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Validation study for germination test of *Carica papaya* L.

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Summary

The objective of this validation test is to introduce a germination method for *Carica papaya* L. into Chapter 5 of ISTA Rules. The experiment was carried out by seven ISTA-accredited laboratories using three seed lots. The papaya seeds were germinated on a sand or between paper medium, using the alternating temperature regime 20<=>30 °C. Light was supplied for 8 hours during the high-temperature phase, and germination counts were made after 12 (first count) and 28 days (final count). Dormancy breaking treatments using seed soaking in water or in a giberrellic acid solution have been tested.

All the methods tested gave comparable results in all the laboratories except on that has been removed from the statistical analysis.

Based on the results of repeatability and reproducibility, the germination method proposed to be included in ISTA Rules for *Carica papaya* seeds is: Sand; 20⇔30°C; 12 - 28 days; with a pre-treatment consisting of soaking the seeds in water, with or without GA_3 added in the substrate.

Introduction

Carica papaya L. is originated from southern Mexico, Central America, and northern South America, and is now cultivated in most tropical countries. In cultivation, it grows rapidly, fruiting within three years. It is, however, highly frost-sensitive, limiting its production to tropical lands. *Carica papaya* L. is an important economical fruit crop in not only in Separate Custom Territory of Taiwan but also other tropical countries.

Preliminary experiment conducted in the lab of Known-You Seed (Separate Custom Territory of Taiwan) confirmed that the optimum germination temperature for *Carica papaya* L. was 20⇔30°C. In addition, seeds have shown physical and physiological dormancy and as a consequence, soaking treatments in water or in gibberelic acid solution have been proposed to overcome seed dormancy.

A multi-laboratory collaborative validation test is proposed in order to develop an ISTA germination method for *Carica papaya* L.

Material and methods

Seed material

Three lots of untreated *Carica papaya* L. seed were obtained from Known-You Seed Co., Ltd. and sent to the participating laboratories. The three seed lots had different level of germination quality: low level (70-80%), intermediate level (80-90%) and high level (above 90%). The seed lots come from three different commercial varieties used both in Asia and Central America.

Participating laboratories

Sample were sent to two ISTA-accredited laboratories in Separate Custom Territory of Taiwan (TW01), Thailand (THDL0102), and five laboratories who have experience in *Carica papaya* L. germination testing in Separate Custom Territory of Taiwan (Known-You Seed Co., Ltd.), Separate Custom Territory of Taiwan (Department of Horticulture, National Chung Hsing University), Thailand (EAST WEST SEED COMPANY LIMITED), Indonesia (PT EAST WEST SEED INDONESIA) and Indonesia (Seed Testing Laboratory of Balai Besar PPMB-TPH).

Pre-treatments

Three different pre-treatments have been applied based on in-house studies done in the Known-You Seed Company laboratory and the results of Bhattacharya and Khuspe (2001).

a. Without soaking in Sand and Between Paper

b. Soaking in water for 16 hours before sowing the seeds in Sand and Between Paper

c. Soaking in water for 16 hours before sowing the seeds in Sand and Between Paper. In this treatment, the germination substrates are moistened with 0.05% solution of GA_3 (prepared by dissolving 500mg GA3 in 1 liter of water).

Germination methods

The three seed lots were tested on sand (S) or between-of-paper (BP) medium, using the alternating temperature regime 20⇔30 °C (20°C maintained for 16 hours, and 30°C for 8 hours). Light was supplied for 8 hours in every 24 hours' cycle during the high temperature (30°C) period. The light intensity was approximately 750-1250 lux from cool white lamps.

For each lot, a total of 400 seeds were tested in replicates of 100 seeds. Germination counts were made after 12 (first count) and 28 days (final count).

The evaluation of the seedlings was done in accordance with the ISTA Seedling Type E – Seedling Group A-2-1-2-3.

Table 1. Germination testing conditions on BP at 20⇔30 °C depending on laboratories

Lab No.	Germination apparatus	Type of box / plate (size)	Type of substrate (size)	Type of water (amount)	No. of seeds per replicate
1	Incubator	Plastic box (38.5 x 30 cm)	Paper (27 x 30 cm)	Deionized water (18 mL per replicate; 3.5 mL per gram paper)	100
2	Room germinator	Germination box (11.5× 19.5 × 7cm)	Filter paper, 2 layers (20 ×13 cm)	Deionized water (11 mL per replicate; 3.1 mL per gram paper)	100
3	Tissue culture ESⅢ-IBM	Plastic box (9 cm x 9 cm x 10.2 cm)	Advantec filter paper NO1. 90 mm	Lower filter paper add 2cc DI water, and upper filter paper add 2cc DI water	100
4	Room germinator	Plastic bag 9'' X 14''	Paper 3 layers (25 x 34cm)	RO water (22.5 mL per replicate)	50
5	Room germinator	Germination box (37 x 30 x 34cm)	Filter paper, 4 layers (33 ×25.5 cm)	Tap water (416ml/15.5gr paper)	100
6	Germinator cabinet	---	Filter, newsprint l x w = 54 x 40 cm	Tap water	100 seeds

7	Room germinator	Paper roll in Plastic basket cover with Plastic bag	Brown Paper (10 x 14 in)	Tap water	50 seeds / rep x 8

Table 2. Germination testing conditions on Sand at 20⇔30 °C depending on laboratories

Lab No.	Germination apparatus	Type of box / plate (size)	Type of substrate	Type of water (amount)	No. of seeds per replicate
1	Incubator	Germination box (26.5 x 16 x 11 cm)	Sand	Deionized water (105 ml per replicate; 0.07 ml per gram sand)	100
2	Room germinator	Germination box (13×21.5 ×6.5cm)	Sand, bottom layer 500g and cover with 250g on upper layer per replicate (at least 90% of the particles pass through a sieve with meshes of 2.0 mm width)	Deionized water (214 ml per kilogram; 160.5 ml per replicate)	100
3	Tissue culture ESⅢ-IBM	Plastic box (22 cm x 13.5 cm x 6.5 cm)	Sand	Add 120 cc deionized water in the box	100
4	Room germinator	Germination box (15.5 x 21.5 x 7.5cm)	Sand (0.05-0.8mm)	RO water (water : sand is 1:7)	50
5	Room germinator	Germination box (37 x 30 x 34cm)	River sand	Tap water (28.9ml/966 gr sand)	100
6	---	Plastic germination box l x w x h: 14x 9 x6 cm	Sand	Tap water	25 seeds
7	Room germinator	Plastic box (20 cm x 28 cm x 10cm)	Sand	Tap water	100 seeds /rep x 4

Statistical analysis of the results

Germination results were checked to make sure that the sum of the percentages was equal to 100%. The performance of the method was assessed by interpreting the fit of a binomial Generalized Linear Model.

Germination results of the three seed lots

The figure 1 presents the data of the percentage of normal seedlings obtained for all the laboratories and all the methods, for each sample.

The sample 1 gives 74.21% of normal seedlings, the sample 2 gives 83.51% of normal seedlings and the sample 3 gives 91.83% of normal seedlings. The three samples were therefore very well chosen to exhibit a range of germination quality of the Papaya seeds.

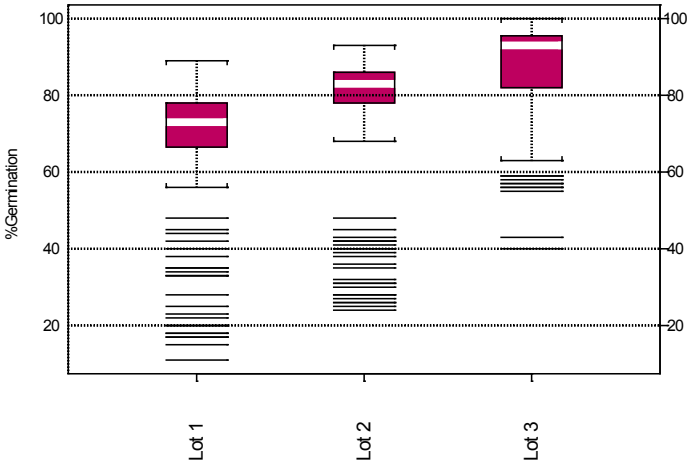

Figure 1. Data (% of normal seedlings) for all the laboratories and all the methods, per sample.

Germination results obtained by the different laboratories

The figure 2 presents the data of the percentage of normal seedlings obtained for all the samples and all the methods, for each laboratory.

The figure shows very clearly that the results obtained from the laboratory 6 are significantly different and lower than the results from the other laboratories. It has therefore been decided to exclude the results from the laboratory 6 for the rest of the analysis.

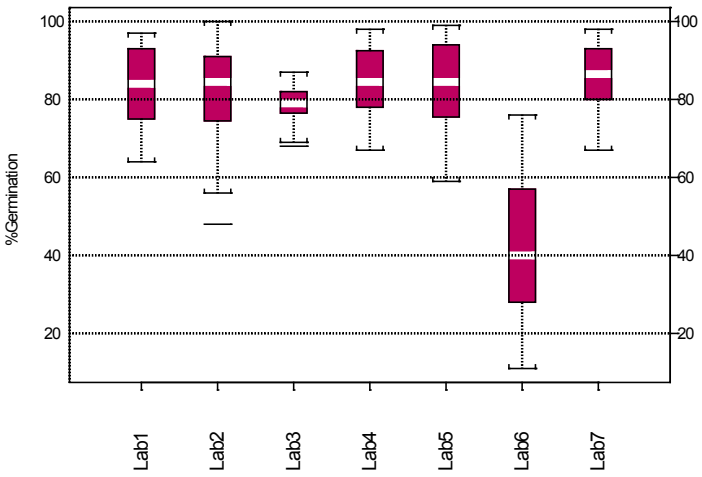

Figure 2. Data (% of normal seedlings) for all the samples and all the methods, per laboratory.

Germination results obtained with the different testing methods

Effect of the testing method

The figure 3 presents the data of the percentage of normal seedlings obtained for all the samples and all the laboratories (without laboratory 6), depending on the seed testing method. The figure 4 presents the same data from each seed lot and the table 3 includes the statistical tests of fixed effects for “lot”, “method” and “lot*method”. All these results show that there is a strong effect of the seed sample but not effect of the testing method.

It means that all the methods tested on these samples are equivalent for testing the seed lots.

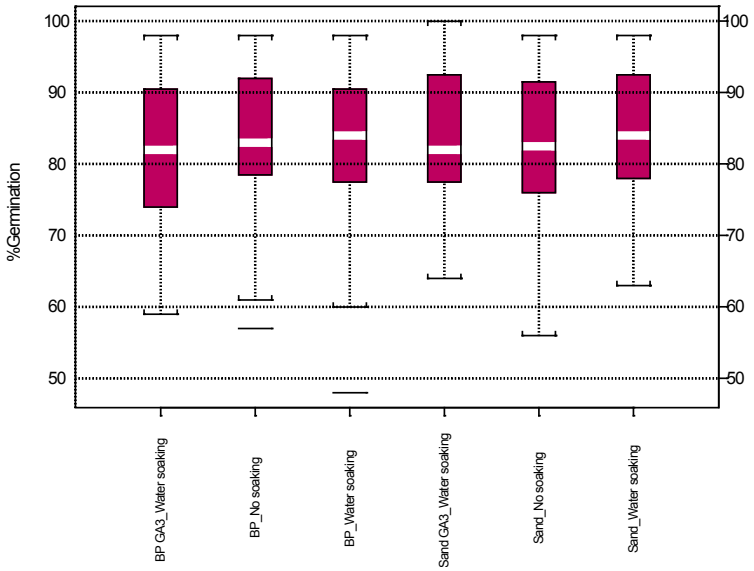

Figure 3 : Effect of the testing method on germination (% of normal seedlings) results. The results coming from the laboratory 6 have been excluded.

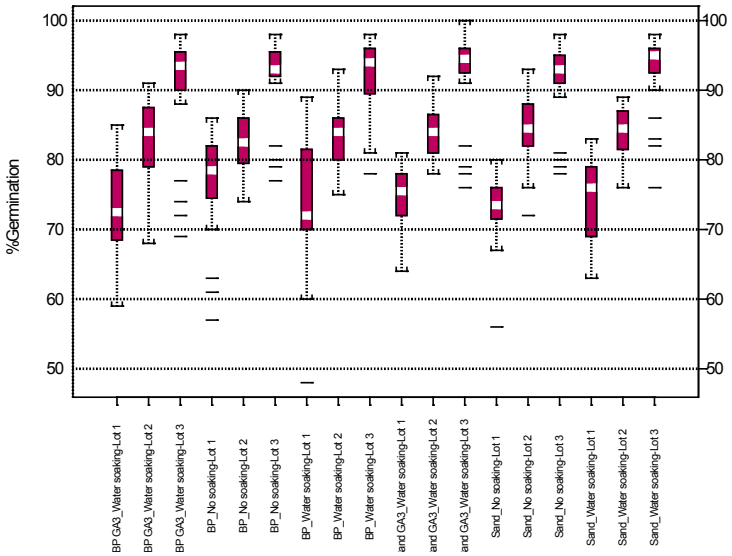

Figure 4 : Effect of the testing method on germination (% of normal seedlings) results, per sample. The results coming from the laboratory 6 have been excluded.

Table 3: Tests of fixed effects for “lot”, “method” and “lot*method”.

Effect	Num DF	Den DF	F Value	Pr > F
LOT	2	10	22.83	0.0002
Method	5	25	0.6	0.6998
LOT*Method	10	374	1.63	0.0956

In order to better evaluate the quality of the testing methods used for the germination of the Papaya seed samples, a statistical analysis has been done to assess the repeatability and the reproducibility of the different methods tested.

Repeatability of the testing methods

For each method, let:

. *I* be the total number of lots

. *J* be the total number of labs

. *K* be the number of reps of *m* seeds for a given lot in a given lab

. p_{ijk} be the percentage of germinated seeds for lot *i*, lab *j* and rep *k*

The repeatability standard-deviation is computed as:

$$S_r = \sqrt{f_r^2 \frac{\overline{p}_{...}\left(100 - \overline{p}_{...}\right)}{m}}$$

where:

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

. f_r^2 is an estimate of the dispersion parameter:

$$f_r^2 = \frac{1}{IJ}\sum_{i,j}\frac{var\_obs_{ij}}{var\_bin_{ij}}$$

where:

$var\_obs_{ij} = \frac{1}{K-1}\sum_k\left(p_{ijk} - \overline{p}_{ij.}\right)^2$ and $var\_bin_{ij} = \frac{\overline{p}_{ij.}\left(100 - \overline{p}_{ij.}\right)}{m}$ with $\overline{p}_{ij.}$ being the average percentage of germinated seeds in lot *i* and lab *j*

If f_r^2 >1 one speaks of over dispersion because the data have larger variance than expected under the assumption of a binomial distribution.

The results of f_r values are presented in the table 4. They indicate that the highest over dispersion is observed for BP GA3_Water soaking and BP_Water soaking. It means that the repeatability is lower for these two methods. The results show also that the repeatability is better when sand methods are used whatever the treatment.

Table 4: Values of f_r obtained for the repeatability of the different germination methods tested for Papaya seeds

BP_No soaking			BP_Water soaking			BP GA3_Water soaking		
$\overline{p}_{...}$	S_r	f_r	$\overline{p}_{...}$	S_r	f_r	$\overline{p}_{...}$	S_r	f_r
83.57	3.83	1.03	82.92	4.37	1.16	82.01	4.36	1.14
Sand_No soaking			Sand_Water soaking			Sand GA3_Water soaking		
$\overline{p}_{...}$	S_r	f_r	$\overline{p}_{...}$	S_r	f_r	$\overline{p}_{...}$	S_r	f_r
83.01	3.73	0.99	83.89	3.48	0.95	83.69	3.97	1.07

Reproducibility of the testing methods

For each method, the reproducibility standard-deviation is computed as: $S_R = \sqrt{\frac{1}{I}\sum_i \sum_j \frac{\left(p_{ij.} - \overline{p}_{i..}\right)^2}{J-1}}$

where:

. $i = 1, 2, \ldots, I$ $j = 1, 2, \ldots, J$

. $p_{ij.}$ is the percentage of germinated seeds out of n in lot i and lab j

. $\overline{p}_{i..} = \frac{\sum_j p_{ij.}}{J}$

Assuming a binomial distribution, the variance of $p_{ij.}$ is:

$$\mathrm{Var}\left(p_{ij.}\right) = \frac{p_{ij.}\left(100 - p_{ij.}\right)}{n}$$

We then compute the following quantity to characterize over dispersion when Lab and Lot by Lab variations are considered:

$$f_R^2 = \frac{n S_R^2}{\overline{p}_{...}\left(100 - \overline{p}_{...}\right)} \text{ where } \overline{p}_{...} = \frac{\sum_{i,j} p_{ij.}}{IJ}$$

The square root of f_R^2 is then compared to the f value defined by Miles (1963) in equation AG4 and which is used to develop ISTA tolerance tables for comparing germination results from different labs.

The results of f_r values are presented in the table 5. All the f_R values are much larger than the f values for all the methods. The lowest values are obtained for the germination methods using sand media suggesting that these testing methods are more reproducible.

Table 5: Values of f_r obtained for the reproducibility of the different germination methods tested for Papaya seeds

BP_No soaking				BP_Water soaking				BP GA3_Water soaking			
$\overline{p}_{...}$	S_R	f_R	f	$\overline{p}_{...}$	S_R	f_R	f	$\overline{p}_{...}$	S_R	f_R	f
83.57	5.38	2.91	1.68	82.92	6.32	3.36	1.69	82.01	6.82	3.55	1.7
Sand_No soaking				**Sand_Water soaking**				**Sand GA3_Water soaking**			
$\overline{p}_{...}$	S_R	f_R	f	$\overline{p}_{...}$	S_R	f_R	f	$\overline{p}_{...}$	S_R	f_R	f
83.01	4.38	2.34	1.69	83.89	4.38	2.38	1.68	83.69	4.16	2.25	1.68

Conclusion

One laboratory among the seven laboratories participating to the comparative test gave lower germination results whatever the method used.

When the results of this laboratory are excluded for the statistical analysis, the data show that all the methods tested are equivalent for evaluating the germination quality of the seed samples, in all the other laboratories.

However, when analyzing the repeatability and the reproducibility of the results, only the methods using sand substrates seem to give the best results, even if results of reproducibility do not strictly fulfill ISTA requirements.

The germination method proposed to be included in ISTA Rules for *Carica papaya* seeds is: Sand ; 20⇔30°C ; 12 - 28 days ; with a pre-treatment consisting of soaking the seeds in water, with or without GA_3 added in the substrate.

References

Bhattacharya J., Khuspe S.S., 2001. In vitro and in vivo germination of papaya (*Carica papaya* L.) seeds. Scientia Horticulturae 91, 39-49.

Miles S.R., 1963. In the “Handbook of tolerances and measures of precision for seed testing” Proceedings of the International Seed Testing Association, 28, 525-686.

Validation of a new method for microsatellite marker analysis for wheat variety verification

Background

This method can be used to generate molecular marker profiles ("DNA fingerprints") of common wheat (*Triticum aestivum*) and durum wheat (*T. durum*). DNA is extracted from individual seeds or seed pools, a prescribed set of microsatellite markers is amplified using the polymerase chain reaction (PCR) and the products are separated and visualized using appropriate electrophoresis techniques. The profile obtained for a seed sample is compared to those of reference samples. Analysis of DNA extracted from seed pools (10 or more seeds per pool) is appropriate for variety verification purposes; for assessment of purity or seed mixtures, analysis of many individual seeds is necessary, with sample size dependent upon the desired level of precision.

The primary purpose of this Comparative Test (CT) was to evaluate the performance of several microsatellite markers in wheat and select a set of markers to be prescribed as a minimum set of markers in an ISTA method for wheat variety verification. These markers will be used in proficiency tests as part of a laboratory accreditation scheme for wheat variety verification. Accreditation will follow a semi-performance based approach. Various aspects of the laboratory methodology such as DNA extraction, PCR conditions and electrophoresis will be performance-based (i.e., laboratories will be free to use in-house validated methodology for these purposes so long as the end result is acceptable); however, use of the prescribed marker set will be mandatory. With this end goal in mind, the comparative tests for wheat followed the same philosophy; the primer sequences of the markers to be examined were prescribed (the only changes permitted related to the manner of fluorescent labeling) and although methods were suggested, participants were free to choose methods and instrumentation for analysis.

It is anticipated that any given marker set will not be sufficient to provide unique profiles for all varieties of wheat and that individual laboratories may need to use additional markers to distinguish among varieties, depending upon their particular circumstances. In this collaborative work we also assessed a supplementary set of markers intended to be recommended for this purpose.

Materials and methods

The microsatellite markers under consideration here were evaluated over a series of four comparative tests (CTs). For each CT, participating laboratories were provided seed samples of selected varieties, a list of microsatellite markers including primer sequences and suggested DNA extraction and PCR protocols (the suggested DNA extraction protocol was adapted from McDonald et al., 1994). Each CT varied somewhat with respect to participants and/or the markers and varieties included. Seeds were generally coarsely crushed prior to shipping to avoid possible import restrictions, with the exception of samples sent to France and within Canada to which whole seeds were sent.

First CT for wheat (CT1)

Four laboratories completed microsatellite analyses in the first CT for wheat (Table 1.1). Primer information for a set of eight microsatellite markers that was amenable to multiplexed amplification was provided to each (Table 1.2); this was the marker set reported by Perry (2004) with one more marker added (Xgwm003). Seed of eight varieties (Table 1.3) was distributed by regular mail to all participants. Seed from Brazil was distributed by Elisa Serra Negra Vieira, from Canada by D. Perry and from Italy by E. Casarini. Each participating laboratory assessed the assigned marker set in 12 individual seeds of each variety with the exception of the lab in France, which analysed 10 individual seeds and two 10-seed bulks per variety.

Table 1.1 Participants in the first CT for wheat

Lab number	Country	Contact	DNA extraction	Instrumentation
1	Canada	Daniel Perry daniel.perry@grainscanada.gc.ca	adapted from McDonald et al., 1994	MJ PTC-200 Li-Cor 4200

2	Canada	Marie-José Coté Marie-Jose.Cote@inspection.gc.ca	QIAGEN DNeasy plant mini kit.	MJ PTC-200 Li-Cor 4200
3	Italy	Emanuela Casarini[1]	QIAGEN DNeasy plant mini kit.	MJ PTC 200 Li-Cor 4300
4	France	David Zhang[1]	adapted from McDonald et al., 1994	ABI GeneAmp 9700 ABI 3130xl

[1]not presently available for further participation

Table 1.2 Microsatellite markers assessed in the first CT for wheat

Marker	Chromosome location	Forward primer[1]	Reverse primer	Source[2]
DuPw167	6A	cggagcaaggacgatagg	caccacaccaatcaggaacc	A
DuPw217	6B	cgaattacacttccttcttccg	cgagcgtgtctaacaagtgc	A
DuPw004	4A	ggtctggtcggagaagaagc	tgggagcgtacgttgtatcc	A
DuPw115	5B	tgtttcttcctcgcgtaacc	cctcgaatctcccagttatcg	A
DuPw205	5B	atccagatcacaccaaacgg	cttccgcttcatcttcttgc	A
Xgwm526	2B	caatagttctgtgagagctgcg	ccaacccaaatacacattctca	B
Xgwm099	1A	aagatggacgtatgcatcaca	gccatatttgatgacgcata	B
Xgwm003	3D	gcagcggcactggtacattt	aatatcgcatcactatccca	B

[1]Sequences provided for forward primers included a 5′-tail sequence (5′-cacgacgttgtaaaacgac-3′) to facilitate fluorescent labeling when used in combination with a labeled M13 FWD(-19) primer having the same sequence.

[2]A = Eujayl *et al*. 2002; B = Röder et al. 1998

Table. 1.3 Varieties included in the first CT for wheat

Variety	Species	Country
CD104	T. aestivum	Brazil
Ônix	T. aestivum	Brazil
AC Avonlea	T. durum	Canada
AC Barrie	T. aestivum	Canada

Mieti	T. aestivum	Italy
Simeto	T. durum	Italy
Iride	T. durum	Italy
Duilio	T. durum	Italy

Second CT for wheat (CT2)

The marker set was modified from that used in CT1; two markers that had complex banding patterns in some varieties were replaced with two other markers, thus keeping the total number of markers at eight (Table 2.1), All were compatible with amplification in a single multiplex PCR. Seed of 16 varieties (Table 2.2) was distributed to the same four participants as in CT1, again sent from Brazil by Elisa Serra Negra Vieira, from Canada by D. Perry, and Italy by E. Casarini. In addition, varieties from France were also included in this CT, with seed distributed by D. Zhang. Each participating laboratory assessed the assigned marker set in 6 individual seeds and two 10-seed bulks of each variety. Laboratories 1 and 4 also examined this modified marker set in the eight CT1 varieties.

Table 2.1 Modified microsatellite marker set assessed in the second CT for wheat

Marker	Chromosome location	Forward primer[1]	Reverse primer	Source[2]
DuPw167	6A	cggagcaaggacgatagg	caccacaccaatcaggaacc	A
DuPw217	6B	cgaattacacttccttcttccg	cgagcgtgtctaacaagtgc	A
DuPw004	4A	ggtctggtcggagaagaagc	tgggagcgtacgttgtatcc	A
DuPw115	5B	tgtttcttcctcgcgtaacc	cctcgaatctcccagttatcg	A
DuPw205	5B	atccagatcacaccaaacgg	cttccgcttcatcttcttgc	A
Xgwm155	3A	caatcatttccccctccc	aatcattggaaatccatatgcc	B[3]
Xgwm413	1B	tgcttgtctagattgcttggg	gatcgtctcgtccttggca	B[3]
Xgwm003	3D	gcagcggcactggtacattt	aatatcgcatcactatccca	B

[1]Sequences provided for forward primers included a 5′-tail sequence (5′-cacgacgttgtaaaacgac-3′) to facilitate fluorescent labeling when used in combination with a labeled M13 FWD(-19) primer having the same sequence.

