.81– 1.00
Lab1 vs Lab2	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%
Lab1 vs Lab3	0.0%	0.0%	0.0%	4.2%	16.7%	79.2%
Lab1 vs Lab4	0.0%	0.0%	0.0%	0.0%	12.5%	87.5%
Lab1 vs Lab5	0.0%	0.0%	4.2%	0.0%	8.3%	87.5%
Lab1 vs Lab6	0.0%	0.0%	0.0%	0.0%	4.2%	95.8%
Lab1 vs Lab7	0.0%	0.0%	0.0%	0.0%	4.2%	95.8%
Lab1 vs Lab8	0.0%	0.0%	0.0%	0.0%	4.2%	95.8%
Lab2 vs Lab3	0.0%	0.0%	0.0%	4.2%	20.8%	75.0%
Lab2 vs Lab4	0.0%	0.0%	0.0%	0.0%	16.7%	83.3%
Lab2 vs Lab5	0.0%	0.0%	4.2%	0.0%	4.2%	91.7%
Lab2 vs Lab6	0.0%	0.0%	0.0%	0.0%	4.2%	95.8%
Lab2 vs Lab7	0.0%	0.0%	0.0%	4.2%	4.2%	91.7%
Lab2 vs Lab8	0.0%	0.0%	0.0%	0.0%	4.2%	95.8%
Lab3 vs Lab4	0.0%	0.0%	0.0%	8.3%	20.8%	70.8%
Lab3 vs Lab5	0.0%	0.0%	0.0%	4.2%	45.8%	50.0%
Lab3 vs Lab6	0.0%	0.0%	0.0%	0.0%	25.0%	75.0%
Lab3 vs Lab7	0.0%	0.0%	0.0%	0.0%	20.8%	79.2%
Lab3 vs Lab8	0.0%	0.0%	0.0%	4.2%	16.7%	79.2%
Lab4 vs Lab5	0.0%	0.0%	0.0%	4.2%	12.5%	83.3%
Lab4 vs Lab6	0.0%	0.0%	0.0%	0.0%	8.3%	91.7%
Lab4 vs Lab7	0.0%	0.0%	0.0%	0.0%	12.5%	87.5%
Lab4 vs Lab8	0.0%	0.0%	0.0%	0.0%	12.5%	87.5%
Lab5 vs Lab6	0.0%	0.0%	4.2%	0.0%	4.2%	91.7%
Lab5 vs Lab7	0.0%	0.0%	0.0%	4.2%	8.3%	87.5%
Lab5 vs Lab8	0.0%	0.0%	0.0%	4.2%	4.2%	91.7%
Lab6 vs Lab7	0.0%	0.0%	0.0%	0.0%	4.2%	95.8%
Lab6 vs Lab8	0.0%	0.0%	0.0%	0.0%	4.2%	95.8%
Lab7 vs Lab8	0.0%	0.0%	0.0%	4.2%	0.0%	95.8%

Table 5: For each pair of laboratories, percentage of varieties having a $\hat{\kappa}_C$ value falling in one of the categories defined by Landis and Koch (1977).

% of alleles for which $\hat{\kappa}_C$ is	< 0	0 – 0.2	0.21 – 0.40	0.41 – 0.60	0.61 – 0.80	0.81– 1.00
Lab1 vs Lab2	0.0%	0.0%	0.0%	0.0%	2.9%	97.1%
Lab1 vs Lab3	0.0%	2.3%	0.0%	15.9%	15.9%	65.9%
Lab1 vs Lab4	0.0%	9.4%	0.0%	0.0%	12.5%	78.1%
Lab1 vs Lab5	4.2%	2.1%	8.3%	4.2%	10.4%	70.8%
Lab1 vs Lab6	0.0%	0.0%	2.1%	2.1%	10.4%	85.4%
Lab1 vs Lab7	0.0%	0.0%	0.0%	2.1%	6.3%	91.7%
Lab1 vs Lab8	0.0%	2.1%	0.0%	2.1%	2.1%	93.8%
Lab2 vs Lab3	0.0%	0.0%	3.1%	6.3%	18.8%	71.9%
Lab2 vs Lab4	0.0%	6.5%	0.0%	0.0%	19.4%	74.2%
Lab2 vs Lab5	0.0%	0.0%	0.0%	2.9%	11.4%	85.7%
Lab2 vs Lab6	0.0%	0.0%	0.0%	0.0%	8.6%	91.4%
Lab2 vs Lab7	0.0%	0.0%	0.0%	5.7%	5.7%	88.6%
Lab2 vs Lab8	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%
Lab3 vs Lab4	0.0%	10.3%	0.0%	3.4%	27.6%	58.6%
Lab3 vs Lab5	2.3%	4.5%	6.8%	11.4%	15.9%	59.1%
Lab3 vs Lab6	0.0%	4.5%	2.3%	11.4%	20.5%	61.4%
Lab3 vs Lab7	0.0%	2.3%	0.0%	13.6%	22.7%	61.4%
Lab3 vs Lab8	0.0%	2.3%	0.0%	13.6%	20.5%	63.6%
Lab4 vs Lab5	0.0%	6.5%	0.0%	3.2%	9.7%	80.6%
Lab4 vs Lab6	0.0%	3.2%	0.0%	0.0%	6.5%	90.3%
Lab4 vs Lab7	0.0%	6.5%	0.0%	0.0%	16.1%	77.4%
Lab4 vs Lab8	0.0%	6.3%	0.0%	0.0%	12.5%	81.3%
Lab5 vs Lab6	4.2%	2.1%	4.2%	6.3%	10.4%	72.9%
Lab5 vs Lab7	4.2%	2.1%	8.3%	4.2%	8.3%	72.9%
Lab5 vs Lab8	4.3%	0.0%	6.4%	6.4%	12.8%	70.2%
Lab6 vs Lab7	0.0%	0.0%	2.1%	6.3%	4.2%	87.5%
Lab6 vs Lab8	0.0%	2.1%	2.1%	6.3%	6.3%	83.3%
Lab7 vs Lab8	0.0%	2.1%	0.0%	6.3%	4.2%	87.5%

Table 6: For each pair of laboratories, percentage of alleles having a $\hat{\kappa}_C$ value falling in one of the categories defined by Landis and Koch (1977).

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