[2]A = Eujayl *et al*. 2002; B = Röder et al. 1998

[3]Xgwm413 and Xgwm155 primers are protected by patents in Europe and the United States; however, it is anticipated that this protection will soon expire (the PCT filing date was June 26, 1996).

Table 2.2 Varieties included in the second CT for wheat

Variety	Species	Country

CD108	T. aestivum	Brazil
IPR85	T. aestivum	Brazil
BRS208	T. aestivum	Brazil
Nova Era	T. aestivum	Brazil
AC Andrew	T. aestivum	Canada
AC Bellatrix	T. aestivum	Canada
AC Readymade	T. aestivum	Canada
Lillian	T. aestivum	Canada
Alberic	T. aestivum	France
Ami	T. aestivum	France
Arbon	T. aestivum	France
Sideral	T. aestivum	France
Claudio	T. durum	Italy
Esperia	T. aestivum	Italy
Palesio	T. aestivum	Italy
Svevo	T. durum	Italy

Third CT for wheat (CT3)

Seed of the 24 varieties (18 *T. aestivum* and 6 *T. durum*) examined in CT1 and CT2 were distributed in the same manner as before to three new laboratories that had not participated in previous wheat CTs (Table 3.1). These participants examined the modified set of eight microsatellite markers (as in CT2) in 6 individual seeds and two 10-seed bulks of each variety.

Table 3.1 New participants joining the third CT for wheat

Lab number	Country	Contact	DNA extraction	Instrumentation
5	Austria	Verena Peterseil verena.peterseil@ages.at	R-Biopharm SureFood PREP Kit	Eppendorf Mastercycler ep Li-Cor 4300
6	Canada	Kim Kenward Kim@2020seedlabs.ca	adapted from McDonald et al., 1994	MJ PTC-100 and Eppendorf MasterCycler ABI 3730, 48 capillary
7	Argentina	Ma. Alicia Loray	adapted from McDonald et	Silver staining

		mloray@inase.gov.ar	al., 1994	

A second objective of CT3 was to examine the modified marker set in a larger number of varieties. To achieve this, three of the original participants (Laboratories 1, 3 and 4) each assessed the markers in additional varieties from their respective countries. The markers were examined in 24 additional Canadian varieties in Lab 1, 12 additional Italian varieties in Lab 3 and 24 additional varieties from France in Lab 4. Identities of these additional varieties were not disclosed.

Fourth CT for wheat (CT4)

The purpose of the fourth CT was to examine additional markers that are intended to be recommended as supplementary to the prescribed marker set. Three participants (Labs 1, 2 and 6) each examined a set of six microsatellite markers (Table 4.1) in the six Canadian varieties (6 seeds and 2 10-seed bulks of each). Lab 1 also examined this marker set in the 24 additional Canadian varieties used in CT3 (identities not disclosed). As was the case for the earlier marker set, this set of six markers was also amenable to amplification in a single multiplex reaction. Instrumentation was as in earlier CTs except that Lab 2 used an ABI 3130xl Genetic analyser.

Table 4.1 Proposed supplementary marker set assessed in the fourth CT for wheat

Marker	Chromosome location	Forward primer[1]	Reverse primer	Source[2]
Xgwm372	2A	aatagagccctgggactggg	gaaggacgacattccacctg	B[3]
Xbarc347	5D	gcgcacctctcctcaccttct	gcgaacatggaaatgaaaactatct	A
Xbarc184	4A	ttcggtgatatctttcccttga	ccgagttgactgtgtgggcttgctg	A
Xbarc074	5B	gcgcttgccccttcaggcgag	cgcgggagaaccaccagtgacagagc	A
Xgwm052	3D	ctatgaggcggaggttgaag	tgcggtgctcttccattt	B[3]
Xgwm095	2A	gatcaaacacacaccctcc	aatgcaaagtgaaaaacccg	B[3]

[1]Sequences provided for forward primers included a 5′-tail sequence (5′-cacgacgttgtaaaacgac-3′) to facilitate fluorescent labeling when used in combination with a labeled M13 FWD(-19) primer having the same sequence.

[2]A = Eujayl *et al*. 2002; B = Röder et al. 1998

[3]Xgwm052, Xgwm095 and Xgwm372 primers are protected by patents in Europe and the United States; however, it is anticipated that this protection will soon expire (the PCT filing date was June 26, 1996).

Results

For each CT, participants reported results in terms of fragment sizes observed for each marker in each variety. They generally included either observations for each individual kernel, or counts of the number of kernels for which a particular sized fragment was observed in each variety as well as the marker fragment sizes observed in the bulk samples. These data were then summarized and compiled to facilitate comparison of results among laboratories.

Proposed prescribed marker set run by seven labs against 24 varieties (CT1, CT2 and CT3)

An example image of a Li-Cor gel of the proposed prescribed marker set run as a multiplex in three individual seeds of each of the eight CT1 varieties is presented in Appendix A (independent file "WheatSSR_AppendixA.tif). Appendix B (independent file "WheatSSR_AppendixB.xlsx) is a compilation of the data showing the consistency of allele calls achieved by seven participants in the

first three CTs. In Appendix B, markers and varieties shaded in blue were introduced in CT1 while those that are shaded in green were introduced in CT2. For individual laboratory results, cells shaded in blue correspond to data collected in CT1, green is data collected in CT2, orange is data collected in CT3. Dashes indicate that no data was reported by a lab for a particular marker/variety combination. Cells shaded in gray correspond to combinations that were not run by Labs 2 and 3 (i.e., the CT1 varieties with markers added in CT2).

As expected, there were minor differences among laboratories regarding the sizes called for specific alleles; however, in general these differences were consistent and for the most part alignment of alleles for summarization and comparison of data was straightforward. In the compiled data, with very few exceptions, there was complete congruence of results reported by the original four laboratories in CT1 and CT2. Exceptions were DuPw115 results reported by Lab 2 for AC Avonlea, Duilio, Iride, Simeto and AC Barrie; DuPw217 results reported by Lab 3 for Alberic and Ami, and the Xgwm413 result reported by Lab 3 for Nova Era. Gel or amplification artifacts are suspected to have caused these few irregularities.

The consistency of results was reduced in CT3 following the addition of three new laboratories. While results reported by Lab 6 were in complete agreement with those of the first four labs, those of labs 5 and 7 deviated quite frequently. The poor agreement of labs 5 and 7 with the others appears likely to have been due to a lack of experience of those participants with these types of analyses. Also, Lab 7 used silver staining for visualization which may have been the most challenging of all the systems used. Some specific observations point to a lack of experience of these two labs. An example Li-Cor gel image was provided by Lab 5 showing results for eight varieties. The run quality was not too bad, but on this one image it was plainly apparent that multiple miscalled alleles had been reported. The alleles visible on the image were in agreement with the data reported by the majority of labs, but the allele calls reported by Lab 5 were not. For example, Lab 5 called an Xgwm155.157 allele for the variety CD104, which was in agreement with calls by other labs. The allele in the next variety on the gel (CD108) was clearly two steps (4 base pairs) larger than the CD104 allele, but Lab 5 reported Xgwm155.159 for CD108 (only 2 base pairs larger than for CD104) whereas all of the other labs reported an allele that would correspond to Xgwm155.161. This miscall and others by Lab 5 were clearly not a fault of the markers.

Lab 7 did not provide an example image, but at least one striking observation indicated that they too may have lacked sufficient experience to make reliable allele calls. For marker DuPw217, the pattern of variation reported by Lab 7 was exactly the same as the pattern they reported for marker DuPw205. Alleles DuPw205.182 and DuPw217.230 were always reported to occur together within a variety as were alleles DuPw205.189 and DuPw217.242. While the DuPw205 data reported by Lab 7 were in good agreement with DuPw205 data reported by other labs, their DuPw217 data bore no resemblance to DuPw217 data of other labs. The cause of this anomaly is not known; perhaps there was some sort of shadow band of DuPw205 appearing higher up in the multiplex or some other artifact on the silver stained gels. In any case, an experienced operator should have recognized a result like this as being unusual and investigated it, or at least flagged it.

Of note in the results reported by Lab 6, all of the missing data corresponded to the smallest allele of marker Xgwm155. Perhaps in that case the size bounds for that marker had been set too narrow in the data analysis software to capture that allele. Similarly, the null/no amplification allele reported by Lab 4 for DuPw004 clearly corresponds to the very large (~310 bp) allele that others report for that marker.

Usually only one allele was observed for a given marker in a variety. However, in a few cases (DuPw167 in Sideral; DuPw217 in AC Bellatrix; DuPw104 in Alberic; DuPw205 in CD104 and CD108; Xgwm003 in Palesio) two alleles were observed by multiple labs within the same variety. The failure of some participants to report both alleles in some of these cases could have been due to sampling effects (the alternate allele not being present in the seeds selected).

Examination of the proposed prescribed marker set in a larger set of varieties (CT3 in Lab 1, Lab 3 and Lab 4).

The proposed prescribed marker set was examined in a total of 84 varieties. These included 24 Canadian varieties examined by Lab 1, 12 Italian varieties examined by Lab 3 and 24 French varieties examined by Lab 4, plus the 24 varieties already considered in the first three CTs. The allele profiles of all 84 varieties are presented in Appendix 3 (independent file WheatSSR_AppendixC.xlsx). All of the French varieties had unique profiles. Two pairs of Canadian varieties (coded as CA09 and CA10; CA17 and CA18) were indistinguishable with this marker set. (Note that these two pairs of varieties were known in advance to Lab 1 as being potentially difficult to distinguish). In addition, a trio of Italian durum wheat varieties (Iride, IT03 and IT06) were not differentiated with this marker set.

For some markers, additional alleles not previously seen in the first 24 varieties were reported. These included 3 new Xgwm413 alleles in the Canadian varieties, one new DuPw167 allele in the Italian varieties. Several new alleles were reportedly found in the French varieties (3 for Xgwm413, 2 for Xgwm155, 1 for DuPw115 and 2 for DuPw167).

The proposed supplementary marker set

Results of the examination of the proposed supplementary marker set were very consistent among the three labs that participated in CT4. Only one deviation was noted (highlighted in yellow in Appendix D, independent file WheatSSR_AppendixD.xlsx): for marker Xgwm372 in AC Avonlea, Lab 1 and Lab 6 both observed an allele that appeared to be one base pair larger than the Xgwm372 allele in AC Barrie or Lillian. Lab 2 did not differentiate these alleles.

An example image of a Li-Cor gel of the proposed supplementary marker set run as a multiplex in two 10-seed bulks of each of eight varieties is presented in Appendix E (independent file WheatSSR_AppendixE.ppt). In this image it is can be seen that CA09 and CA10, which were indistinguishable using the first marker set were differentiated with the proposed supplementary marker set; CA17 and CA18 were not. Subsequent testing also indicated that the Italian varieties Iride and IT03 were also distinguishable with this marker set (IT06 was not tested).

Final comments and conclusions

According to the statistical analysis of the combined CT results for the proposed prescribed marker set (independent file WheatSSR_AppendixF.docx) the agreement for scoring varieties across laboratories 1, 2, 3, 4 and 6 was good and it was also good for allele's scoring. Although laboratories 5 and 7 were clearly not in agreement with the others, those discrepancies appeared to be due to insufficient experience of Labs 5 and 7 in these types of analyses. Overall the performance of the selected marker set was good and it is proposed to be prescribed as a minimum set of markers in an ISTA method for wheat variety verification.

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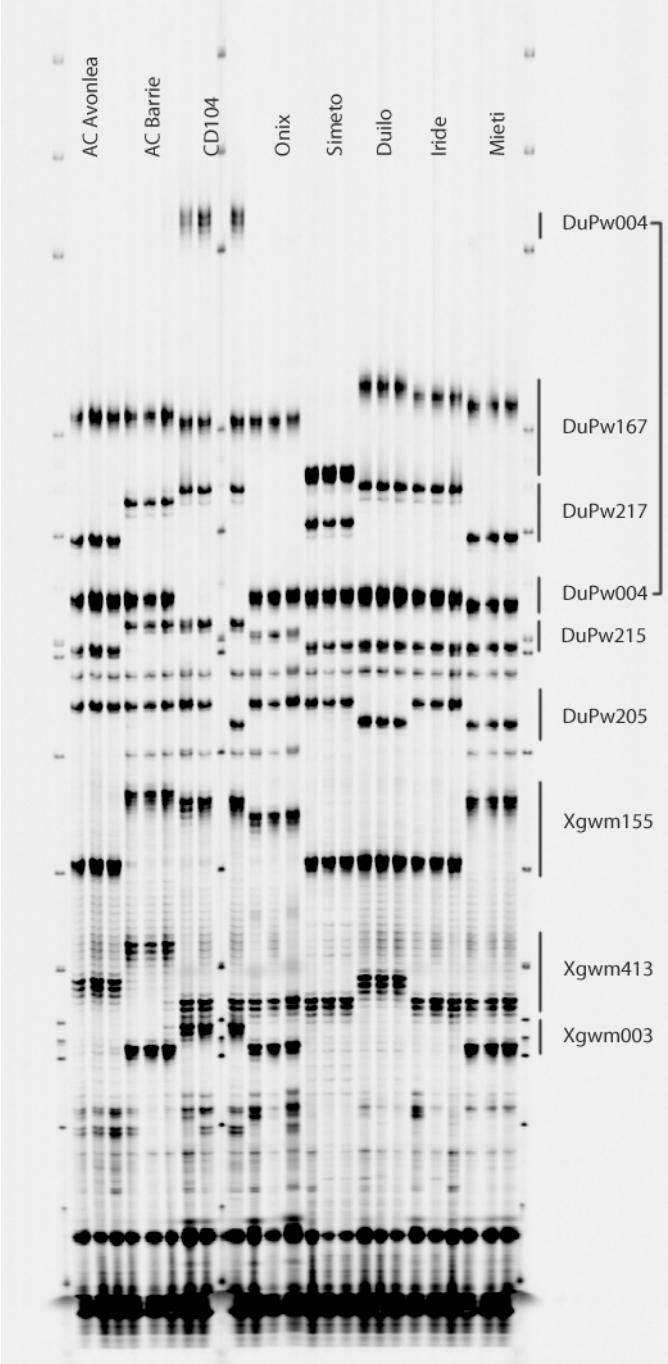
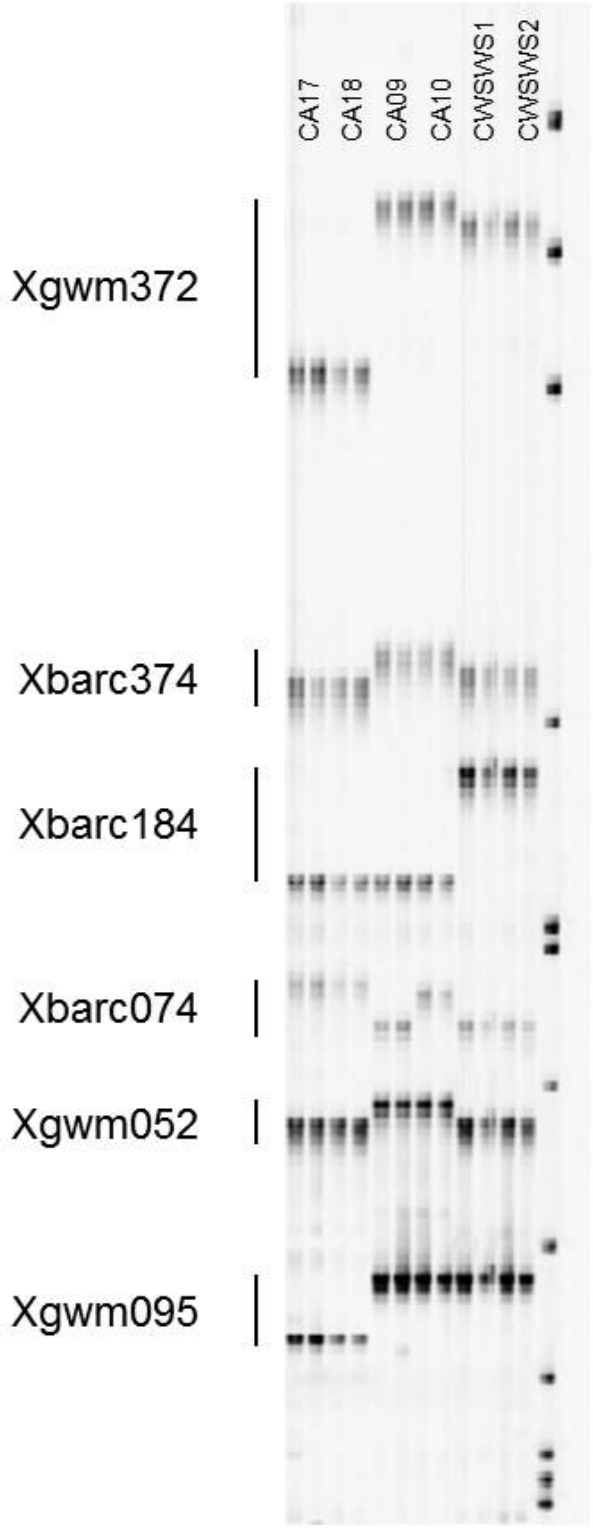
Appendix A


AC Avonlea
AC Barrie
CD104
Onix
Simeto
Duilo
Iride
Mieti
DuPw004
DuPw167
DuPw217
DuPw004
DuPw215
DuPw205
Xgwm155
Xgwm413
Xgwm003

Appendix E


CA17
CA18
CA09
CA10
CWSWS1
CWSWS2
Xgwm372
Xbarc374
Xbarc184
Xbarc074
Xgwm052
Xgwm095

Appendix F

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

Jean-Louis Laffont, ISTA Statistics Committee

Materials and methods

Allele results (0 or 1) from three Comparative Tests (CT) for evaluating the performance of several microsatellite markers are available for 7 laboratories and 24 varieties. Figure 1 summarizes the structure of the data.

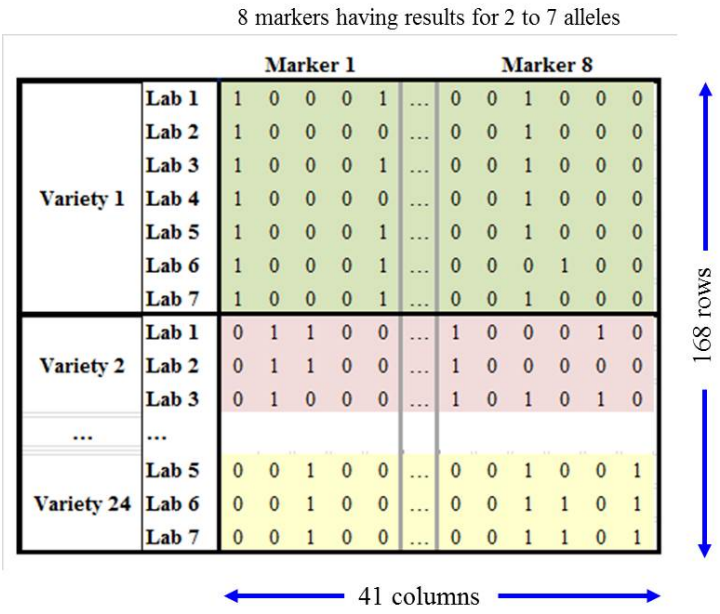


Figure 1: Structure of the data analyzed.

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).

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 and 3 visualize with dot plots the overall percentages agreements; Figures 4 and 5 visualize Cohen's kappas. Tables 4 and 5 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 1, 2, 3, 4 and 6 is good (Fleiss's kappa between 0.913 and 1). It is also good for allele's scoring (overall agreement percentages all above 80%). Laboratories 5 and 7 are clearly not in agreement with the others. Providing the reasons for this failure will be necessary before validating the method.

		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 $\hat{\kappa}_C$.

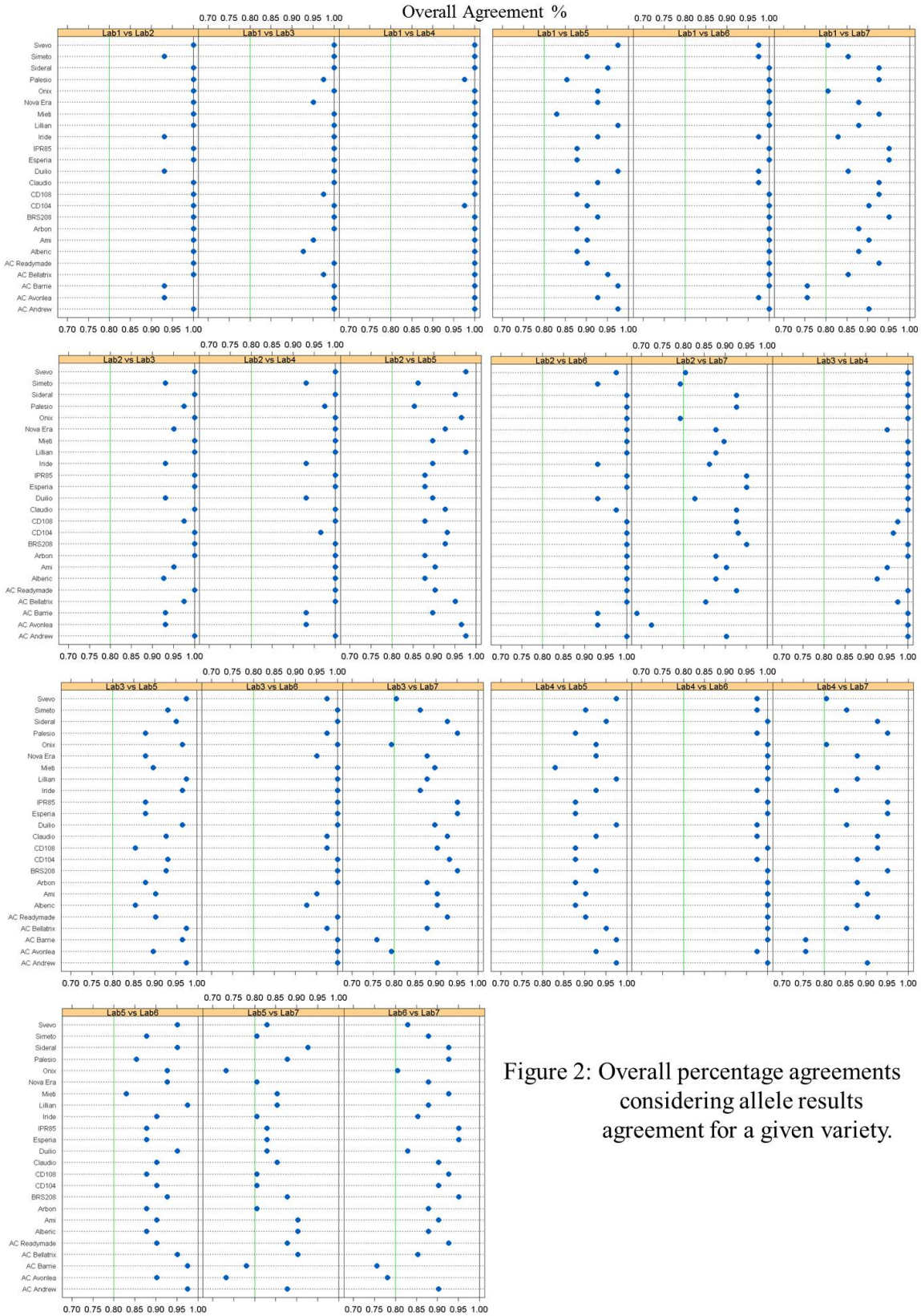


Figure 2: Overall percentage agreements considering allele results agreement for a given variety.

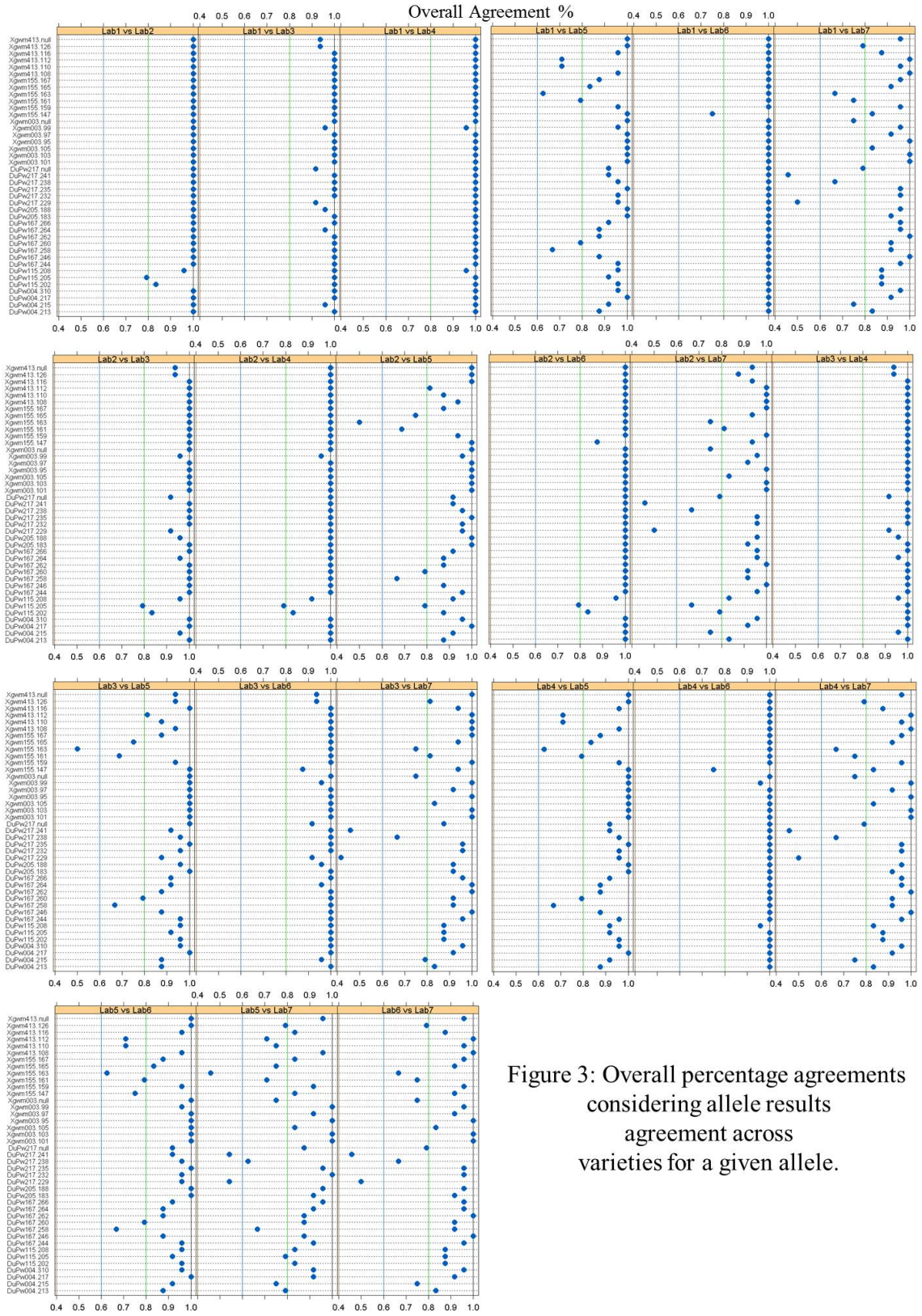


Figure 3: Overall percentage agreements considering allele results agreement across varieties for a given allele.

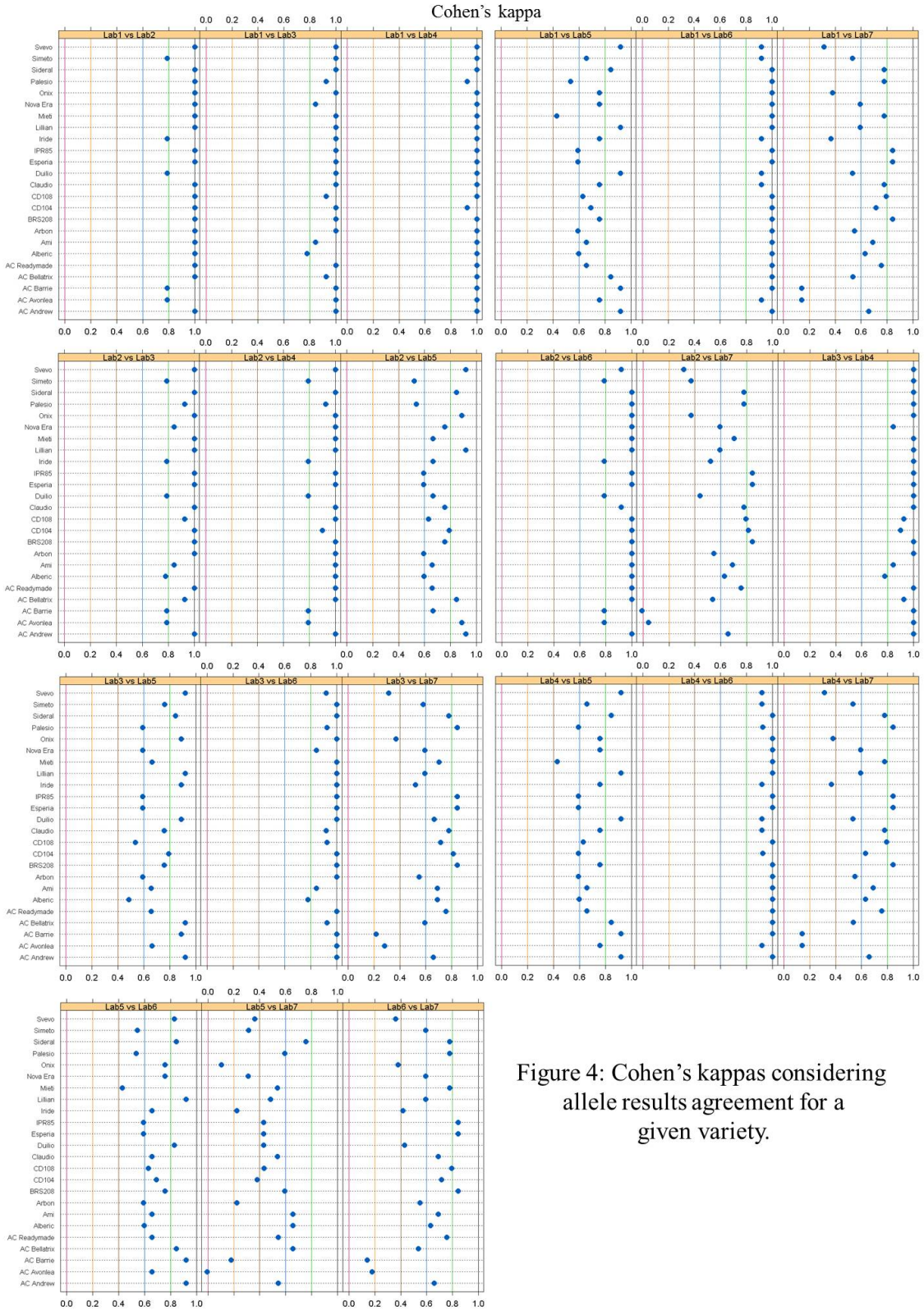


Figure 4: Cohen's kappas considering allele results agreement for a given variety.

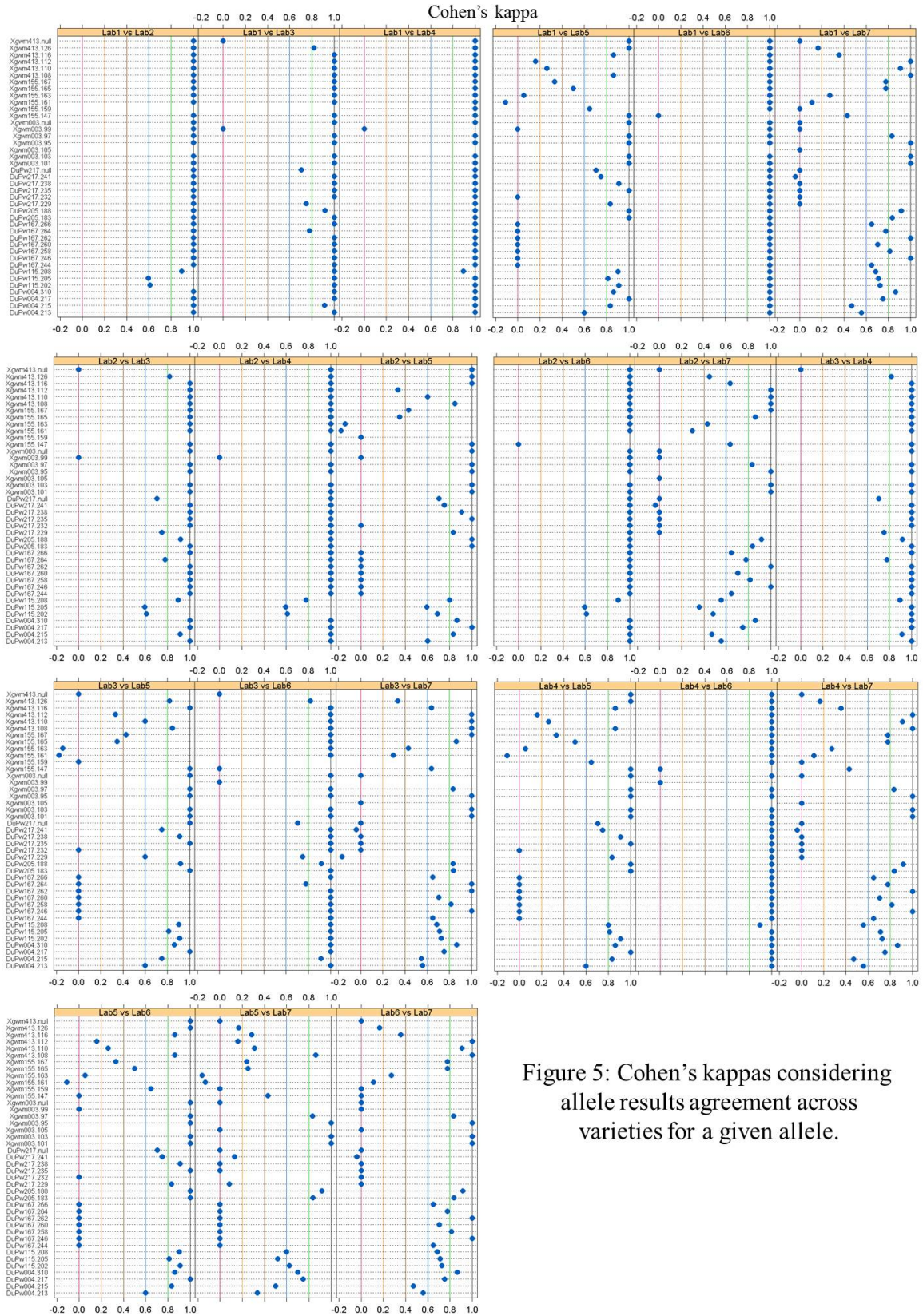


Figure 5: Cohen's kappas considering allele results agreement across varieties for a given allele.

% 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%	20.8%	79.2%
Lab1 vs Lab3	0.0%	0.0%	0.0%	0.0%	4.2%	95.8%
Lab1 vs Lab4	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%
Lab1 vs Lab5	0.0%	0.0%	0.0%	25.0%	45.8%	29.2%
Lab1 vs Lab6	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%
Lab1 vs Lab7	0.0%	8.3%	12.5%	25.0%	41.7%	12.5%
Lab2 vs Lab3	0.0%	0.0%	0.0%	0.0%	25.0%	75.0%
Lab2 vs Lab4	0.0%	0.0%	0.0%	0.0%	20.8%	79.2%
Lab2 vs Lab5	0.0%	0.0%	0.0%	25.0%	45.8%	29.2%
Lab2 vs Lab6	0.0%	0.0%	0.0%	0.0%	20.8%	79.2%
Lab2 vs Lab7	4.2%	4.2%	12.5%	25.0%	37.5%	16.7%
Lab3 vs Lab4	0.0%	0.0%	0.0%	0.0%	4.2%	95.8%
Lab3 vs Lab5	0.0%	0.0%	0.0%	29.2%	33.3%	37.5%
Lab3 vs Lab6	0.0%	0.0%	0.0%	0.0%	4.2%	95.8%
Lab3 vs Lab7	0.0%	0.0%	16.7%	25.0%	37.5%	20.8%
Lab4 vs Lab5	0.0%	0.0%	0.0%	29.2%	41.7%	29.2%
Lab4 vs Lab6	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%
Lab4 vs Lab7	0.0%	8.3%	12.5%	25.0%	37.5%	16.7%
Lab5 vs Lab6	0.0%	0.0%	0.0%	29.2%	41.7%	29.2%
Lab5 vs Lab7	4.2%	8.3%	25.0%	45.8%	16.7%	0.0%
Lab6 vs Lab7	0.0%	8.3%	8.3%	29.2%	41.7%	12.5%

Table 4: 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%	2.6%	2.6%	94.9%
Lab1 vs Lab3	0.0%	5.1%	0.0%	0.0%	7.7%	87.2%
Lab1 vs Lab4	0.0%	2.5%	0.0%	0.0%	0.0%	97.5%
Lab1 vs Lab5	2.5%	27.5%	5.0%	5.0%	7.5%	52.5%
Lab1 vs Lab6	0.0%	2.5%	0.0%	0.0%	0.0%	97.5%
Lab1 vs Lab7	2.4%	29.3%	4.9%	7.3%	24.4%	31.7%
Lab2 vs Lab3	0.0%	5.1%	0.0%	2.6%	10.3%	82.1%
Lab2 vs Lab4	0.0%	2.6%	0.0%	2.6%	5.1%	89.7%
Lab2 vs Lab5	5.0%	25.0%	5.0%	10.0%	10.0%	45.0%
Lab2 vs Lab6	0.0%	2.6%	0.0%	2.6%	2.6%	92.3%
Lab2 vs Lab7	2.5%	22.5%	5.0%	15.0%	17.5%	37.5%
Lab3 vs Lab4	0.0%	2.6%	0.0%	0.0%	7.9%	89.5%
Lab3 vs Lab5	5.1%	25.6%	5.1%	10.3%	5.1%	48.7%
Lab3 vs Lab6	0.0%	7.7%	0.0%	0.0%	7.7%	84.6%
Lab3 vs Lab7	5.3%	15.8%	5.3%	7.9%	23.7%	42.1%
Lab4 vs Lab5	2.6%	25.6%	5.1%	5.1%	10.3%	51.3%
Lab4 vs Lab6	0.0%	5.0%	0.0%	0.0%	0.0%	95.0%
Lab4 vs Lab7	2.5%	27.5%	5.0%	10.0%	22.5%	32.5%
Lab5 vs Lab6	2.5%	30.0%	5.0%	5.0%	7.5%	50.0%
Lab5 vs Lab7	5.1%	46.2%	12.8%	7.7%	10.3%	17.9%
Lab6 vs Lab7	2.4%	31.7%	4.9%	4.9%	24.4%	31.7%

Table 5: 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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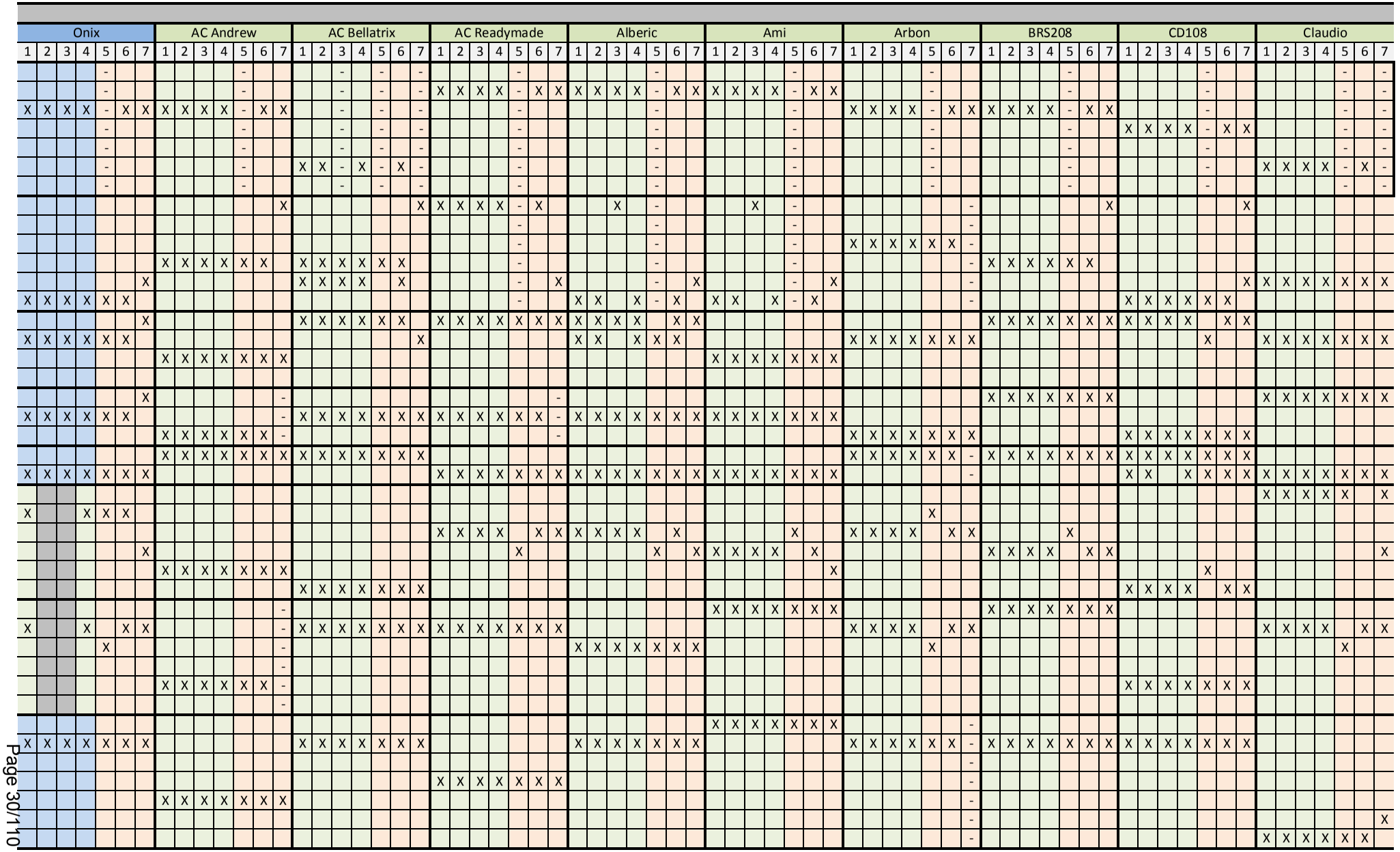
Wheat SSR Appendix B -- Summary of individual lab results for first three comparative tests.

Marker	Allele	Laboratory and allele scoring 1	2	3	4	5	6	7
DuPw167	1	244	244	244	244	-	244	260
	2	246	246	245	246	-	245	265
	3	258	258	258	258	-	257	274
	4	260	260	260	260	-	259	277
	5	262	262	262	262	-	261	280
	6	264	264	264	264	-	263	-
	7	266	266	266	266	-	265	285
DuPw217	1	229	229	229	227	224	228	230
	2	232	232	232	231	-	231	-
	3	235	235	235	235	228	234	-
	4	238	238	238	237	237	237	-
	5	241	241	241	241	241	240	242
	6	null	null	null	null	null	null	-
DuPw004	1	213	213	213	212	212	211	208
	2	215	215	215	214	213	213	209
	3	217	217	216	216	216	215	213
	4	310	310	310	null	306	308	315
DuPw115	1	202	203	202	202	202	200	170
	2	205	205	205	204	204	203	173
	3	208	208	208	208	206	206	175
DuPw205	1	183	183	183	183	180	180	182
	2	188	188	188	187	186	186	189
Xgwm155	1	147	147	147	148	141	-	141
	2	159			161	153	157	-
	3	161	161	160	163	155	159	159
	4	163	163	164	165	157	161	161
	5	165	165	165	167	159	163	163
	6	167	167	168	169	162	165	167
Xgwm413	1	108	109	108	106	107	105	133
	2	110	111	111	108	110	107	135
	3	112	113	112	110	112	109	137
	4	116	117	117	114	115	113	142
	5	126	127	124	124	126	123	185
	6	null	null	-	null	null	null	-
Xgwm003	1	95	96	95	94	95	92	101
	2	97	98	97	96	96	94	103
	3	99	100	-	98	-	96	-
	4	101	102	101	100	100	98	107
	5	103	104	103	102	101	100	109
								111
	6	null	null	null	null	null	null	-

Variety and Laboratory result

Marker	Allele	AC Avonlea 1	2	3	4	5	6	7	Duilio 1	2	3	4	5	6	7	Iride 1	2	3	4	5	6	7
DuPw167	1					-		-					-							-		-
	2					-		-					-							-		-
	3					-		-					-							-		-
	4	X	X	X	X	-	X	-					-							-		-
	5					-		-					-							-		-
	6					-		-					-			X	X	X	X	-	X	-
	7					-		-	X	X	X	X	-	X	X					-		-
DuPw217	1	X	X	X	X	X	X								X							
	2																					
	3																					
	4																					
	5							X	X	X	X	X	X	X		X	X	X	X	X	X	X
	6																					
DuPw004	1							X														
	2	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X
	3																					
	4																					
DuPw115	1	X		X	X		X	X	X		X	X	X	X	X	X		X	X	X	X	
	2		X			X				X							X					
	3																					
DuPw205	1								X	X	X	X	X	X	X							-
	2	X	X	X	X	X	X	X								X	X	X	X	X	X	-
Xgwm155	1	X			X	X	-		X			X	X	-	X	X			X	X	-	
	2						-							-							-	
	3						-							-							-	X
	4						-							-	X						-	
	5						-							-							-	
	6						-	X						-							-	
Xgwm413	1																					-
	2															X			X		X	-
	3																			X		-
	4	X			X	X	X		X			X	X	X								-
	5							X							X							-
	6																					-
Xgwm003	1							-							-							
	2							-							-							
	3							-							-							
	4							-							-							
	5							-							-							
								-							-							X
	6	X	X	X	X	X	X	-	X	X	X	X	X	X	-	X	X	X	X	X	X	

Marker	Allele	Simeto 1	2	3	4	5	6	7	AC Barrie 1	2	3	4	5	6	7	CD104 1	2	3	4	5	6	7	Mieti 1	2	3	4	5	6	7
DuPw167	1	X	X	X	X	-	X	X					-							-							-		X
	2					-							-							-							-		
	3					-							-		X	X	X	X	X	-	X	X					-		
	4					-			X	X	X	X	-	X						-							-		
	5					-							-							-			X	X	X	X	-	X	X
	6					-							-							-							-		
	7					-							-							-							-		
DuPw217	1					-																X	X	X	X	X	X	X	X
	2	X	X	X	X	-	X																						
	3					-																							
	4					-			X	X	X	X	X	X															
	5					-		X							X	X	X	X	X	X	X	X							
	6					-																							
DuPw004	1																			-			X	X	X	X		X	
	2	X	X	X	X	X	X	X	X	X	X	X	X	X						-							X		X
	3														X					-									
	4															X	X	X	X	-	X	X							
DuPw115	1	X		X	X	X	X	X											-				X	X	X	X	X	X	X
	2		X							X									-										
	3								X		X	X	X	X	X	X	X	X	-	X	X	X							
DuPw205	1															X	X	X	X	X	X		X	X	X	X	X	X	X
	2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X							
Xgwm155	1	X			X	X	-																						
	2						-																						
	3						-	X																					
	4						-								X	X			X	X	X	X	X			X		X	X
	5						-		X			X	X	X															
	6						-																				X		
Xgwm413	1														-														
	2	X			X		X	X							-	X			X		X	X	X			X		X	X
	3					X									-												X		
	4														-					X									
	5								X			X	X	X	-														
	6														-														
Xgwm003	1														-														
	2								X	X	X	X	X	X	-								X	X	X	X	X	X	X
	3														-														
	4														-														
	5														-	X	X	X	X	X	X	X							
								X							-														
	6	X	X	X	X	X	X								-														


Onix
AC Andrew
AC Bellatrix
AC Readymade
Alberic
Ami
Arbon
BRS208
CD108
Claudio

Esperia							IPR85							Lillian							Nova Era							Palesio							Sideral							Svevo						
1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7
				-							-							-							-							-							-							-		-
				-							-							-							-							-							-							-		-
X	X	X	X	-	X	X					-							-			X	X	X	X	-	X	X					-			X	X	X	X	-	X						-		-
				-			X	X	X	X	-	X	X	X	X	X	X	-	X	X					-							-							-							-		-
				-							-							-							-			X	X	X	X	-	X	X	X	X	X	X	-	X	X					-		-
				-							-							-							-							-							-							-		-
				-							-							-							-							-							-			X	X	X	X	-	X	-
						X																					X							X							X	X	X	X	X	X	X	X
X	X	X	X	X	X		X	X	X	X	X	X		X	X	X	X	X	X		X	X	X	X	X	X						X																	
													X							X								X	X	X	X		X																
																																		X	X	X	X	X	X									
																																									X	X	X	X	X	X	X
																											X	X	X	X	X	X	X	X	X	X	X	X	X	X	X							
X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X																						
							X	X	X	X	X	X	X																														X	X	X	X	X	X	
X	X	X	X		X	X														X	X	X	X	X	X	X	X								X	X	X	X	X	X	X							
				X										X	X	X	X	X	X									X	X	X	X	X	X	X														X
X	X	X	X	X	X	X															X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
							X	X	X	X	X	X	X	X	X	X	X	X	X	X																												
																																									X	X	X	X	X	-	
																																														-	
																																														-	X
				X							X			X	X	X	X	X	X	X					X			X	X	X	X		X	X													-	
X	X	X	X		X	X	X	X	X	X		X	X								X	X	X	X		X	X								X	X	X	X	X	X	X						-	
																															X															-	
							X	X	X	X		X	X							-							-	X	X	X	X	X	X	X	X	X	X	X	X	X	X							-
																				-							-																					-
											X									-							-																					-
X	X	X	X	X	X	X														-							-															X	X	X	X	X	X	-
														X	X	X	X	X	X	-			X				-																					-
																				-	X	X		X	X	X	-																					-
X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X							
																												X	X				X															
																																															X
																																									X	X	X	X	X	X	

Wheat SSR Appendix C -- Genotypes obtained for 84 varieties usingthe proposed prescribed marker set.

Source	Variety Name / Coded ID	Xgwm003 lab scoring	Xgwm413 lab scoring	Xgwm155 lab scoring	DuPw205 lab scoring	DuPw115 lab scoring	DuPw004 lab scoring	DuPw217 lab scoring	DuPw167 lab scoring
		1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4
		A = 95 96 95 94	A = 108 109 108 106	A = 144	A = 183 183 183 183	A = 202 203 202 202	A = 213 213 213 212	A = 229 229 229 227	A = 244 244 244 244
		B = 97 98 97 96	B = 110 111 111 108	B = 147 147 147 148	B = 188 188 188 187	B = 205 205 205 204	B = 215 215 215 214	B = 232 232 232 231	B = 246 246 245 246
		C = 99 100 98	C = 112 113 112 110	C = 159 161		C = 208 208 208 208	C = 217 217 216 216	C = 235 235 235 235	C = 250
		D = 101 102 101 100	D = 114	D = 161 161 160 163		D = na	D = 310 310 310 na	D = 238 238 238 237	D = 252
		E = 103 104 103 102	E = 116 117 117 114	E = 163 163 164 165				E = 241 241 241 241	E = 258 258 258 258
		F = null null null na	F = 118	F = 165 165 165 167				F = null null null na	F = 260 260 260 260
			G = 122 120	G = 167 167 168 169					G = 262 262 262 262
			H = 126 127 127 124	H = 172					H = 264 264 264 264
			I = 126						I = 266 266 266 266
			J = 128						J = na
			K = null na						
CT1	AC Avonlea	F	E	B	B	A	B	A	F
CT1	AC Barrie	B	H	F	B	C	B	D	F
CT1	CD104	E	B	E	A B	C	D	E	E
CT1	Duilio	F	E	B	A	A	B	E	I
CT1	Iride	F	B	B	B	A	B	E	H
CT1	Nieti	B	B	E	A	A	A	A	G
CT1	Ônix	B	B	C	B	B	B	F	E
CT1	Simeto	F	B	B	B	A	B	B	A
CT2	AC Andrew	E	H	F	A	C	C	D	E
CT2	AC Bellatrix	B	B	G	A B	B	A	D E	H
CT2	AC Readymade	D	B	D	B	B	A	A	B
CT2	Alberic	B	C	D	B	B	A B	F	B
CT2	Ami	A	A	E	B	B	C	F	B
CT2	Arbon	B	B	D	A	C	B	C	E
CT2	BRS208	B	A	E	A	A	A	D	E
CT2	CD108	B	H	G	A B	C	A	F	F
CT2	Claudio	F	B	B	B	A	B	E	H
CT2	Esperia	B	E	F	A	B	D	D	E
CT2	IPR85	B	A	F	B	A	D	D	F
CT2	Lillian	B	H	E	B	C	D	D	F
CT2	Nova Era	B	H K	F	A	B	D	D	E
CT2	Palesio	B C	A	E	A	C	C	E	G
CT2	Sideral	B	A	F	A	B	C	F	E G
CT2	Svevo	F	E	B	A	A	B	A	I
Canada	CA01	B	B	E	B	A	B	A	F
Canada	CA02	B	A	E	A	C	B	A	F
Canada	CA03	E	H	E	A	C	B	E	E
Canada	CA04	B	A	E	A B	C	B	E	B
Canada	CA05	E	H	F	A	C	B	D	F
Canada	CA06	B	A	E	B	A	B	A	E
Canada	CA07	E	B	F	A	C	D	E	E
Canada	CA08	B	B	F	B	C	D	E	F
Canada	CA09	E	H	E	A	C	D	E	E
Canada	CA10	E	H	E	A	C	D	E	E
Canada	CA11	B	B	F	B	C	D	D	F
Canada	CA12	E	H	E	A	A	B D	E	F
Canada	CA13	F	D	B	A	A	B	A	F
Canada	CA14	F	E	B	A	A	B	E	F
Canada	CA15	F	B	B	B	A	C	E	F
Canada	CA16	B E	B	E	A	C	A D	E	G
Canada	CA17	E	B	E	A	C	D	F	G
Canada	CA18	E	B	E	A	C	D	F	G
Canada	CA19	D	B	G	B	C	D	D	F
Canada	CA20	B	B	G	B	B C	A D	D	E
Canada	CA21	D	C	D	B	B	D	E	F
Canada	CA22	B	B	E	A	C	D	F	B E
Canada	CA23	B	G	E	A	C	D	E F	B
Canada	CA24	E	B F	E	A	A C	B D	D	F
France	FR01	A	E	C	A	B	D	F	F
France	FR02	A	B	D	B	A	C	E	F
France	FR03	B	I	D	A	A	C	E	E
France	FR04	B	E	A	A	B	A	F	E
France	FR05	F	H	C	B	C	D	D	J
France	FR06	B	B	H	A	B	B	D	B
France	FR07	A	A	E	B	A	B	E	G
France	FR08	B	C	D	A	B	B	E	F
France	FR09	B	J	C	A	A	B	D	F
France	FR10	A	A	B D	A	A	D	D	E
France	FR11	B	A	F	A	B	D	F	J
France	FR12	B	B	G	B	A	A	F	B E
France	FR13	B	B C	D	A	B	A	A	B E
France	FR14	B	B	D	B	A	C	C	E
France	FR15	B	H	D	A	A	C	E	D
France	FR16	A	B	D	B	B	B	D	F
France	FR17	A	K	D	B	B	C	D	E
France	FR18	F	B	D	A	D	D	C	J
France	FR19	B	A H	E	A	B	D	F	B
France	FR20	F	B	F	B	B	D	D	E
France	FR21	F	B	D	A	A	B	C	E
France	FR22	B	B	D	B	A	B	D	E
France	FR23	B	B	A	B	A	B	E	G
France	FR24	B	A	D	B	C	D	D	F
Italy	IT01	B	B	E	B	B	A	D	G
Italy	IT02	F	E	B	B	A	B	A	H
Italy	IT03	F	B	B	B	A	B	E	H
Italy	IT04	B	B	E	A	B	D	E	G
Italy	IT05	B	B	E	B	B	A	D	G
Italy	IT06	F	B	B	B	A	B	E	H
Italy	IT07	B	E+H	F	A	B	B	C	C
Italy	IT08	F	E	B	B	A	B	A	I
Italy	IT09	B	B	E	A	B	C	E	G
Italy	IT10	A F	A	F	A	C	D	E	G
Italy	IT11	A	A	D	A	C	D	D	B
Italy	IT12	B	A	D	A	A	A	A	F

Appendx D -- Proposed supplementary marker set examined by three laboratories in six Canadian varieties from CT1 and CT2

SSR	Allele No.[1]	Lab allele scoring			Variety and Lab																	
		Lab 1	Lab 2	Lab 6	AC Andrew			AC Avonlea			AC Barrie			AC Bellatrix			Lillian			AC Readymade		
					1	2	6	1	2	6	1	2	6	1	2	6	1	2	6	1	2	6
Xgwm095	1	127																				
	2	137																				
	3	139	136	135	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	4	141																				
Xgwm052	1	167	168	164	X	X	X							X	X	X						
	2	169	170	166							X	X	X				X	X	X	X	X	X
	3	171																				
	4	null	null	null				X	X	X												
Xbarc074	1	185	187	183	X	X	X										X	X	X			
	2	187	189	185																X	X	X
	3	189	191	187							X	X	X									
	4	191	193	189				X	X	X												
	5	193	195	191										X	X	X						
	6	195																				
	7	197																				
	8	199																				
	9	203																				
Xbarc184	1	211	214	209							X	X	X				X	X	X			
	2	229	232	227																X	X	X
	3	231	234	229	X	X	X							X	X	X						
	4	233																				
	5	235																				
	6	null	null					X	X	X												
Xbarc347	1	247																				
	2	249																				
	3	251	250	250										X	X	X				X	X	X
	4	253	252	252							X	X	X									
	5	255	254	254													X	X	X			
	6	259	258	258	X	X	X															
	7	null	null					X	X	X												
Xgwm372		304																				
		322																				
		328																				
		330	332	329	X	X	X															
		332																				
		334	335	333					X		X	X	X				X	X	X			
		335		334				X		X												
		336	338	335																X	X	X
		340	341	339										X	X	X						
		352																				

[1]Alleles shaded in grey were observed by Lab 1 in a larger set of 24 Canadian Varieties

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

Jean-Louis Laffont, ISTA Statistics Committee

1. Materials and methods

Allele results (0 or 1) from three Comparative Tests (CT) for evaluating the performance of several microsatellite markers are available for 7 laboratories and 24 varieties. Figure 1 summarizes the structure of the data.

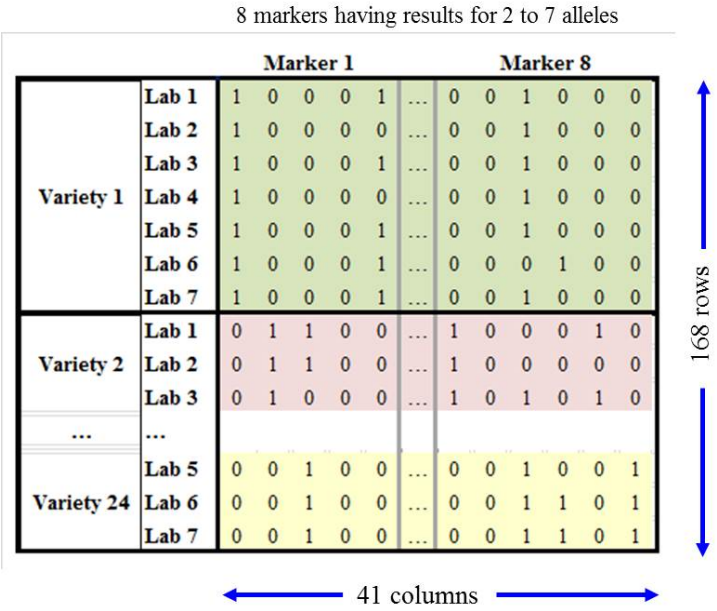


Figure 1: Structure of the data analyzed.

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 and 3 visualize with dot plots the overall percentages agreements; Figures 4 and 5 visualize Cohen's kappas. Tables 4 and 5 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 1, 2, 3, 4 and 6 is good (Fleiss's kappa between 0.913 and 1). It is also good for allele's scoring (overall agreement percentages all above 80%). Laboratories 5 and 7 are clearly not in agreement with the others. Providing the reasons for this failure will be necessary before validating the method.

		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 $\hat{\kappa}_C$.

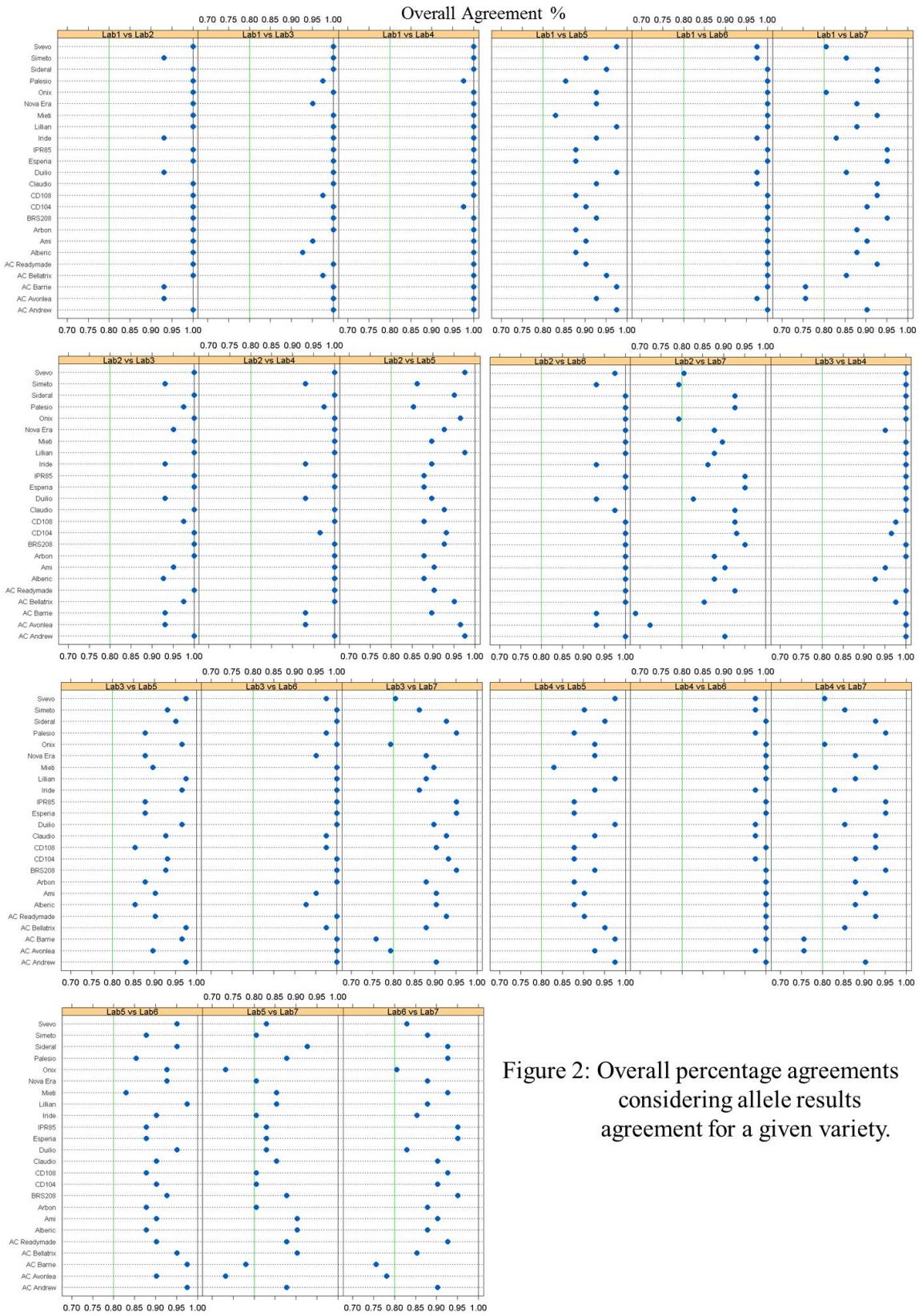


Figure 2: Overall percentage agreements considering allele results agreement for a given variety.

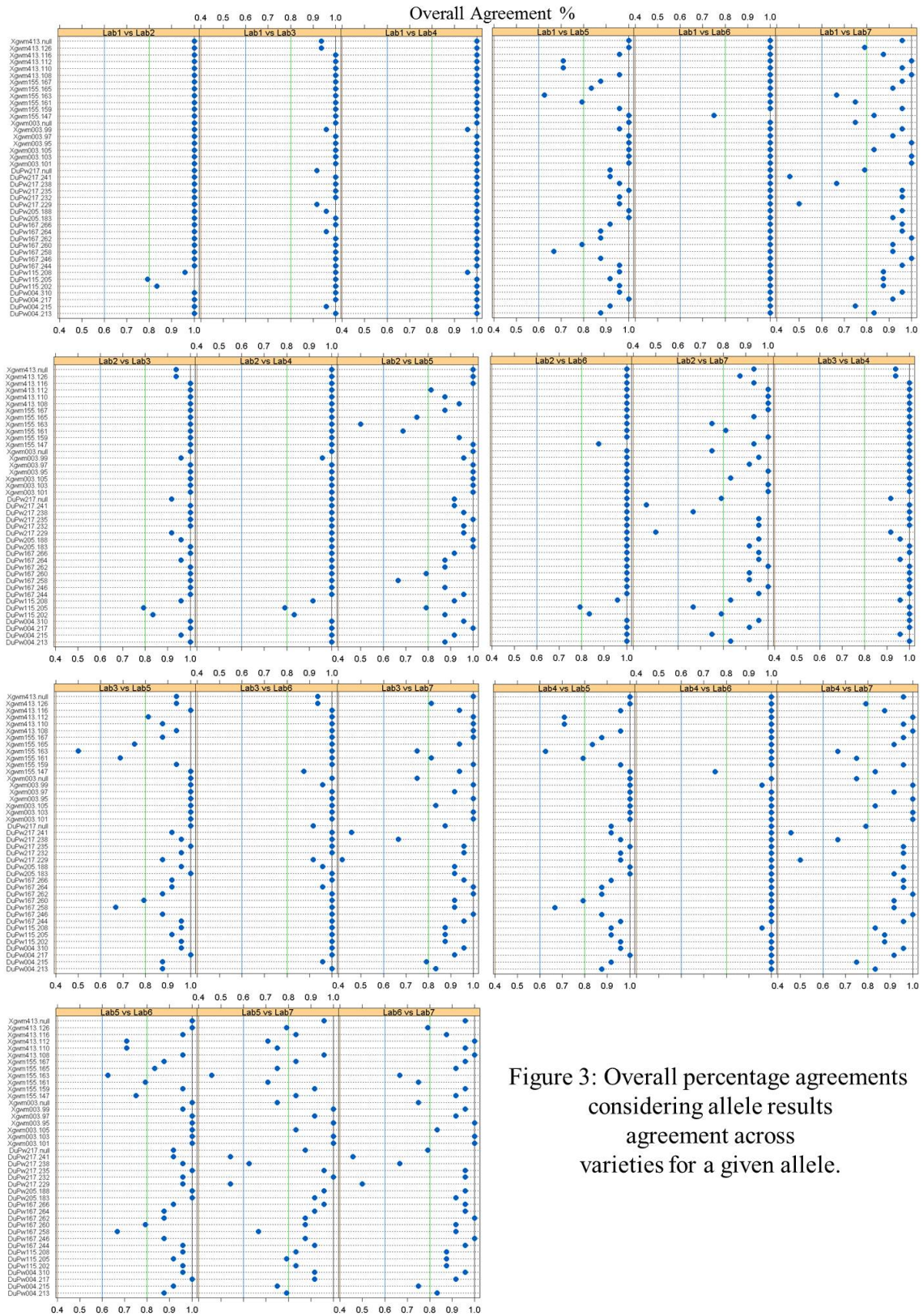


Figure 3: Overall percentage agreements considering allele results agreement across varieties for a given allele.

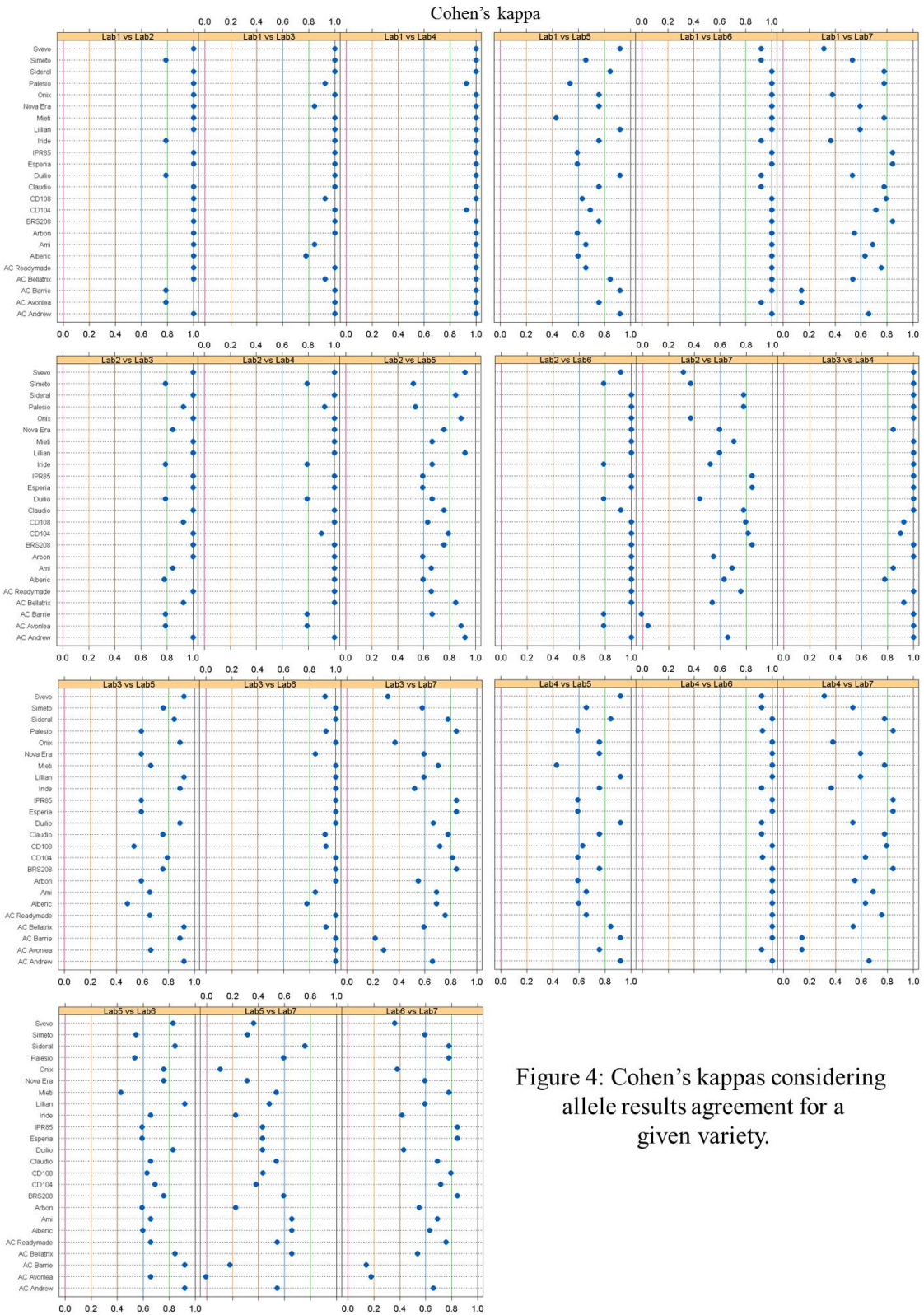


Figure 4: Cohen's kappas considering allele results agreement for a given variety.

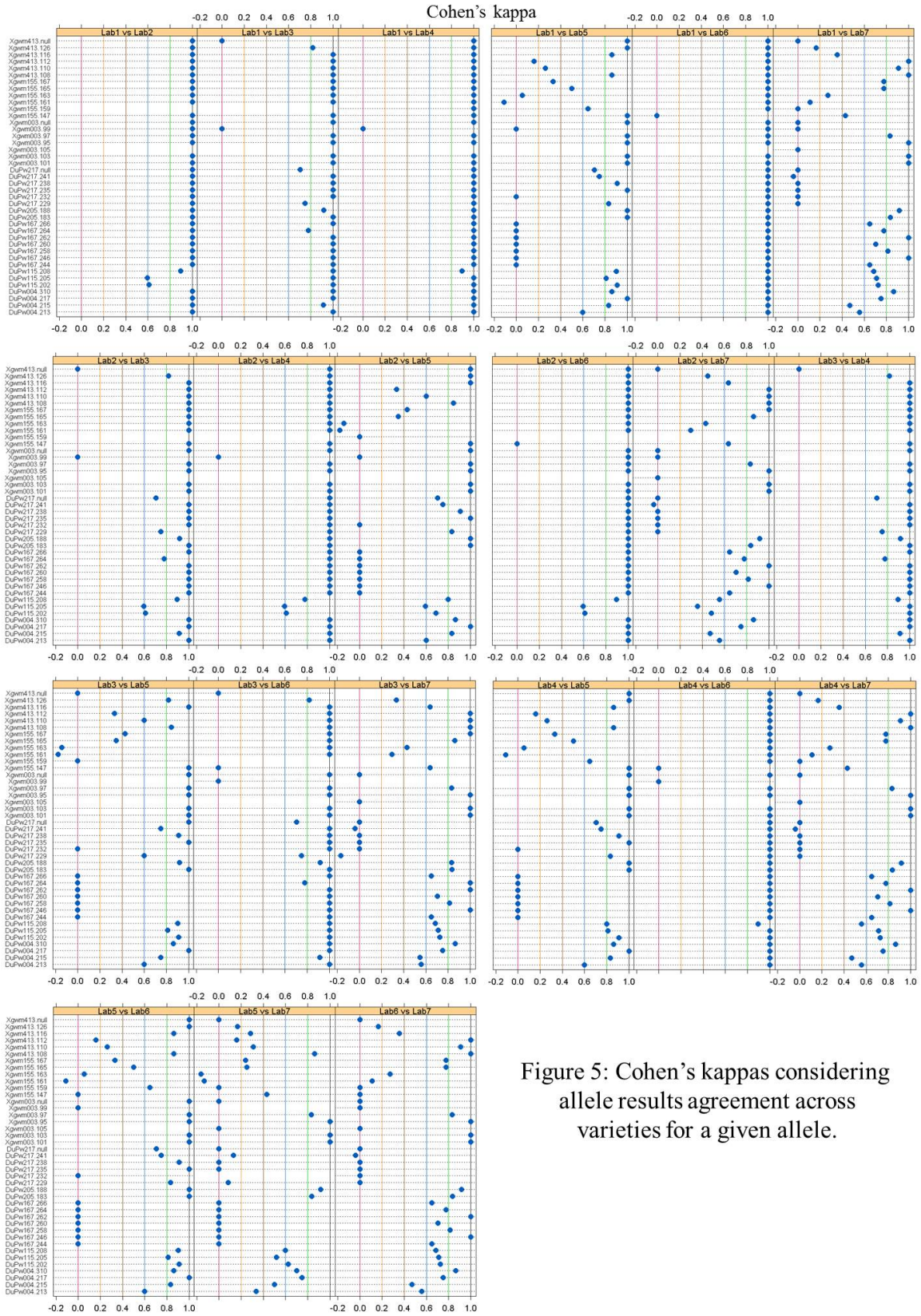


Figure 5: Cohen's kappas considering allele results agreement across varieties for a given allele.

% 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%	20.8%	79.2%
Lab1 vs Lab3	0.0%	0.0%	0.0%	0.0%	4.2%	95.8%
Lab1 vs Lab4	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%
Lab1 vs Lab5	0.0%	0.0%	0.0%	25.0%	45.8%	29.2%
Lab1 vs Lab6	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%
Lab1 vs Lab7	0.0%	8.3%	12.5%	25.0%	41.7%	12.5%
Lab2 vs Lab3	0.0%	0.0%	0.0%	0.0%	25.0%	75.0%
Lab2 vs Lab4	0.0%	0.0%	0.0%	0.0%	20.8%	79.2%
Lab2 vs Lab5	0.0%	0.0%	0.0%	25.0%	45.8%	29.2%
Lab2 vs Lab6	0.0%	0.0%	0.0%	0.0%	20.8%	79.2%
Lab2 vs Lab7	4.2%	4.2%	12.5%	25.0%	37.5%	16.7%
Lab3 vs Lab4	0.0%	0.0%	0.0%	0.0%	4.2%	95.8%
Lab3 vs Lab5	0.0%	0.0%	0.0%	29.2%	33.3%	37.5%
Lab3 vs Lab6	0.0%	0.0%	0.0%	0.0%	4.2%	95.8%
Lab3 vs Lab7	0.0%	0.0%	16.7%	25.0%	37.5%	20.8%
Lab4 vs Lab5	0.0%	0.0%	0.0%	29.2%	41.7%	29.2%
Lab4 vs Lab6	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%
Lab4 vs Lab7	0.0%	8.3%	12.5%	25.0%	37.5%	16.7%
Lab5 vs Lab6	0.0%	0.0%	0.0%	29.2%	41.7%	29.2%
Lab5 vs Lab7	4.2%	8.3%	25.0%	45.8%	16.7%	0.0%
Lab6 vs Lab7	0.0%	8.3%	8.3%	29.2%	41.7%	12.5%

Table 4: 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%	2.6%	2.6%	94.9%
Lab1 vs Lab3	0.0%	5.1%	0.0%	0.0%	7.7%	87.2%
Lab1 vs Lab4	0.0%	2.5%	0.0%	0.0%	0.0%	97.5%
Lab1 vs Lab5	2.5%	27.5%	5.0%	5.0%	7.5%	52.5%
Lab1 vs Lab6	0.0%	2.5%	0.0%	0.0%	0.0%	97.5%
Lab1 vs Lab7	2.4%	29.3%	4.9%	7.3%	24.4%	31.7%
Lab2 vs Lab3	0.0%	5.1%	0.0%	2.6%	10.3%	82.1%
Lab2 vs Lab4	0.0%	2.6%	0.0%	2.6%	5.1%	89.7%
Lab2 vs Lab5	5.0%	25.0%	5.0%	10.0%	10.0%	45.0%
Lab2 vs Lab6	0.0%	2.6%	0.0%	2.6%	2.6%	92.3%
Lab2 vs Lab7	2.5%	22.5%	5.0%	15.0%	17.5%	37.5%
Lab3 vs Lab4	0.0%	2.6%	0.0%	0.0%	7.9%	89.5%
Lab3 vs Lab5	5.1%	25.6%	5.1%	10.3%	5.1%	48.7%
Lab3 vs Lab6	0.0%	7.7%	0.0%	0.0%	7.7%	84.6%
Lab3 vs Lab7	5.3%	15.8%	5.3%	7.9%	23.7%	42.1%
Lab4 vs Lab5	2.6%	25.6%	5.1%	5.1%	10.3%	51.3%
Lab4 vs Lab6	0.0%	5.0%	0.0%	0.0%	0.0%	95.0%
Lab4 vs Lab7	2.5%	27.5%	5.0%	10.0%	22.5%	32.5%
Lab5 vs Lab6	2.5%	30.0%	5.0%	5.0%	7.5%	50.0%
Lab5 vs Lab7	5.1%	46.2%	12.8%	7.7%	10.3%	17.9%
Lab6 vs Lab7	2.4%	31.7%	4.9%	4.9%	24.4%	31.7%

Table 5: 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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Proposal for the addition of Tetrazolium Method as a Vigour Test to *Glycine max* seeds.

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SUMMARY

The objective of this study was to demonstrate that the Tetrazolium Test is a Vigour Method for *Glycine max,* repeatable within laboratories and reproducible between them. Three seed lots of *Glycine max*, with a laboratory germination of ≥ 80% (lot 1: 92%; lot 2: 89% and lot 3: 80%), were tested by six laboratories using the Tetrazolium Test. The seeds were classified into four categories: high vigour, medium vigour, low vigour and other staining (viable non-vigorous seeds plus non-viable seeds). The proportion of seeds in high, medium and low vigour categories was summarised as TZ-vigour (%) . The values for TZ-vigour (%) fell within the tolerance levels established by ISTA. The TZ-vigour data were analysed separately using calculation of *z-scores*, *h-values* and *k-values*. Calculation of the *z-scores* revealed that TZ-vigour data did not exceed the value 2, therefore the results are considered satisfactory. The *h-values* showed that five laboratories neither overestimated nor underestimated the results for all the lots. The *k-values* showed the variability between replicates of each lot within each laboratory. The replicates were in tolerance for all lots and all laboratories. The test was both repeatable within laboratories and reproducible in different laboratories. We conclude that the data for TZ-vigour (%) shows acceptable variation and therefore that the Tetrazolium test can be applied as a vigour method for soybean.

INTRODUCTION

The estimated diagnosis of seed vitality, evaluated with Tetrazolium technique, was called by Lakon "vitality of the seed", and so it was assimilated to the term "germinating power" (ISTA, Working Sheets, 2003). This term refers to the fact that seeds could be evaluated with a tetrazolium dye to verify their potential to produce new seedlings.

The Tetrazolium Test also provides a rapid evaluation of the vigour of viable seeds (Moore,1985), and it was quickly accepted in USA, South America and Europe, where it was applied for numerous commercial species. Many investigations were carried out to explain the phenomenon of "how alive live seeds are". This led to the use of viability testing not only to find a correspondence with the germination power of the seed lot, but also to create a tool capable of clarifying essential aspects related to seed vigour. Therefore, the Tetrazolium Test as a vigour method was described for cereals in general by Lakon (1950)

and presented for wheat seed by Perry (1987). In addition, it has been used on maize (Dias and Barros, 1995), cotton (Santos *et al*., 1992; Vieira & Von Pinho, 1999), peanut (Gelmond, 1962; Moore, 1972; Bittencourt, 1995), soybean (França-Neto *et al*., 1988; Craviotto *et al*., 1995; Costa *et al*., 1998; Craviotto *et al*., 2009), pea, pine and clover (AOSA, 1983).

The reliability and precision of the tetrazolium test has been revealed in work by 41 seed laboratories that tested several samples of soyabean seeds in the standard germination, accelerated aging and Tetrazolium tests, and for emergence in sand (França-Neto et al., 1986). The Tetrazolium test ranked as the second most precise test with respect to repeatability after the standard germination test. Subsequently França-Neto et al. (2001, 2002) performed a referee test involving 27 seed laboratories. They concluded that the Tetrazolium test was as precise as the standard germination test for determining viability and more precise than the accelerated aging test for vigour determination. In 2004, França-Neto et al. (2004), concluded, on the basis of evaluating 1117 soybean lots from 1997 to 2002 in Brazil, that the vigour value determined by the Tetrazolium test can be reliably used to estimate the potential emergence of seedlings in field conditions (PFE) when the test is performed three or four weeks before sowing. An equation was developed to predict potential field emergence on the basis of seed vigour tested using the Tetrazolium test (PFE = 0.6165 * TZ Vigor + 35.716). When this equation was tested for 100 seed lots in 2002 the coefficient of regression of 0.79 was highly significant (P< 0.001). This equation is currently being evaluated by soybean seed companies in Brazil." (Seed Vigor Testing Handbook, AOSA 2009)

The Tetrazolium Test is based on the activity of certain enzymes called dehydrogenases, which participate in the respiration reactions of the mitochondria of live cells. These enzymes are present in the live seed tissues, and they reduce the colourless solution of 2,3,5-triphenyl tetrazolium chloride to an insoluble red/rose colour generically called formazan. When the seeds are soaked in a tetrazolium solution, the live cells of the tissues suffer a chemical reaction of oxide reduction where are present the enzymes participant (Glenner, 1990).

The staining of the soybean seed shows the respiratory activity of the different tissues. As a consequence of the vitality condition of the seed structures, different colours and intensities appear, reflecting the differences in respiratory activity. Therefore, according to its physiological condition, a topographic image of the seed is produced on its external and internal surfaces (Moore, 1985; Craviotto *et al*., 2008; França-Neto *et al*., 1998).

The objective of this study was to demonstrate that the tetrazolium test is a vigour method to *Glycine max,* repeatable within laboratories and reproducible between them.

MATERIALS AND METHODS

Samples of three seed lots of *Glycine max* no GMO were obtained from Parana Experimental Station of National Institute of Agricultural Research, Argentina. All seed lots had standard germination above 80% (lot 1: 92%; lot 2: 89% and lot 3: 80%). Coded samples of the seed lots were sent from Oliveros, Argentina to the participating laboratories, namely Francomano & Picardi Seed Lab, Argentina; LEA Seed Lab, Argentina; EMBRAPA Soybean, Brazil; Sementes Adriana, Brazil; COCARI Seed, Brazil; National Institute of Agricultural Investigation, Uruguay and National Institute of Agricultural Research, Argentina.

The laboratories participating from Argentina, Brazil and Uruguay are accredited by MERCOSUR Rules. Laboratories of Argentina and Uruguay are also accredited by the Seed National Institutes of both countries. The Seed National Institutes are accredited by ISTA. The private laboratories participating were selected by their prestige, high training, responsibility and experience on Tetrazolium test, especially on soybean seeds.

The three selected seed lots, identified by the numbers in Table 1 were sent out to all participating laboratories by Seed Laboratory of the Institute of Agricultural Research in Argentina in December 2013. The data were received in February 2014. Each laboratory received the three lots codified by seed lot number with the corresponding protocol without any mention of the germination values nor the names of other participants.

Recipients were asked to store the seed in a moisture-proof container (polythene bags) at a low temperature (10ºC) prior to use.

Test method

Preparation of tetrazolium solution: The 2,3,5 triphenyl-tetrazolium chloride salt was dissolved in buffer solution according to the ISTA Rules (ISTA, 2014).

Pre-treatment: Tetrazolium test was conducted on two replicates of 100 seeds to each seed lot. The seeds were soaked overnight during 16-18 hours between rolled filter paper at 20ºC ± 2ºC within sealed plastic bags to avoid evaporation. Then, if necessary, the seeds were soaked in water during 30 to 60 minutes at 20ºC ± 2ºC to complete additional imbibition. Hard seeds may be present at the end of imbibition period and these seeds must be incised at the cotyledonar area opposite the embryo. Then, the hard seeds were soaked overnight during 16-18 hours between rolled filter paper at 20ºC ± 2ºC.

Staining: The intact imbibed seeds were placed in a 0.1% 2, 3, 5 triphenyl tetrazolium chloride solution in dark, during 3 hours at 35ºC ± 2ºC.

Preparation for evaluation: Before evaluation, the tetrazolium solution was decanted and the seeds were rinsed with water. The seeds remained submerged in water during the evaluation to avoid dehydration and discoloration. The seed coat was removed by hand (Figures 1 and 2) and then the embryo was exposed to cut carefully down the middle of the cotyledons, and the hypocotyl axis with a razor blade (Figures 3 and 4).

Figure 1. Soybean seed coat removal

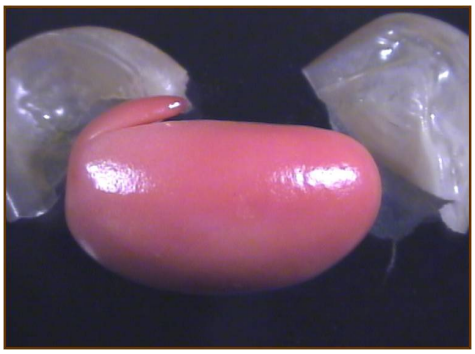

Figure 2. Seed coats totally removed

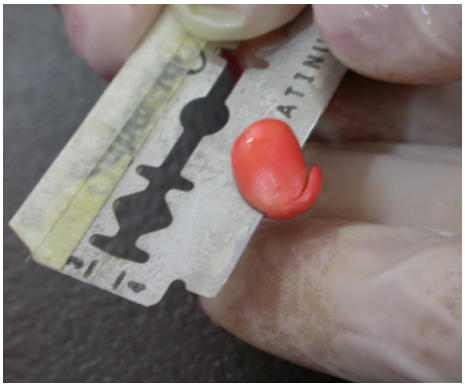

Figure 3. Cutting down through middle of the cotyledons and embryo axis using a razor blade.

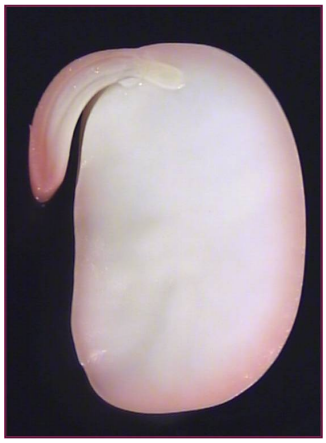

Figure 4. Detail of the inner area of the seed. Longitudinal cut of the embryonic axis and cotyledon.

Evaluation: The stained seeds were observed internally and externally. Seeds with close fitting seed coats were directly related with sound and vigorous tissues which always appeared turgid, externally and internally in pink colour (Figure 5). In addition, in this kind of seed, the Tetrazolium solution penetrated only to a shallow depth, and internally this seed showed a white brilliant colour surrounded by an area of pink colour (Figure 6).

Deteriorated tissues always appeared in red colour and dead tissues in a white dull colour (Figure 7).

Other colours like yellowish, and/or greyish and/or purplish-red and green were considered as non- living tissues (Figure 8).

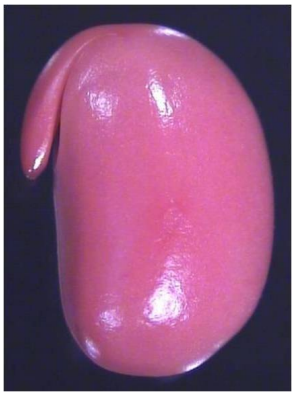

Figure 5. External view. Vigorous seed in pink colour (Category A)

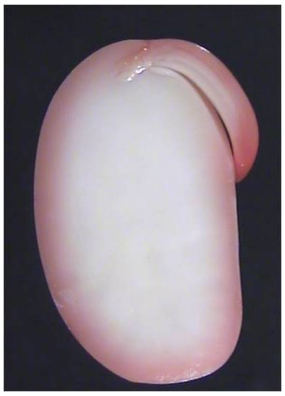

Figure 6: Internal view. Vigorous seed in brilliant white surrounded by pink area (Category A).

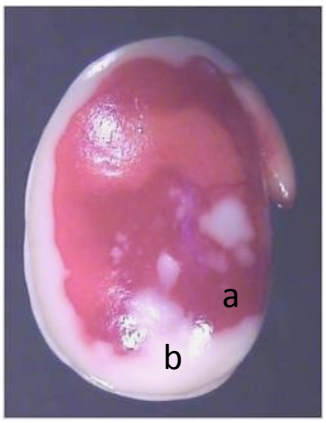

Figure 7. External view.
Seed with deteriorated red tissue (a) and dead tissues in white dull colour (b).

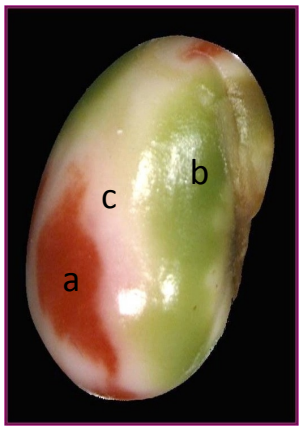

Figure 8. External view.
Seed with deteriorated red tissue (a) and green (b) and white dull dead tissues (c).

The seeds were classified in different categories according to the colour, tissue turgidity and the location (extension and depth) of damaged areas on the seed (Figure 9).

- *High vigour (category A)*: Completely turgid and stained seed of a normal pink colour (Figures 5 and 6).
- *Medium vigour (category B)*: Presence of minor area of red colour, unstained, flaccid or necrotic tissues with limited extension and superficial depth localised at any site of the seed (including embryo axis and joining area on the embryo axis and the cotyledons) (Figure 9 and 10).
- *Low vigour (category C)*: Presence of major or multiple areas of red colour, unstained, flaccid or necrotic tissues with an extension of 1/3 of the cotyledon area to 3/3 of the cotyledonar area at the distal end of the cotyledon(s); and a depth of ½ of the cotyledon to entire cotyledon (Figure 9 and 11).
- *Other staining*: This included **Viable- Non Vigorous Seeds (Figure 12A-E)**: Radicle with tissues up to 1/3 deteriorated, unstained or lost (A); joining area embryo axis-cotyledons with deteriorated red tissues (B); cotyledons with tissues up to ½ deteriorated, unstained or lost (C); cotyledons with tissues up to ¼ deep deteriorated or unstained (D); cotyledon with tissues up to ¾ deteriorated, unstained or lost (E).
  Non Viable Seeds: Radicle with more than 1/3 of deteriorated, unstained or lost tissues (Figure 12F); joining area embryo axis-cotyledons unstained (Figure 12G); plumule deteriorated or lost (Figure 12H); cotyledons with ½ or more deteriorated, unstained or lost tissues (Figure 12I); cotyledons with more than ¼ deep deterioration or unstained tissues (Figure 12J); cotyledon with more than ¾

deteriorated, unstained or lost tissues (Figure 12K); entire seed unstained (Figure 12L).

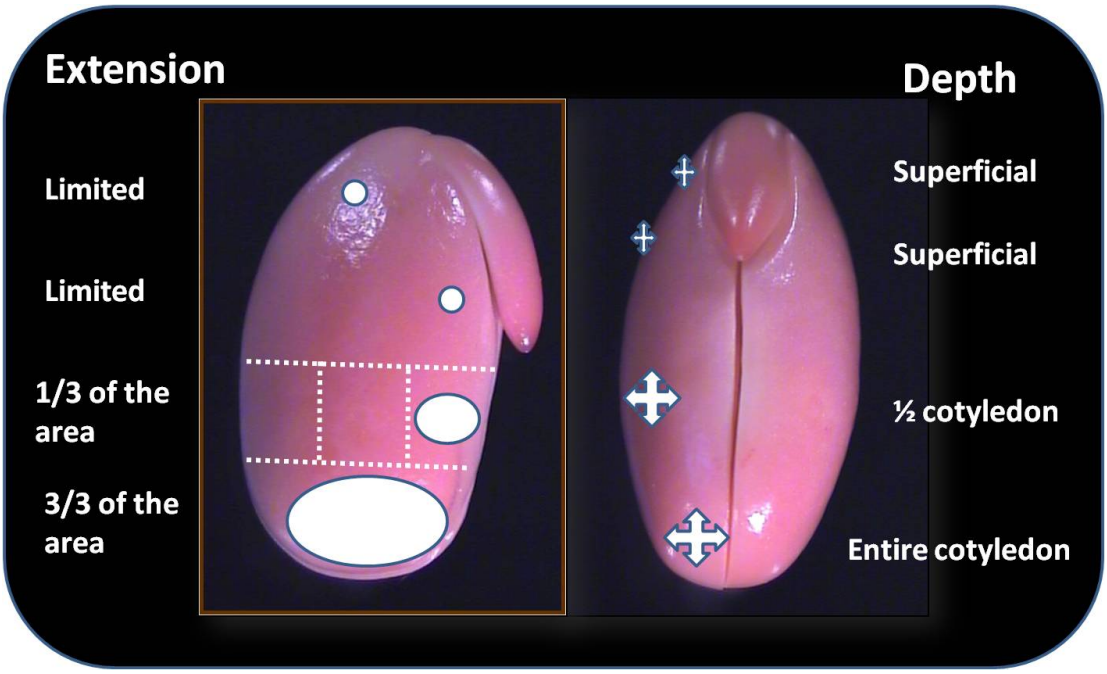

Figure 9. Location of damaged areas on the seed.

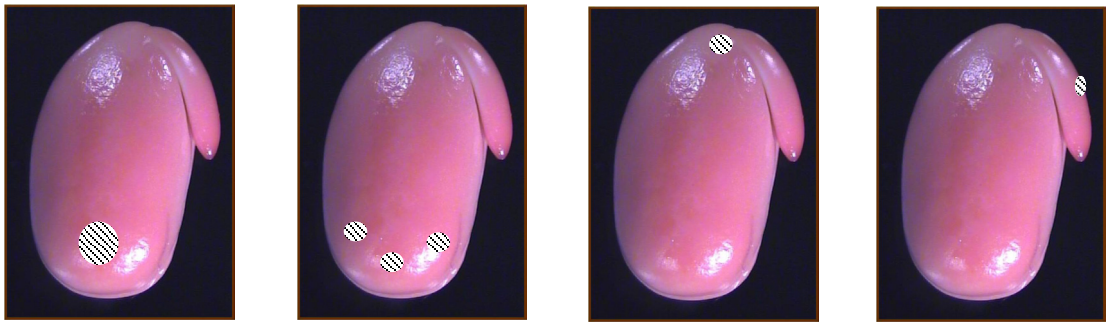

Figure 10. **Medium Vigour Seeds (Category B)**: minor areas of red colour, unstained, flaccid or necrotic tissues with limited extension and superficial depth.

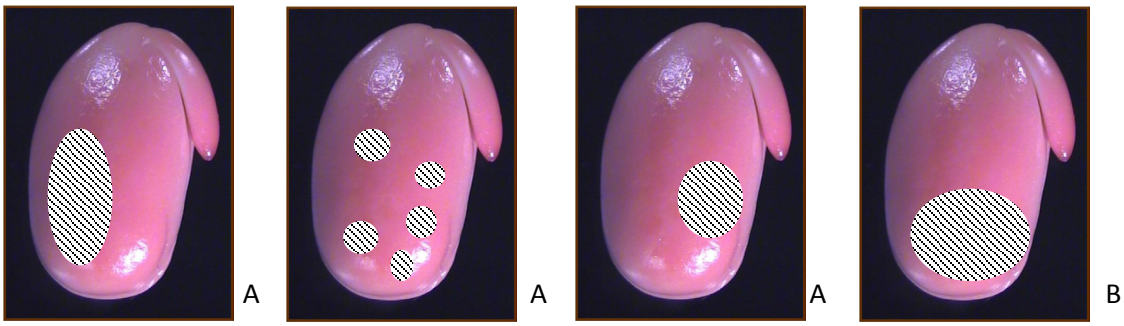

Figure 11. **Low Vigour Seeds (Category C)**: major or multiple areas of red colour, unstained, flaccid or necrotic tissues with an extension of 1/3 of the cotyledonar area (A) to 3/3 of the cotyledonar area at the distal end of the cotyledon(s) (B); and a depth of ½ of the cotyledon to entire cotyledon.

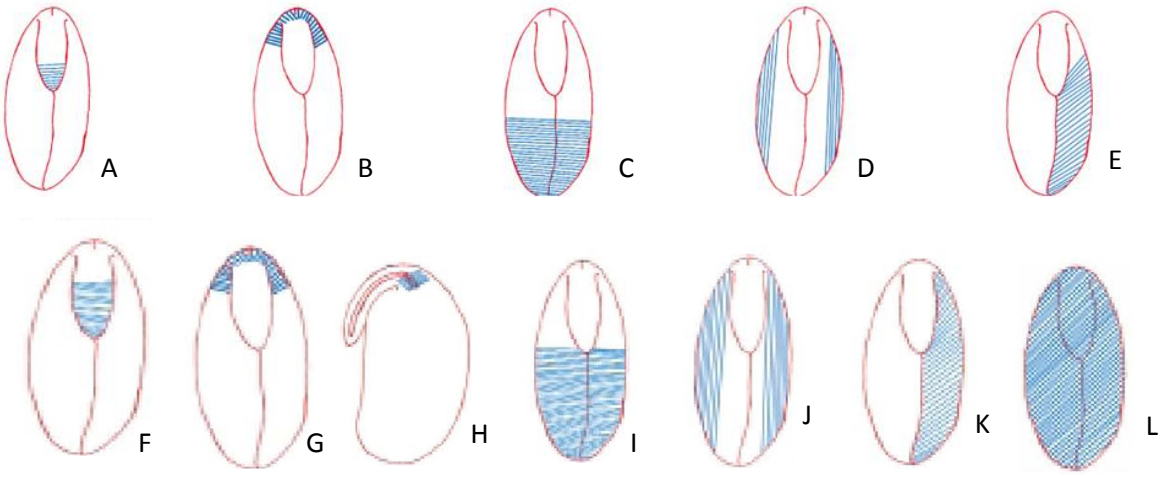

Figure 12. **Other Staining** : *Viable- Non Vigorous Seeds*: Radicle with tissues up to 1/3 deteriorated, unstained or lost (A); joining area embryo axis-cotyledons with deteriorated red tissues (B); cotyledons with tissues up to ½ deteriorated, unstained or lost (C); cotyledons with tissues up to ¼ deep deteriorated or unstained (D); cotyledon with tissues up to ¾ deteriorated, unstained or lost (E).
Non Viable Seeds: Radicle with more than 1/3 of deteriorated, unstained or lost tissues (Figure 12F); joining area embryo axis-cotyledons unstained (Figure 12G); plumule deteriorated or lost (Figure 12H); cotyledons with ½ or more deteriorated, unstained or lost tissues (Figure 12I); cotyledons with more than ¼ deep deterioration or unstained tissues (Figure 12J); cotyledon with more than ¾ deteriorated, unstained or lost tissues (Figure 12K); entire seed unstained (Figure 12L).

Appendix 1 shows an example of the table used to record the data. In this report, the vigour of a lot is summarised as the TZ-vigour (%), which is the sum of seeds in the three categories of vigour: A (high vigour) +B (medium vigour) +C (low vigour), expressed as a percentage. The proportion of seed in each category can be used for 'in-house' assessment

of seed vigour. However, this report will focus only on the overall (TZ-vigour (%)) assessment.

Statistical analysis

The TZ-vigour (%) data from Tetrazolium Test were analysed separately using calculation of **z-scores** and the statistical tool developed by S. Grégoire according to **ISO 5725-2** to calculate **h-values** and **k-values**. The statistical tool is available for download at the ISTA website:

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

The tolerance range (Appendix 2) was used to compare the TZ-vigour (%) for the two replicates of 100 seeds analyzed for each seed lot).

Statistical analysis of the test results was done by Linear Modelling. Tolerances were also used to compare the results from the participating labs: both within labs and between labs.

The participants did not provide information on their experiences regarding the testing of the samples of *Glycine max.*

Reporting results

The results of this method were reported as a unique value called: the TZ-vigour (%).

RESULTS

All the laboratories sent the results of the analysis in time. In this validation no data/laboratory was excluded from the analysis.

Results from all laboratories were expressed as the TZ-vigour (%) (Table 2). Lot 3 had a low TZ-vigour (%) (37, Table 2) compared to lots 1 and 2 which had the same value (66, Table 2). Lots 1 and 2 also showed very similar mean values for the percentage of seed in categories A, B and C across all six laboratories: Lot 1 – A, 14.1; B, 25.9; C, 24.3; Lot 2 – A, 8.0; B, 29.9; C, 27.0. Since the data for the categories A, B and C are not reported, further analysis was limited to the TZ-vigour (%) **Appendix 3** shows the raw data of three seed lots obtained from Tetrazolium Test by six laboratories.

Calculation of the z-scores (**Table 2**) revealed that all data for the TZ-vigour (%) did not exceed the value 2, therefore all results are considered satisfactory.

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

Figure 13 indicates that the laboratory E overestimated the results of lot 1 if we consider the critical h value at 5% significance level, whereas if we consider the critical h value at 1%

significance level this laboratory does not overestimate the results of the same lot. The remaining laboratories neither overestimated nor underestimated the results for all the lots.

Figure 14 shows the variability between replicates of each lot. The results obtained for the lot 1 and 2 in the laboratory A were significantly higher than the variability for the same lots of the other laboratories. Even so, the replicates were in tolerance for all lots and all laboratories (Proceedings of the ISTA Tetrazolium Workshop, Edinburgh, 1997).

Table 3 revealed the repeatability and reproducibility values obtained by all laboratories to three seed lots. These values are within a similar range to previously validated vigour tests (controlled deterioration, radicle emergence, conductivity for *Phaseolus vulgaris* and *Glycine max*)

DISCUSSION

The test clearly revealed that lot 3 had an overall lower level of vigour, expressed as the TZ-vigour (%), than lots 1 and 2 which had similar values. Previous work has shown that such differences relate closely to the ability of seeds to emergence in the field (França-Neto et al 2004). Thus the TZ-vigour (%) predicts one of the expressions of vigour in a seed lot, field emergence ability.

The TZ-vigour (%) expresses the level of vigour amongst the vigorous seeds; non-vigorous and non-viable seeds are excluded. Further separation of seed lots with similar TZ-vigour (%) can be achieved in-house by examination of the proportion of seeds within each category (A, B, C). However, since separation of seeds into these categories can be subject to experience of the analyst, these categories are not reported.

The test was both repeatable within laboratories and reproducible in different laboratories. In addition, the replicates within the laboratories and the mean values obtained for each lot in different laboratories fell within tolerance, using the tolerance tables in the Proceedings of the ISTA Tetrazolium Workshop, Edinburgh, 1997. This provides evidence in support of the addition of Tetrazolium Test to the ISTA Rules as a Vigour Test method for *Glycine max*

ACKNOWLEDGEMENTS

I would like to thank Ing. Agr. M.Sc. Diego Santos to provide the seed lots and Lic. Luciana Magnano for contributing to data analysis.

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Table 1: TZ-vigour (%) data of two replicates of Tetrazolium Test for each of three lots taken in six laboratories.

	Replicate	*LAB*					
		A	***B***	***C***	***D***	***E***	***F***
Lot 1	1	68	58	63	63	76	65
	2	55	59	64	60	72	67
	Mean	**62**	**59**	**64**	**62**	**74**	**66**
Lot 2	1	70	66	61	57	67	66
	2	73	65	62	58	67	66
	Mean	**72**	**66**	**62**	**58**	**67**	**66**
Lot 3	1	26	43	27	37	32	38
	2	27	44	27	36	30	36
	Mean	**27**	**44**	**27**	**37**	**31**	**37**

Table 2: Comparison of means, standard deviations (SD) and z-scores of TZ- Vigour (%) for three seed lots of soybean tested by six laboratories using the Tetrazolium Test.

	Lot 1	**Lot 2**	**Lot 3**
LAB A	61,5	71,5	26,5
LAB B	58,5	65,5	43,5
LAB C	63,5	61,5	27
LAB D	61,5	57,5	36,5
LAB E	74	67	31
LAB F	66	66	37
Mean	**64,167**	**64,833**	**33,583**
Standard Dev	**5,419**	**4,813**	**6,614**

z-score	**Lot 1**	**Lot 2**	**Lot 3**
LAB A	-0,492	1,385	-1,071
LAB B	-1,046	0,139	1,499
LAB C	-0,123	-0,693	-0,995
LAB D	-0,492	-1,524	0,441
LAB E	1,815	0,450	-0,391
LAB F	0,338	0,242	0,517

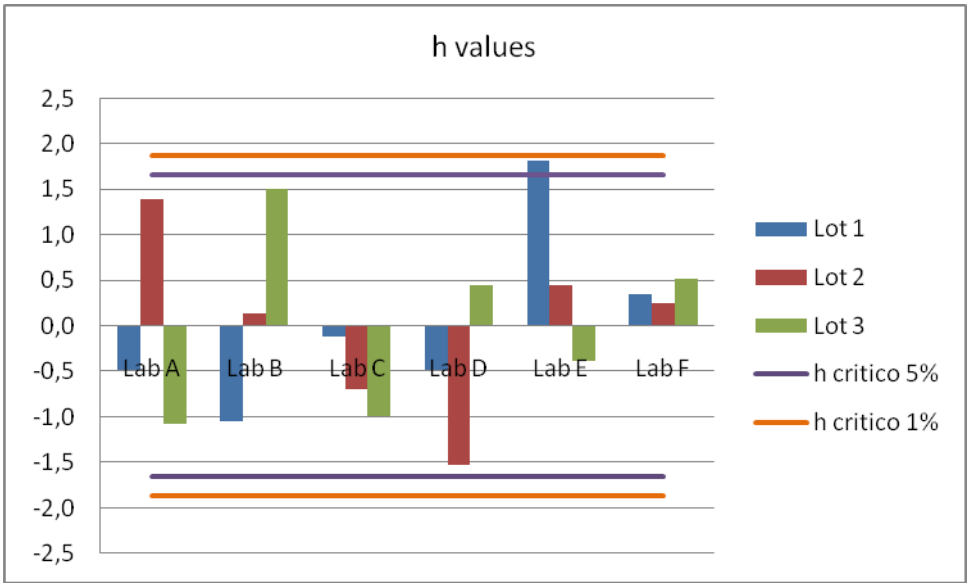

Figure 13: TZ-vigour (%): h-values for three seed lots of *Glycine max* tested using the Tetrazolium test in six laboratories.

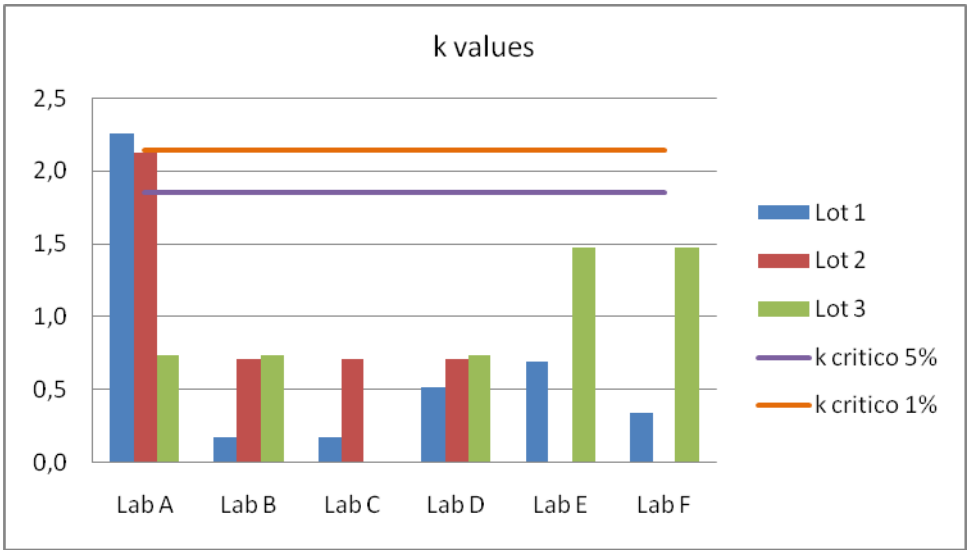

Figure 14: TZ-vigour (%): k-values for three seed lots of *Glycine max* tested using the Tetrazolium test in six laboratories.

Table 3: TZ-vigour (%) - repeatability and reproducibility of results from the Tetrazolium Test

Lot	Repeatability	Reproducibility
Lot 1	4,082	6,140
Lot 2	1,000	4,865
Lot 3	0,957	6,648

Appendix 1. Example of the data of two replicates of Tetrazolium Test to illustrate how the final TZ- vigour (%) is obtained.

Tetrazolium Vigour Test results

LOT Nº 1				
Vigour Levels	Replicates (Number of seeds)			
	I	II	Average (Number of seeds)	Average (%)
High Vigour (A)	2	4	3	3
Medium Vigour (B)	25	21	23	23
Low Vigour (C)	59	57	58	58
TZ-vigour (A + B + C)	86	82	84	84
Other Staining	14	18	16	16

Appendix 2. Tolerance Ranges

Taken from International Seed Testing Association, Proceedings of the ISTA Tetrazolium Workshop, Edinburgh, 1997 (Compiled and Edited by Don, R.; Leist, N; Steiner, A.M.)

Tolerance Ranges employed to compare results of tetrazolium analyses (P= 0,05)

Average Number of Viable Seeds in the Sample		Number of Seeds Analyzed 2 x 100
A	B	Tolerance
99	2	3
98	3	4
97	4	5
96	5	5
95	6	6
94	7	7
93	8	7
92	9	7
91	10	8
90	11	8
89	12	9
88	13	9
87	14	9
86	15	9
85	16	10
84	17	10
83	18	10

Average Number of Viable Seeds in the Sample		Number of Seeds Analyzed 2 x 100
A	B	Tolerance
82	19	10
81	20	11
80	21	11
79	22	11
78	23	11
77	24	11
76	25	12
75	26	12
74	27	12
73	28	12
72	29	12
71	30	12
70	31	12
69	32	13
68	33	13
67	34	13
66	35	13
65	36	13
64	37	13
63	38	13
62	39	13
61	40	13
60	41	13
59	42	13
58	43	13
57	44	13
56	45	13
55	46	13
54	47	14
53	48	14
52	49	14
51	50	14

Appendix 3. Raw data of two replicates of Tetrazolium Test for each of three lots taken in six laboratories.

LAB A

LOT 1			
	REPLICATES		
VIGOUR LEVEL	I	II	MEAN (%)
HIGH VIGOUR (A)	29	16	22,5
MEDIUM VIGOUR (B)	10	27	18,5
LOW VIGOUR (C)	29	12	20,5
ACCUMULATED VIGOUR (A+B+C)	68	55	62
OTHER STAINING	32	45	39

LOT 2			
	REPLICATES		
VIGOUR LEVEL	I	II	MEAN (%)
HIGH VIGOUR (A)	16	13	14,5
MEDIUM VIGOUR (B)	40	32	36
LOW VIGOUR (C)	14	28	21
ACCUMULATED VIGOUR (A+B+C)	70	73	72
OTHER STAINING	30	27	28

LOT 3			
	REPLICATES		
VIGOUR LEVEL	I	II	MEAN (%)
HIGH VIGOUR (A)	5	1	3
MEDIUM VIGOUR (B)	11	5	8
LOW VIGOUR (C)	10	21	15,5
ACCUMULATED VIGOUR (A+B+C)	26	27	27
OTHER STAINING	74	73	73

LAB B

LOT 1			
	REPLICATES		
VIGOUR LEVEL	I	II	MEAN (%)
HIGH VIGOUR (A)	2	2	2
MEDIUM VIGOUR (B)	24	20	22
LOW VIGOUR (C)	32	37	35
ACCUMULATED VIGOUR (A+B+C)	58	59	59
OTHER STAINING	42	41	41

LOT 2			
	REPLICATES		
VIGOUR LEVEL	I	II	MEAN (%)
HIGH VIGOUR (A)	3	2	3
MEDIUM VIGOUR (B)	32	31	32
LOW VIGOUR (C)	31	32	31
ACCUMULATED VIGOUR (A+B+C)	66	65	66
OTHER STAINING	34	35	34

LOT 3			
	REPLICATES		
VIGOUR LEVEL	I	II	MEAN (%)
HIGH VIGOUR (A)	1	0	1
MEDIUM VIGOUR (B)	11	9	10
LOW VIGOUR (C)	31	35	33
ACCUMULATED VIGOUR (A+B+C)	43	44	44
OTHER STAINING	57	56	56

LAB C

LOT 1			
	REPLICATES		
VIGOUR LEVEL	I	II	MEAN (%)
HIGH VIGOUR (A)	24	18	21
MEDIUM VIGOUR (B)	16	26	21
LOW VIGOUR (C)	23	20	21,5
ACCUMULATED VIGOUR (A+B+C)	63	64	63,5
OTHER STAINING	37	36	36,5

LOT 2			
	REPLICATES		
VIGOUR LEVEL	I	II	MEAN (%)
HIGH VIGOUR (A)	15	9	12
MEDIUM VIGOUR (B)	22	31	26,5
LOW VIGOUR (C)	24	22	23
ACCUMULATED VIGOUR (A+B+C)	61	62	61,5
OTHER STAINING	39	38	38,5

LOT 3			
	REPLICATES		
VIGOUR LEVEL	I	II	MEAN (%)
HIGH VIGOUR (A)	0	0	0
MEDIUM VIGOUR (B)	9	11	10
LOW VIGOUR (C)	18	16	17
ACCUMULATED VIGOUR (A+B+C)	27	27	27
OTHER STAINING	73	73	73

LAB D

LOT 1			
	REPLICATES		
VIGOUR LEVEL	I	II	MEAN (%)
HIGH VIGOUR (A)	3	4	4
MEDIUM VIGOUR (B)	20	23	22
LOW VIGOUR (C)	40	33	36
ACCUMULATED VIGOUR (A+B+C)	63	60	62
OTHER STAINING	37	40	38

LOT 2			
	REPLICATES		
VIGOUR LEVEL	I	II	MEAN (%)
HIGH VIGOUR (A)	0	0	0
MEDIUM VIGOUR (B)	12	12	12
LOW VIGOUR (C)	45	46	46
ACCUMULATED VIGOUR (A+B+C)	57	58	58
OTHER STAINING	43	42	42

LOT 3			
	REPLICATES		
VIGOUR LEVEL	I	II	MEAN (%)
HIGH VIGOUR (A)	1	1	1
MEDIUM VIGOUR (B)	10	5	8
LOW VIGOUR (C)	26	30	28
ACCUMULATED VIGOUR (A+B+C)	37	36	37
OTHER STAINING	63	64	63

LAB E

LOT 1			
	REPLICATES		
VIGOUR LEVEL	I	II	MEAN (%)
HIGH VIGOUR (A)	2	4	3
MEDIUM VIGOUR (B)	49	47	48
LOW VIGOUR (C)	25	21	23
ACCUMULATED VIGOUR (A+B+C)	76	72	74
OTHER STAINING	24	28	26

LOT 2			
	REPLICATES		
VIGOUR LEVEL	I	II	MEAN (%)
HIGH VIGOUR (A)	1	1	1
MEDIUM VIGOUR (B)	40	42	41
LOW VIGOUR (C)	26	24	25
ACCUMULATED VIGOUR (A+B+C)	67	67	67
OTHER STAINING	33	33	33

LOT 3			
	REPLICATES		
VIGOUR LEVEL	I	II	MEAN (%)
HIGH VIGOUR (A)	0	0	0
MEDIUM VIGOUR (B)	14	14	14
LOW VIGOUR (C)	18	16	16
ACCUMULATED VIGOUR (A+B+C)	32	30	31
OTHER STAINING	68	70	69

LAB F

LOT 1			
	REPLICATES		
VIGOUR LEVEL	I	II	MEAN (%)
HIGH VIGOUR (A)	32	31	32
MEDIUM VIGOUR (B)	21	27	24
LOW VIGOUR (C)	12	9	10
ACCUMULATED VIGOUR (A+B+C)	65	67	66
OTHER STAINING	35	33	34

LOT 2			
	REPLICATES		
VIGOUR LEVEL	I	II	MEAN (%)
HIGH VIGOUR (A)	18	18	18
MEDIUM VIGOUR (B)	32	31	32
LOW VIGOUR (C)	16	17	16
ACCUMULATED VIGOUR (A+B+C)	66	66	66
OTHER STAINING	34	34	34

LOT 3			
	REPLICATES		
VIGOUR LEVEL	I	II	MEAN (%)
HIGH VIGOUR (A)	1	0	1
MEDIUM VIGOUR (B)	20	23	21
LOW VIGOUR (C)	17	13	15
ACCUMULATED VIGOUR (A+B+C)	38	36	37
OTHER STAINING	62	64	63

Application of the radicle emergence test to radish (*Raphanus sativus*) seed

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Summary

Radicle emergence (RE) of six seed lots of radish was assessed after 48h at 20^{o} C by each of four laboratories. Clear and significant differences were observed between lots in all laboratories. All results were within tolerance and both repeatability and reproducibility were good, there being no evidence of over-dispersion. It is proposed that the RE test be validated as a vigour test for oilseed rape and include in the ISTA Rules.

Introduction

Early counts of radicle emergence are closely related to the rate of germination, as expressed by the mean germination time (MGT), and to vigour, reflected in the rate of and final field emergence, in a range of species (Matthews and Powell, 2011). This has led to the development of the radicle emergence (RE) test which is included in the ISTA Rules for maize and oilseed rape (ISTA, 2014). In radish, MGT, calculated from regular counts of RE, was related to both field emergence ($r = -0.93^{***}$) and storage potential ($r = -0.96^{***}$) (Mavi *et al*., 2014). The count of RE at 48h taken from this data is also highly predictive of field emergence ($r - 0.948^{***}$; $r^2 = 0.90$) and germination of the lots after storage ($r = .937^{***}$, $r^2 = 0.88$) (unpublished data). Thus the single 48h count of RE test also predicted differences in vigour of radish seed lots.

A comparative test was therefore carried out to determine if the RE test applied to radish is repeatable and reproducible in different laboratories and hence radish could be included in the ISTA Rules as a species to which this test can be applied.

Materials and Methods:

Seeds of 10 seed lots of radish (*Raphanus sativus*) were obtained from different seed suppliers in Turkey. The seed lots were tested for laboratory germination and radicle emergence after 48h at 20^{o}C and six lots showing clear differences in RE, and hence vigour, were selected for the comparative test. The six seed lots selected had standard germinations above 80% (table 1).The lots were sealed in foil packets and coded before sending to the participating laboratories (table 2). On receipt of the seed, they were kept <10^{o}C before use.

The general guidelines for completing the radicle emergence test (ISTA Rules 2015) were followed with modifications as described below.

Each of four replicates of 50 seeds of each seed lot were placed on two germination papers (Whatman No: 5 or similar) moistened with 4 ml distilled water in 90 mm-diameter Petri dishes. When germination papers other than Whatman No. 5 were used the volume of water

added was adjusted; in this case care was taken to ensure that all the water was absorbed into the germination paper and there was no free water on the surface. In addition, care was taken to ensure that the papers were completely flat after addition of water, so that the same surface area of each seed was in contact with the paper.

The Petri dishes were placed into plastic bags in order to prevent water loss during the test and held at 20 ± 1°C. Stacking the Petri dishes on top of each other was avoided as far as possible and where this could not be avoided stacking was limited to two dishes.

Radicle emergence for each replicate was counted after 48 hours. The criterion of germination was 2 mm radicle protrusion. The mean percentage RE was calculated for each seed lot.

The data was analysed by ANOVA and possible outliers were assessed using side by side boxplots (figure 1) and by computing tolerances for germination test replicates..

R**esults**

All laboratories returned data for the RE of six seed lots of radish. However, one laboratory had problems maintaining the temperature during the test and therefore the data from this laboratory has not been included in this validation report.

Data exploration with side-by-side boxplots

There were clear differences between the lots with no outliers (figure 1a), and only small differences between laboratories with only one outlier (figure 1b). Comparison of labs x lots (figure 1c) revealed a number of outliers, most of which were associated with lot 1 where germination was close to 100% (appendix 1)

Comparison of laboratories and lots

There were clear and significant differences between the lots, ranging from a mean of 100% for lot 1 (high vigour) to 44% for lot 4 (low vigour). The differences were consistent between the laboratories, particularly for lots 1 and 2 (high vigour) and lots 3 and 4 (low vigour). Lots with intermediate RE counts were not always clearly different. There were small, although significant differences, between the means for the four laboratories. The z-scores (calculated excluding the outliers) were within the range 2.0 and -2.0 (table 3) and all data was in tolerance (table 4).

Repeatability and Reproducibility

Repeatability and reproducibility were analysed with the statistical tool developed by S. Grégoire, based on ISO 5725-2; this allows the calculation of k-values, which give a measure of the variability of the repeats. Higher values indicate greater variability between replicates (k-values).

There were only two significant k-values at $p< 0.05$ for lot 4, lab 1 and $p<0.01$ for lot 2 lab 2 (figure 2).

Discussion

The range of RE test results from 44 to 100%, clearly and consistently distinguished between seed lots, with lots 1 and 2 having high vigour and lots 3 and 4, low vigour (table 3). The replication of the data (Appendix 1), and the means and z-scores from the laboratories (table 3) all indicated that the test is repeatable and reproducible, as did k-values. Similar data on radish (Mavi *et al*., 2014) has predicted both field emergence and storage potential, i.e. vigour, of seed lots. This data therefore supports the proposal that the RE test could be applied as a vigour test for radish seed lots

Acknowledgements

We are am grateful to all participants in this comparative test, namely Kazim Mavi and Stan Matthews as WG leaders, Ibrahim Demir, Hulya Ilbi, Gillian McLaren and Marie-Hélène Wagner

References

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Mavi, K., Mavi, F., Demir, I. and Matthews, S. (2014). Electrical conductivity of seed soak water predicts seedling emergence and seed storage potential in commercial seed lots of radish. *Seed Science and Technology*, **42**, 1-11

Table 1: Seed lots of radish used in the radicle emergence comparative test

Seed lot	Standard germination (normal seedlings %)
1	100
2	98
3	91
4	80
5	93
6	93

Table 2: Participants in radicle emergence comparative test for radish

Laboratory	**Participant**
Department of Horticulture, Ege Unversity, Izmir, Turkey	Hulya Ilbi
Department of Horticulture, Ankara University, Turkey	Ibrahim Demir
SNES, GEVES, Angers, France	Marie-Helene Wagner
Mustafa Kemal University, Hatay, Turkey	Kazim Mavi
OSTS, SASA, Edinburgh, UK	Gillian McLaren

Table 3: Mean RE data and z-scores obtained in four laboratories for six seed lots of radish. The seed lots are ranked from the highest RE (high vigour) at the top, to the lowest RE (low vigour) at the bottom.

Lot	Laboratory					
	A	B	C	D	Mean	SD
RE data						
1	100	100	100	100	100^{A}	0
2	95	95	95	96	95^{B}	0.82
6	90	88	95	88	90^{C}	3.30
5	89	85	95	88	89^{C}	4.19
3	64	60	69	75	67^{D}	6.24
4	62	21	52	39	44^{E}	18.02
Mean	83^{ab}	75^{c}	84^{a}	81^{b}		
Z scores						
1	0	0	0	0		
2	-1.23	0	0	1.23		
6	0	-0.61	1.51	-0.61		
5	0	-0.95	1.43	-0.24		
3	-0.16	-1.12	0.32	1.28		
4	0.61	1.33	0.55	0.17		

Table 4: Mean germinations and tolerance ranges (4 replicates x 50 seeds) for six lots of radish tested in four laboratories.

Seed lot		Lab A	Lab B	Lab C	Lab D
1	Maximum tolerance range	4	4	4	4
	Observed range	2	2	2	2
	Mean	100	100	100	100
2	Maximum tolerance range	13	13	13	13
	Observed range	2	8	10	4
	Mean	95	95	95	96
3	Maximum tolerance range	27	27	27	24
	Observed range	14	24	10	20
	Mean	64	60	69	75
4	Maximum tolerance range	27	22	28	27
	Observed range	22	16	8	14
	Mean	62	21	52	37
5	Maximum tolerance range	89	20	13	18
	Observed range	10	8	6	8
	Mean	18	85	95	88
6	Maximum tolerance range	17	18	13	18
	Observed range	10	14	4	8
	Mean	90	88	95	88

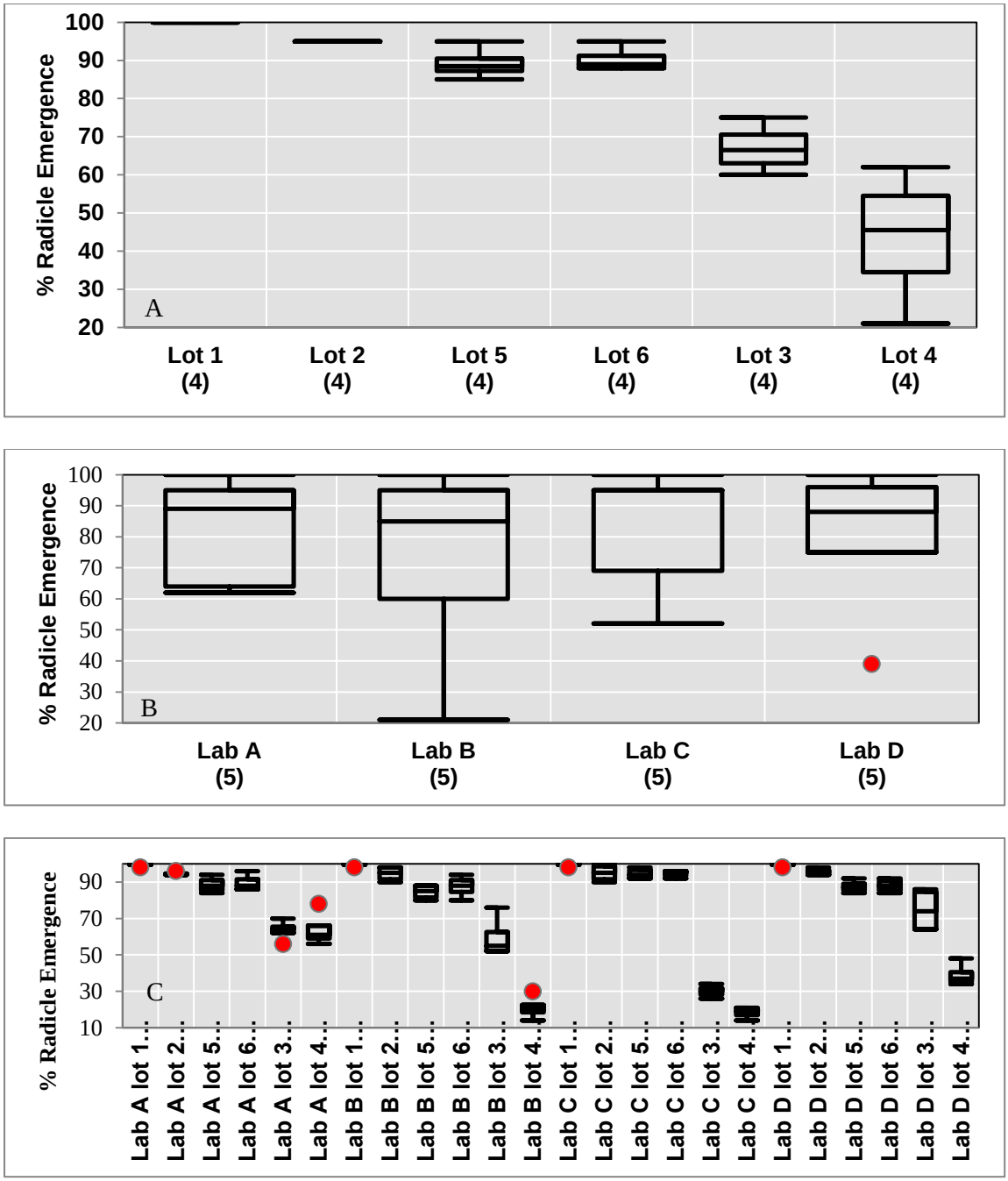

Figure 1: Box plot comparisons of the EC data from seed lots (A), laboratories (B) and seed lot x laboratory (C)

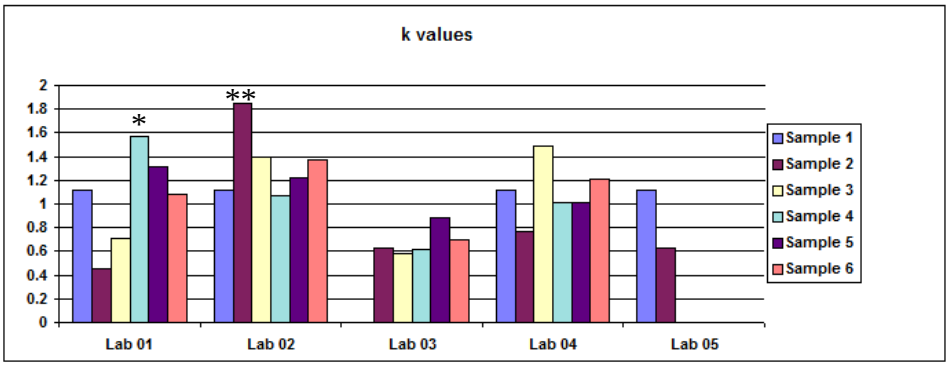

Figure 2 k-values for six seed lots of *Raphanus vulgaris* following the radicle emergence test in five laboratories. * indicates a significant difference at $p < 0.05$; ** indicates a significant difference at $p < 0.01$

Appendix 1: Summary of all raw data for radish radicle emergence test carried out on six seed lots in four laboratories

Lot	Rep	Laboratory			
		A	B	C	D
1	1	100	100	100	98
	2	100	100	100	100
	3	100	100	98	100
	4	98	98	100	100
	Mean	100	100	100	100
2	1	94	92	98	94
	2	94	98	100	98
	3	96	98	92	94
	4	94	90	90	96
	Mean	95	95	95	96
3	1	56	52	64	64
	2	70	52	74	86
	3	64	58	72	84
	4	64	76	66	64
	Mean	64	60	69	75
4	1	60	14	52	39
	2	56	20	46	48
	3	78	20	54	34
	4	62	30	54	36
	Mean	62	21	52	39
5	1	94	82	98	84
	2	90	80	92	86
	3	84	88	96	92
	4	86	88	92	88
	Mean	89	85	95	88
6	1	86	94	94	90
	2	86	80	92	84
	3	96	86	96	86
	4	90	90	96	92
	Mean	90	88	95	88

Alternative method for seed moisture content adjustment in vigour testing, as applied in the CD test.

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Summary

An alternative method to the established filter paper (FP) method of raising the seed moisture content (SMC) during the CD test has been developed. In this method, the volume of water required to achieve the desired moisture content is added to seed in a glass vial, then the seed rolled at <10^{o}C overnight (Added Water, Rolled; AWR method). When applied to 15 seed lots of oilseed rape, similar raised SMCs were achieved to the FP method and only small differences in germination after CD (mean 2% for 15 lots) seen between the two methods. A comparative test using the AWR method was subsequently completed using five seed lots of oilseed rape in five laboratories. Clear and significant differences in germination were observed between lots in all laboratories after CD using the AWR method. All results were within tolerance and both repeatability and reproducibility were good, there being no evidence of over-dispersion.

Introduction

Since 2010, the method for controlled deterioration in the ISTA Rules has combined a first step of seed moisture content (SMC) adjustment before a second step in which seeds are aged at high temperature (ISTA, 2013). Adjustment of SMC currently requires a period of moisture raising in which seeds are imbibed to the desired SMC on filter paper which takes up to two hours per sample depending on the initial seed SMC, followed by a period of overnight equilibration of the SMC. Thus, this step is time consuming. As a result this two-step method could limit the number of samples which can be tested at the same time and the use of CD test in seed testing laboratories.

The French seed testing station has compared the three methods described in the Vigour ISTA Handbook (filter paper, water added and high relative humidity; Hampton and Tekrony, 1995) for SMC adjustment on several species (Wagner *et al*., 2004). The present work has improved the quickest of these methods, namely the addition of a specified volume of water to seeds to achieve the desired increase in SMC, and hence increased its repeatability on Brassica seeds. In this approach the seeds are continuously rotated overnight on a laboratory roller following the addition of water, so that raising the SMC and moisture equilibration take place at the same time,

The promising results obtained in one laboratory (Wagner *et al*., 2013) have recently been tested by four other laboratories in a comparative test on five samples of oilseed rape.

The present report aimed to shorten the method for raising seed moisture content that precedes the deterioration stage of the CD test, thus increasing the throughput of the CD test.

Materials and Methods

Seed Material

Fifteen seed lots of winter oilseed rape were selected from samples of seed lots from different seed companies. The standard germinations of the lots were all high, 93% or above for all (table 1). Assessments of the 1000 seed weight of ten lots were carried out following ISTA Rules (2013), while for five lots the 1000 seed weights are estimates, based on the weight of 100 seeds (table 1). A second set of seed lots were provided by Syngenta Seeds SAS for a comparative test on CD test with 5 laboratories (table 2; in this case 1000 seed weights were supplied by the seed company

Table 1: Details of fifteen lots of winter oilseed rape from different cultivars determined in one laboratory: 1000 seed weight, seed moisture content and standard germination.

Seed lot	Harvest year	Thousand seed weight (g)	Seed moisture content (%)	Standard germination (%)
1	2005	4.744	6.6	95.5
2	2002	4.870	6.2	97.0
3	2002	4.995	6.2	96.5
4	2003	3.897	6.4	96.5
5	2003	3.608	6.0	97.0
6	2003	3.793	5.9	95.0
7	2003	3.797	6.4	93.5
8	2007	4.3	7.0	97.5
9	2007	3.7	6.2	94.0
10	2007	4.7	6.7	96.0
11	2007	4.4	6.9	95.5
12	2006	4.7	6.1	94.0
13	2005	4.890	6.5	95.0
14	2003	5.421	6.3	95.0
15	2008	4.020	6.4	98.0

Table 2: Details of five lots of winter oilseed rape used for the comparative ring test determined in one laboratory: 1000 seed weight (provided by the seed company), seed moisture content and standard germination.

Seed lot	Harvest year	Thousand seed weight (g)	Seed moisture content (%)	Standard germination (%)
A	2012	4.63	6.7	95
B	2012	4.29	6.2	74
C	2012	4.10	6.8	99
D	2012	4.28	7.5	96
E	2012	3.90	6.3	94

For the comparative test, samples of the five lots were distributed in April 2013 in moisture-proof bags. On receipt the laboratories were directed to store the bags at low temperature (4 to 10°C). The samples were coded independently of the test participants. The laboratory tests reported were concluded in April and May 2013.

Controlled deterioration

General directions are those described in ISTA Rules for *Brassica* (ISTA, 2013): seeds adjusted to 20% MC and aged at 45°C ± 0.5°C for 24 hours.

Seed moisture content adjustment: The ISTA prescribed method for adjusting seed MC (filter paper method; FP) was compared to an alternative method for seed moisture content adjustment to 20%, namely the 'Added Water, Rolled' (AWR) method. The AWR method consists of placing seeds in glass vials, adding the volume of water necessary for seeds to reach 20% MC, sealing the vials and rolling them overnight at <10°C using a tube roller (Wagner et al., 2013). This method has been developed by GEVES in comparison to the filter paper method since 2010 and was tested by five labs in a 2013 ring test for CD test.

Four replicates of 100 seeds were adjusted for both the FP and AWR method and the SMC after raising was calculated as:

Raised seed MC = 100-[(initial seed weight/adjusted seed weight) x (100 – initial seed MC)]

The seeds with raised SMC were deteriorated for 24 hours at 45°C before sowing 2 x 100 seeds for a standard germination test.

Analysis of the data accumulated by one lab, comparing the filter paper method of raising seed moisture content with the AWR method is provided in the first part of this report. In the second

part of the report, the results of a comparative CD test using the alternative method are presented.

Moisture content determination

Seed moisture content was determined on 2 replicates of 4-5g seed by drying for 17 hours at 103°C as prescribed in ISTA Rules (ISTA, 2015).

Statistical analysis

Analyses of variance (ANOVA) were carried out with the Generalised Linear Model from the SAS GLM procedure. Possible outliers were assessed by computing tolerances for germination test replicates.

The performance of the method was also assessed though the estimation of repeatability and reproducibility parameters according to ISO5725-2, available from the ISTA website.

Results

Part 1: Initial work establishing the potential for use of the alternative AWR method of raising seed MC

SMC accuracy with the adding water + rolling method (AWR)

Both the AWR and filter paper methods of raising SMC achieved similar levels of accuracy, with a mean moisture content of 19.9 ±0.4 achieved by the AWR method (table 3) and 19.9 ±0.2 by the filter paper method (appendix 1).

Table 3: Accuracy of SMC adjustment on 4 replicates of around 100 seeds using the adding water + rolling method (AWR) to raise seed to 20% MC. MC was calculated following weighing seeds just after equilibration.

Sample	**Weight (g)**	**Water volume added (µl)**	**Moisture content reached (%) after cold equilibration**
1	0.47	64	20.1± 0.3
2	0.50	66	20.2± 0.1
3	0.53	72	20.3± 0.2
4	0.41	54	19.6± 1.1
5	0.37	52	19.7± 0.5
6	0.42	57	19.7± 0.1
7	0.38	49	19.8 ± 0.1
8	0.45	62	19.9± 0.0
9	0.39	51	19.8± 0.1
10	0.50	69	19.8± 0.0
11	2.34	375	20.0± 0.0
12	2.76	430	20.0± 0.1
13	2.60	420	19.9± 0.3
14	2.37	370	19.9± 0.2
15	1.97	310	20.0± 0.2
General MC mean with adding water			**19.9 ±0.4**
MC mean with filter paper method (appendix 1)			**19.9 ±0.2**

Comparison of the results of controlled deterioration test following use of the two SMC raising methods.

Slight, but significant, differences were found in the results of the CD test due to the moisture raising method (appendix 2, table B). The overall mean difference for the 15 lots was 2% of normal germination (AWR = 70% and Filter paper method = 72%). The significant differences were mainly due to two seed lots (9 and 11) as revealed by the interaction plot for SG after CD (figure 1). Nevertheless, low vigour lots (low CD germination) such as 4, 7, 13 and 14 were clearly distinguished from high vigour lots e.g. 2, 3, 10, and 15, with high CD germination, using both SMC adjustment methods (figure 2).

Thanks to these promising results and a tube roller being common and cheap equipment, other ISTA laboratories tested the alternative AWR method to adjust SMC to 20% on more samples simultaneously.

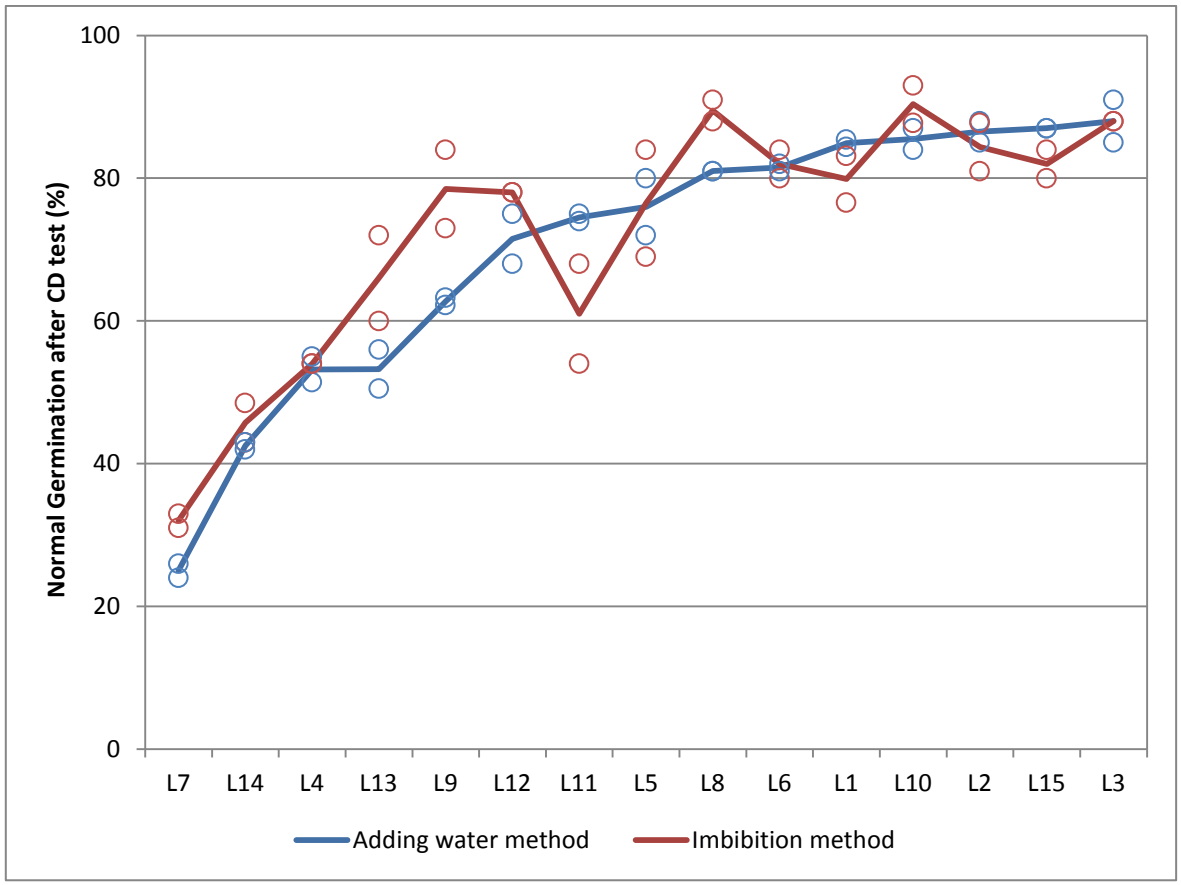

Figure 1: Comparison of the normal germination results after CD at 20% of seed moisture content using the adding water and rolling method or the filter paper imbibition method on 15 seed lots ranked from low normal germination (NG) to high NG after CD test (2 x 100 seed replicates per method).

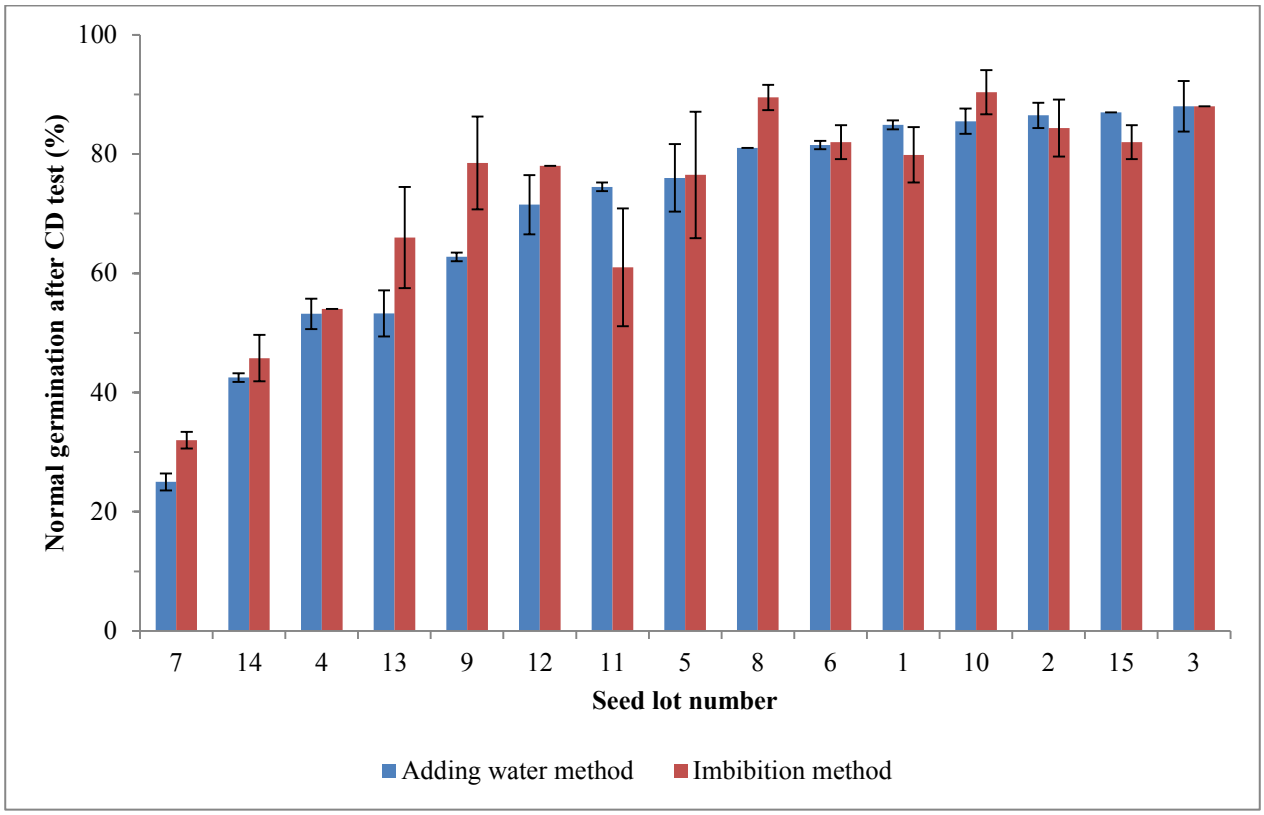

Figure 2: Comparison of normal germination after CD test (%) means (± SD in vertical bars) for 15 OSR samples previously adjusted at 20% of SMC by two methods (Appendix 3). Seed lots are placed in order of increasing CD test result from low (lot 7) to high (lot 3)

Part 2: Comparative test on the use of the alternative AWR method of raising seed MC in the CD test

In 2013, a comparative test was organized through the VIG Committee in which five laboratories evaluated the repeatability and reproducibility of the alternative AWR method of raising SMC. The results are presented below.

Normal germination results after CD revealed clear differences between the seed lots in all laboratories: lot D was the most vigorous and lot B was the worst (table 4).

Table 4: Normal germination (%) after CD test of five lots of oilseed rape in each of four 100-seed replicates in five laboratories.

Lab	**Lot A**	**Lot B**	**Lot C**	**Lot D**	**Lot E**	**Overall lab mean**
1	79.8c	67.8d	87.5b	94.0a	63.5d	78.5b
2	52.3c	48.5c	80.5b	86.8a	47.5c	63.1d
3	69.5b	54.5d	74.0b	86.8a	60.5c	69.0c
4	91.0a	75.0b	95.3a	95.5a	75.5b	86.5a
5	68.0b	49.3d	84.5a	79.8a	60.5c	68.4c
Overall lot mean	72.1c	59.0e	84.3b	88.5a	61.5d	
Lab	**Z-scores**					
1	0.57	0.77	0.38	0.82	0.28	
2	-1.63	-0.93	-0.46	-0.48	-1.62	
3	-0.19	-0.40	-1.24	-0.48	-0.10	
4	1.40	1.41	1.24	1.00	1.78	
5	-0.43	-0.86	0.02	-1.73	-0.28	

Data in columns (lot and lab means) having the different lower-case letters are significantly different ($p < 0.05$).
Within a laboratory, different letters indicate that lots are significantly different ($p < 0.05$).

All laboratories clearly identified lots D and C as having the highest CD germinations (high vigour), with lots E and B having the lowest vigour (table 4), even though both seed sample and laboratory main effects were significant for the CD test germination results (appendix 2, table D). Lot A, having intermediate vigour, had the greatest variability in lab ranking.

Calculated Z-scores (table 4) revealed that all data were within + 2.0 and - 2.0 which is the acceptable range for ISTA proficiency tests.

Repeatability and reproducibility of the CD test using the AWR method for SMC adjustment were also analysed using the ISTA ISO5725 software. No h-values were critical for normal germination after CD test using the alternative method for raising 20% of seed moisture content (figure 3). There were only two significant k-values for two lots (C and D) in laboratory 5 indicating that there was greater variability between replicates (figure 4). For lot C, this heterogeneity was already highlighted by ISTA tolerance tables (appendix 3).

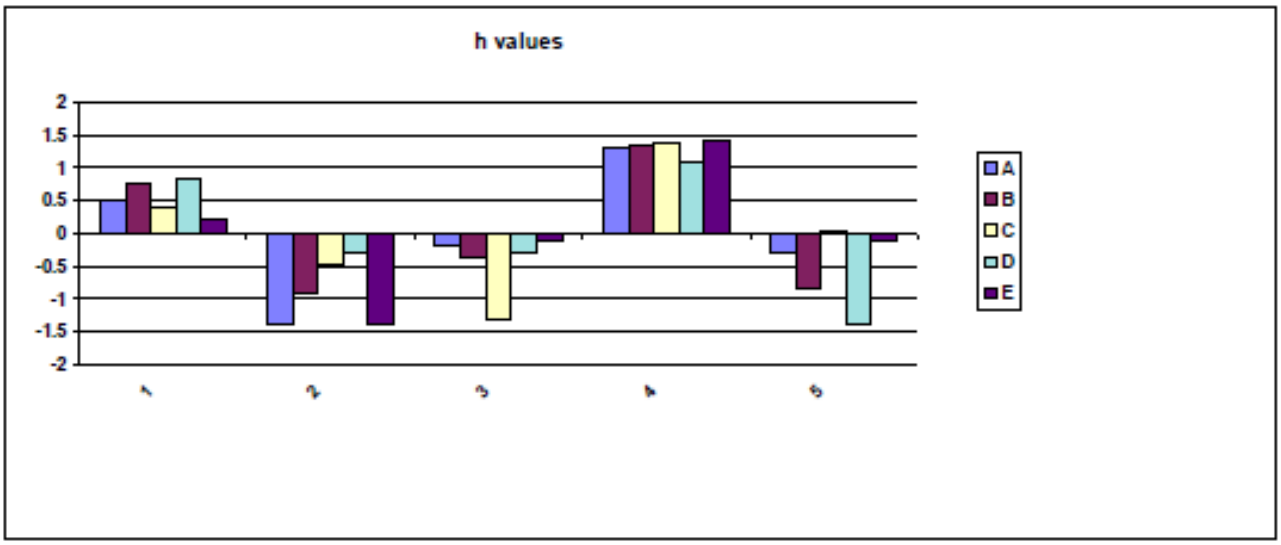

Figure 3: h values for the normal germination assessed after CD test in five laboratories.

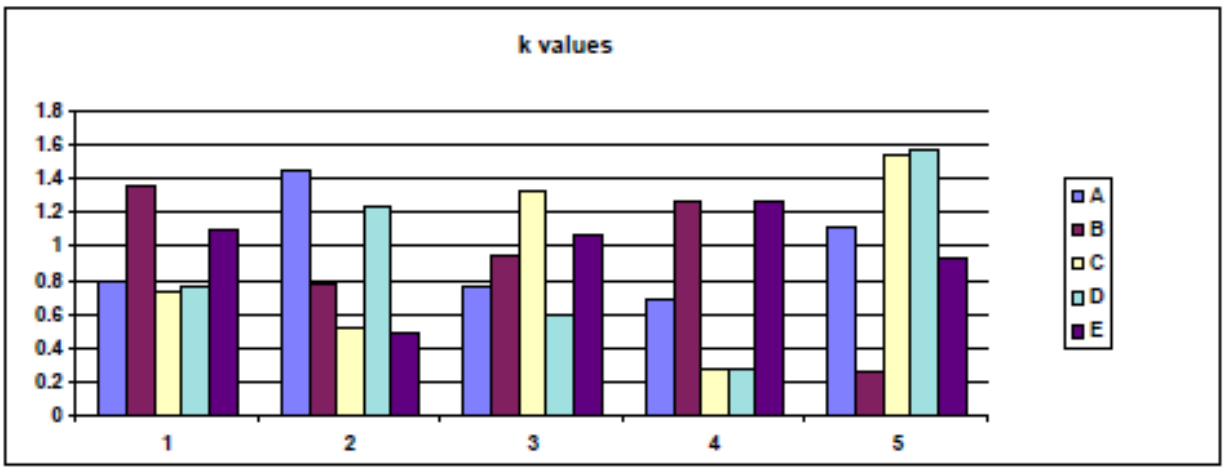

Figure 4: k values for the normal germination assessed after CD test in five laboratories.

Data exploration with side-by-side boxplots

There was a wide range in the CD test assessments from the five seed lots (figure 5A), especially with lot B, but with few outliers. There were no outliers between the mean values obtained by the five laboratories (figure 5B). A seed lot x lab interaction was exhibited in the side-by side boxplots (figure 5C) with 6 outliers.

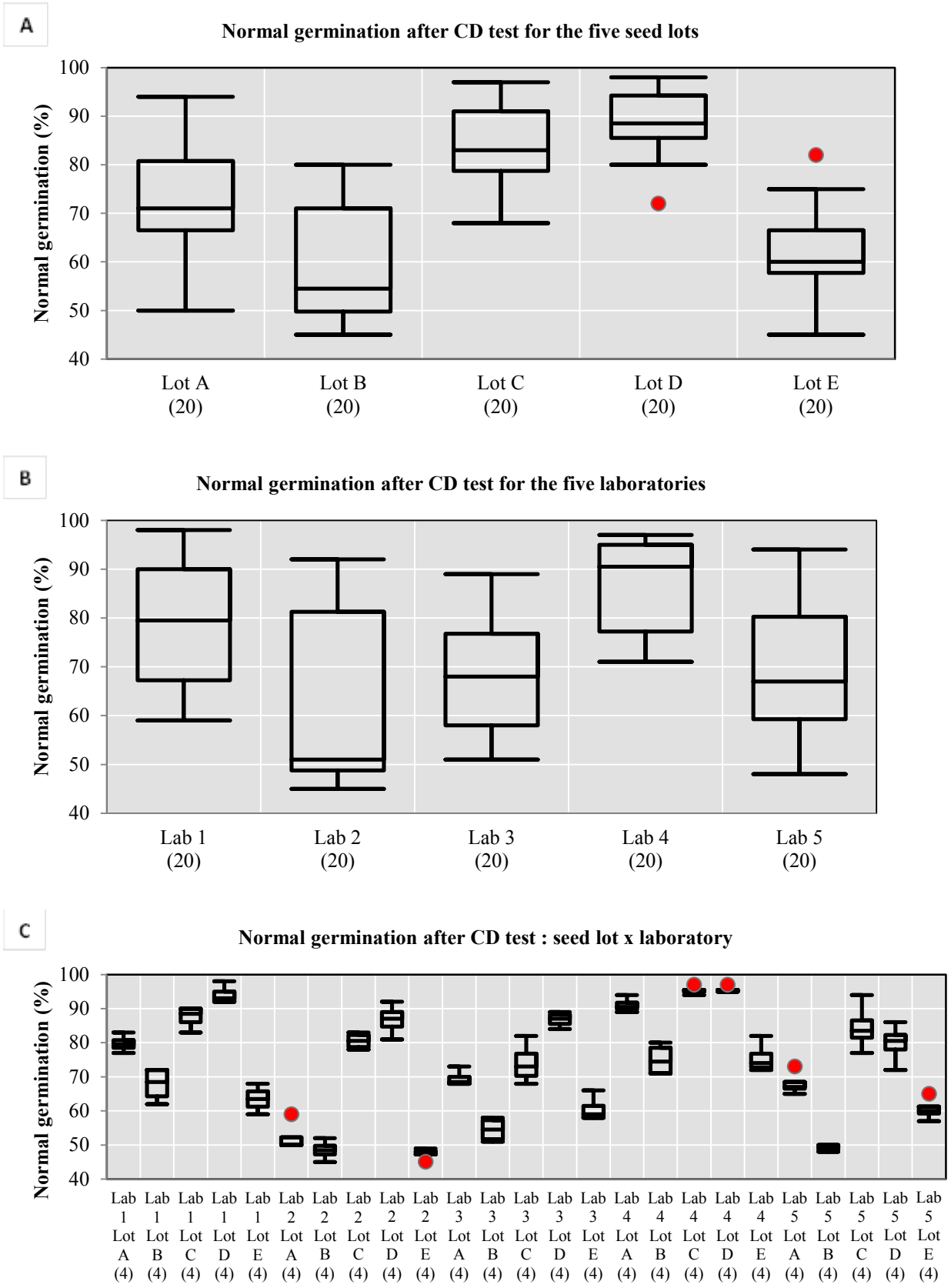

Figure 5: Comparisons of the normal germination after CD test (SG) data from seed lots (A) , laboratories (B) and seed lot x laboratory (C)

Repeatability and Reproducibility

The five laboratories which used the rolling method to adjust seed moisture content had good repeatability and reproducibility. These fell within a similar range to previously validated vigour tests (controlled deterioration, Powell, 2009; radicle emergence, Matthews *et al.* 2011; conductivity test applied to *Phaseolus vulgaris* and *Glycine max,* Powell, 2009a,b).

Table 5: Values for repeatability and reproducibility of results from the conductivity and germination after controlled deterioration test on oilseed rape

	Normal germination	
Lot	**Repeatability**	**Reproducibility**
A	3.125	14.685
B	3.719	12.244
C	4.573	8.852
D	3.699	7.123
E	3.568	10.443

Discussion

The controlled deterioration test, performed using a quicker method of seed moisture content adjustment (water added and rolling, AWR), showed consistent results to identity vigour differences between seed lots in each of five laboratories. The CD test after AWR was both repeatable within laboratories and reproducible in several laboratories.

The water added and rolling method could be an alternative to the current filter paper method for raising seed to the required moisture content during controlled deterioration test. This method is less time consuming as it eliminates the need for frequent re-weighing of seeds as required in the filter paper method. It also completes both moisture raising and equilibration of the SMC at the same time.

Acknowledgements

We sincerely thank Solène Boizard, Laura Bowden, Cathy Colombano, for their contribution to this work. We are also grateful to Mustafa El-Yakhlifi for helping to complete the statistical analysis and the whole ISTA Statistics Committee for the practical and free tools they have developed on line.

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Appendix 1

Moisture content reached after the filter paper imbibition method on 15 samples of oilseed rape

Table A: Accuracy of SMC adjustment on 4 replicates of around 100 seeds using the filter imbibition method to raise 20% of SMC.

Sample	Initial moisture content (%)	Adjusted Weight (g)	Moisture content reached (%) after cold equilibration
1	9.2	0.47	19.7 ± 0.1
2	9.6	0.50	19.7 ± 0.1
3	9.2	0.53	19.8 ± 0.3
4	9.4	0.41	19.9 ± 0.3
5	8.8	0.37	20.0 ± 0.3
6	9.0	0.41	20.1 ± 0.4
7	9.7	0.39	20.1 ± 0.3
8	9.0	0.45	19.8 ± 0.4
9	9.5	0.39	19.7 ± 0.1
10	9.0	0.50	19.8 ± 0.3
11	9.0	0.55	20.1 ± 0.2
12	8.9	0.59	19.9 ± 0.1
13	9.8	0.61	19.9 ± 0.3
14	9.5	0.67	20.0 ± 0.1
15	9.2	0.45	19.9 ± 0.1
General MC mean			**19.9 ± 0.2**

Appendix 2: ANOVA tables

Table B: Results of the Generalised Linear Model on normal germination after CD test in one laboratory comparing two methods for seed moisture content adjustment.

Dependent Variable: SG

Source	DF	Sum of squares	Mean square	F Value	Pr > F
Model	29	18552.39333	639.73770	35.02	<.0001
Error	30	548.10000	18.27000		
Corrected Total	59	19100.49333			

Source	DF	Type I SS	Mean square	F Value	Pr > F
lot	14	17705.39333	1264.67095	69.22	<.0001
method	1	80.73600	80.73600	4.42	0.0440
lot*method	14	766.26400	54.73314	3.00	0.0057

Source	DF	Type III SS	Mean square	F Value	Pr > F
lot	14	17705.39333	1264.67095	69.22	<.0001
method	1	80.73600	80.73600	4.42	0.0440
lot*method	14	766.26400	54.73314	3.00	0.0057

Table C: Results of the Generalised Linear Model on conductivity data after CD test in one laboratory comparing two methods for seed moisture content (SMC) adjustment.

Dependent Variable: EC

Source	DF	Sum of squares	Mean square	F Value	Pr > F
Model	29	33234.35933	1146.01239	91.58	<.0001
Error	30	375.42000	12.51400		
Corrected Total	59	33609.77933			

Source	DF	Type I SS	Mean square	F Value	Pr > F
lot	14	32683.00933	2334.50067	186.55	<.0001
method	1	22.81667	22.81667	1.82	0.1870
lot*method	14	528.53333	37.75238	3.02	0.0054

Source	DF	Type III SS	Mean Square	F Value	Pr > F
lot	14	32683.00933	2334.50067	186.55	<.0001
method	1	22.81667	22.81667	1.82	0.1870
lot*method	14	528.53333	37.75238	3.02	0.0054

Table D: Results of the generalised linear model for CD test (normal germination) in five laboratories using the water added and rolling method for SMC adjustment

Dependent Variable: SG

Source	DF	Sum of squares	Mean square	F Value	Pr > F
Model	24	22801.00000	950.04167	66.97	<.0001
Error	75	1064.00000	14.18667		
Corrected Total	99	23865.00000			

Source	DDL	Type I SS	Mean square	F Value	Pr > F
lab	4	6917.50000	1729.37500	121.90	<.0001
sample	4	13992.70000	3498.17500	246.58	<.0001
lab*sample	16	1890.80000	118.17500	8.33	<.0001

Source	DDL	Type III SS	Mean square	F Value	Pr > F
lab	4	6917.50000	1729.37500	121.90	<.0001
sample	4	13992.70000	3498.17500	246.58	<.0001
lab*sample	16	1890.80000	118.17500	8.33	<.0001

Appendix 3: Germination results (means and SD of 2 x 100 replicates) obtained after controlled deterioration of 15 seed lots adjusted to 20%MC with two methods: adding water and rolling (AWR) or filter paper imbibition (FP).

Seed lot	Mean AWR	SD	Mean FP	SD
1	84.9	0.7	79.9	4.6
2	86.5	2.1	84.4	4.8
3	88.0	4.2	88.0	0.0
4	53.2	2.5	54.0	0.0
5	76.0	5.7	76.5	10.6
6	81.5	0.7	82.0	2.8
7	25.0	1.4	32.0	1.4
8	81.0	0.0	89.5	2.1
9	62.8	0.7	78.5	7.8
10	85.5	2.1	90.4	3.7
11	74.5	0.7	61.0	9.9
12	71.5	4.9	78.0	0.0
13	53.3	3.9	66.0	8.5
14	42.5	0.7	45.8	3.9
15	87.0	0.0	82.0	2.8

Appendix 4

Mean germinations (NG = normal germination, TG = total germination) and tolerance ranges (4 replicates x 100 seeds) for five lots of oilseed rape tested in five laboratories.

Seed lot		Lab 1		Lab 2		Lab 3		Lab 4		Lab 5	
		NG	**TG**	**NG**	**TG**	**NG**	**TG**	**NG**	**TG**	**NG**	**TG**
A	Maximum tolerance range	16	10	20	15	18	10	11	5	18	10
	Observed range	6	5	9	5	5	10	5	3	8	4
	Mean	**79.75**	**93.25**	**52.25**	**82.75**	**69.5**	**94.5**	**91**	**99**	**68**	**94**
B	Maximum tolerance range	18	15	20	18	20	16	17	13	20	16
	Observed range	10	11	7	10	7	11	9	8	2	7
	Mean	**67.75**	**81.5**	**48.5**	**71.25**	**54.5**	**80**	**75**	**88**	**49.25**	**80.25**
C	Maximum tolerance range	13	5	16	7	17	6	9	5	14	6
	Observed range	7	2	5	3	14	4	3	1	17*	3
	Mean	**87.5**	**98.75**	**80.5**	**97.25**	**74**	**98**	**95.25**	**99.5**	**84.5**	**98.25**
D	Maximum tolerance range	10	6	13	6	13	7	9	5	16	5
	Observed range	6	4	11	6	5	3	2	2	14	2
	Mean	**94**	**98.25**	**86.75**	**98**	**86.75**	**97.5**	**95.5**	**99.5**	**79.75**	**98.75**
E	Maximum tolerance range	19	12	20	17	19	13	17	10	19	9
	Observed range	9	8	4	4	8	9	10	8	8	5
	Mean	**63.5**	**89.5**	**47.5**	**77.25**	**60.5**	**87**	**75.5**	**94.25**	**60.5**	**94.75**

* Out of tolerance

Shortening the controlled deterioration (CD) test for Brassica by replacing the germination test with a conductivity measurement.

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Summary

The controlled deterioration test was carried out on five seed lots of oilseed rape by eight laboratories. Following the deterioration stage, a standard germination test was conducted as described in the ISTA Rules and in addition an electro-conductivity (EC) measurement on 4 replicates of 100 seeds was taken after soaking in 100ml deionised water for 16 hours at 20°C. Clear and significant differences were observed between lots in all laboratories in both germination and EC after CD. All results were within tolerance and both repeatability and reproducibility were good, there being no evidence of over-dispersion. The EC measurement after CD was significantly correlated with the CD germination suggesting that an EC measurement after the deterioration phase of the CD test could be an alternative to a standard germination test. This would shorten the time taken to complete the CD test.

Introduction

Since 2010, a method for controlled deterioration (CD) has been included in the ISTA Rules which combines a first step of seed ageing followed by a standard germination test (ISTA, 2013). This two-step method requires time to both raise the seed moisture content and to complete a germination test. This could limit the number of samples which can be tested at the same time and the use of CD test in seed testing laboratories.

Work on Brassica species has, however, shown that following periods of ageing under different conditions, both normal and total germination correlated with the conductivity of seeds after 24h soaking (Mirdad *et al*., 2006). In addition Matthews *et al*. (2009) showed that after CD of cabbage seed lots, an EC measurement was significantly correlated with both their germination after CD and emergence in transplant modules. Thus EC after CD was related to both another measure of vigour (CD germination) and outcome of vigour (emergence). Furthermore Wagner *et al.*, (2012) highlighted the use of conductivity measurements for seed quality assessment within a short frame time as it is the quickest vigour testing method.

The present work aimed to examine the potential of replacing the germination test that follows the deterioration stage of the CD test with an assessment of seed leachate conductivity, thus

reducing the time needed to complete the CD test.

Materials and Methods

Seed Material

Five seed lots of winter oilseed rape were selected from samples of seed lots supplied by Syngenta. The standard germinations of the lots were all high, 94% or above for all except one lot (lot B) with a standard germination of 74% (table 1).

Table 1: Details of five lots of winter oilseed rape determined in one laboratory: 1000 seed weight (provided by the seed company), seed moisture content and standard germination.

Seed lot	Thousand seed weight (g)	Seed moisture content (%)	Standard germination (%)
A	4.63	6.7	95
B	4.29	6.2	74
C	4.10	6.8	99
D	4.28	7.5	96
E	3.90	6.3	94

Participating laboratories

Samples of the lots were distributed to the participating laboratories (table 2) in April 2013 in moisture-proof bags. On receipt the laboratories were instructed to store the bags at low temperature (4 to 10^{o}C). The samples were coded independently of the test participants. The laboratory tests reported were concluded in April and May 2013, except one lab which conducted the tests in July because of difficulties and delay in delivering samples.

Table 2*:* Laboratories participating in the comparative test

Frédéric Corre Syngenta Seeds SAS FRANCE	Malavika Dadlani Indian Agricultural Research Institute (IARI) INDIA
Ibrahim Demir University of Ankara, Faculty of Agriculture TURKEY	Gillian Mc Laren Science and Advice for Scottish Agriculture (SASA) UNITED KINGDOM
Yan Rong Wang College of Pastoral Agriculture Science and Technology - Lanzhou University CHINA	Enrico Noli Laboratorio di Ricerca e Analisi Sementi (LaRAS) – Università di Bologna ITALY
Carey Matthiessen	Marie-Hélène Wagner

20/20 SeedLabs Inc. CANADA	GEVES-Station Nationale d'Essais de Semences FRANCE

Controlled deterioration

General directions were those described in ISTA Rules for *Brassica*: seeds were adjusted to 20% MC and aged at 45°C ± 0.5°C for 24 hours.

Two laboratories (2 and 3) used the ISTA prescribed method for adjusting seed MC whereas an alternative method for seed moisture content adjustment to 20% was used by six laboratories. The alternative method consisted of placing a known weight of seeds in a glass vial, adding the volume of water necessary for the seeds to reach 20% MC, sealing the vial and rolling the seeds overnight at <10°C using a tube roller. Lab 4 did not roll seeds after adding water but gave them a quick shake before equilibration at 5°C overnight. Variance analysis revealed no significant differences in the mean conductivity measurements when conductivity measurements were used in place of a germination test. Analysis of data comparing the filter paper method of raising the seed moisture content with the roller method is provided within another validation test report (Alternative method for seed moisture content adjustment in seed vigour testing, Wagner *et al*. 2015).

Germination and conductivity were both measured after the CD period.

Electroconductivity

General directions (conductivity meter, water quality, checking etc…) were those described in ISTA Rules (2013) for pea, soyabean and bean (Chapter 15: detailed methods in 15.8.1).

Specifications for *Brassica* seeds: 4 replicates of a weight of seed equivalent to the TSW divided by 10 (i.e. approximately 100 weighed seeds) were imbibed 16 hours ± 15 minutes at 20°C ± 2°C in 50 ml of deionised or distilled water per replicate.

Statistical analysis

Analyses of variance (ANOVA) were carried out with the Generalised Linear Model from the SAS GLM procedure. Possible outliers were assessed using side by side boxplots for conductivity test and by computing tolerances for germination test replicates.

The performance of the method was also assessed though the estimation of repeatability and reproducibility parameters according to ISO5725-2, available from the ISTA website.

Results

Controlled deterioration test

The normal germination results after CD revealed clear differences between the seed lots in all laboratories: lots C and D were the most vigorous and lot B was the worst (table 3). All laboratories identified clear differences between the high vigour (high CD germination, e.g. lot C) and low vigour lots e.g. lots B and E even though there were some differences in the normal germination CD results between labs (figure 1). The low vigour of Lot B was perhaps not surprising, given that it had a lower SG than other lots (74%). Even so, the differences in vigour of the lots with similarly high SG were clearly different, with the mean CD germination ranging from 56% to 81% normal germination (table 3).

Differences between laboratories were also apparent in the total germination after CD test (table 4); the overall lab mean for labs 3 and 4 was less than 60% germination whereas all the others were above 85%. Germination methods for labs 3 and 4 may be responsible for their lower results since this species was not a familiar one for them. All germination test results (normal and total germination) were within tolerance, with only one exception (appendix 1).

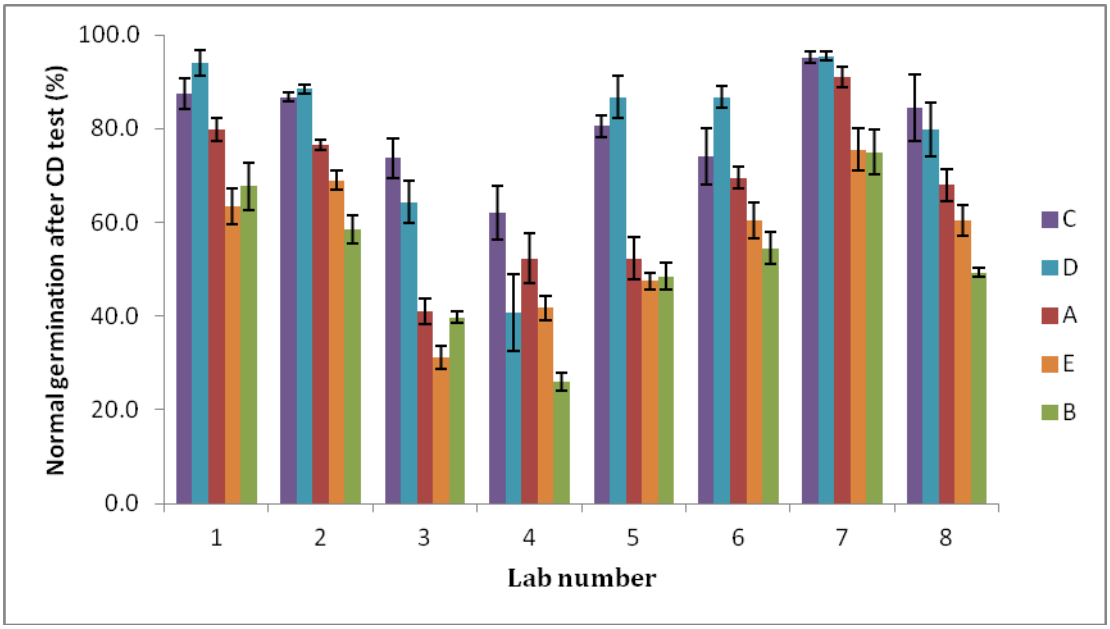

Figure 1: Normal germination mean results after controlled deterioration test for the eight labs according to the seed lot.

Table 3: Normal germination (%) after CD test of five lots of oilseed rape in each of four 100-seed replicates in eight laboratories. Z-scores were calculated after removing outliers (10/160) shown by the side-by-side boxplots exploration (appendix 2).

Lab	**Lot A**	**Lot B**	**Lot C**	**Lot D**	**Lot E**	**Overall lab mean**
1	80c	68d	87.5b	94a	63.5d	**79^{b}**
2	76.5b	58.5e	87a	88.5a	69c	**76^{c}**
3	41c	40c	74a	64b	31d	**50^{f}**
4	52b	26d	62a	41c	42c	**45^{g}**
5	52c	48.5c	80.5b	87a	47.5c	**63^{e}**
6	69.5b	54.5d	74b	87a	60.5c	**69^{d}**
7	91a	75b	95a	95.5a	75.5b	**86^{a}**
8	68b	49d	84.5a	80a	60.5c	**68^{d}**
Overall lot mean	**66^{b}**	**52^{d}**	**81^{a}**	**80^{a}**	**56^{c}**	
SD	16.7	15.4	10.4	18.5	14.8	
Lab z-scores	Lot A	Lot B	Lot C	Lot D	Lot E	
1	0.84	1.03	0.66	0.82	0.51	
2	0.67	0.41	0.59	0.39	0.89	
3	-1.57	-0.81	-0.64	-1.47	-1.74	
4	-0.87	-1.77	-1.75	-2.98	-1.01	
5	-1.01	-0.26	0.00	0.30	-0.55	
6	0.20	0.14	-0.62	0.30	0.30	
7	1.54	1.51	1.34	0.89	1.35	
8	0.00	-0.21	0.37	-0.20	0.20	

Data in columns (lot and lab means) having the different lower-case letters are significantly different ($p < 0.05$). Within a laboratory, different letters indicate that lots are significantly different ($p < 0.05$) as both seed sample and laboratory main effects were both significant on CD test germination results (appendix 3).

Table 4: Total germination (%) after CD test of five lots of oilseed rape in each of four 100-seed replicates in eight laboratories.

Lab means	**Lot A**	**Lot B**	**Lot C**	**Lot D**	**Lot E**	**Overall lab mean**
1	93.3b	81.5c	98.8a	98.3a	89.5b	**92b**
2	97.3a	88.5c	100a	98.3a	93.0b	**95a**
3	45.3c	50.3c	78.5a	71.3b	37.3d	**56d**
4	64.5ab	39.0c	71.3a	54.0b	55.3b	**57d**
5	82.8b	71.3d	97.3a	98.0a	77.3c	**85c**
6	94.5a	80.0c	98.3a	97.5a	86.8b	**91b**
7	99.0a	88.0c	99.5a	99.5a	94.3b	**96a**
8	94.0b	80.3c	98.3a	98.8a	94.8b	**93b**
Overall lot mean	**83.8c**	**72.3e**	**92.7a**	**89.4b**	**78.5d**	

Data in columns (lot and lab means) having the different lower-case letters are significantly different ($p < 0.05$).

Electro-conductivity after CD test

The detailed results are presented in appendix 4. The mean EC results gave similar variability between seed lots (figure 2) or labs (figure 3) as seen for the germination results. However, variability between labs was lower using the EC measurement after CD than using germination. Seed lots were in general ranked in the same order for conductivity as for CD germination (figure 1) although there were some changes in rank order between labs in the medium class of vigour.

As seen for the CD data, the lowest vigour (highest EC) occurred for lot B which had a relatively low SG (74%). However the differences in vigour of the remaining lots were clear, with lot E having the consistently highest EC, followed by lot A, while lots C and D had similar levels of EC.

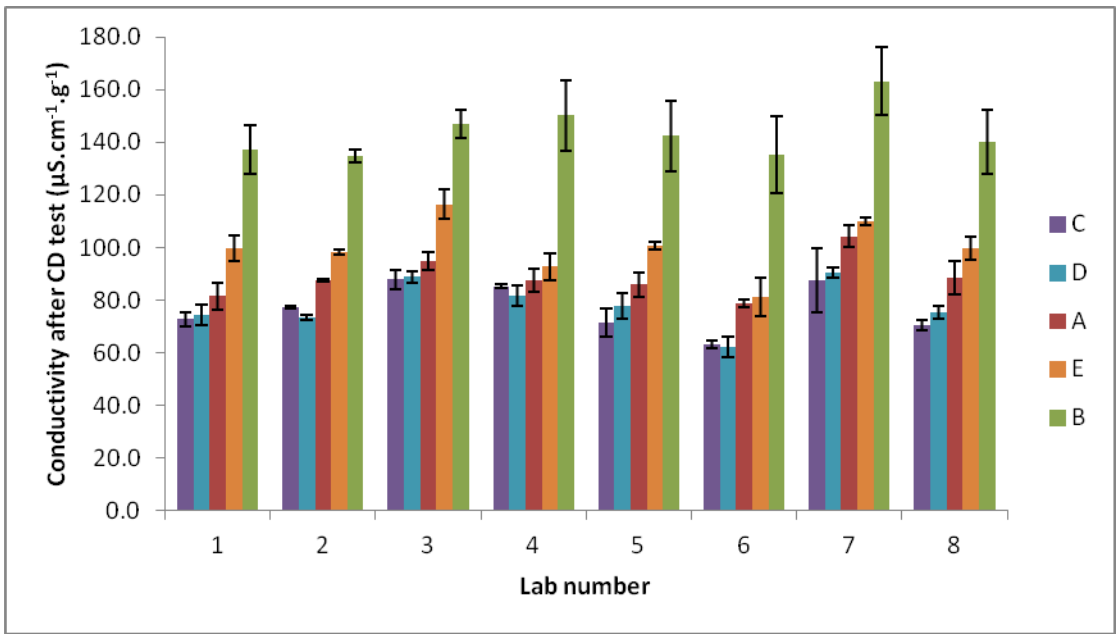

Figure 2: Conductivity results after controlled deterioration test for the eight labs.

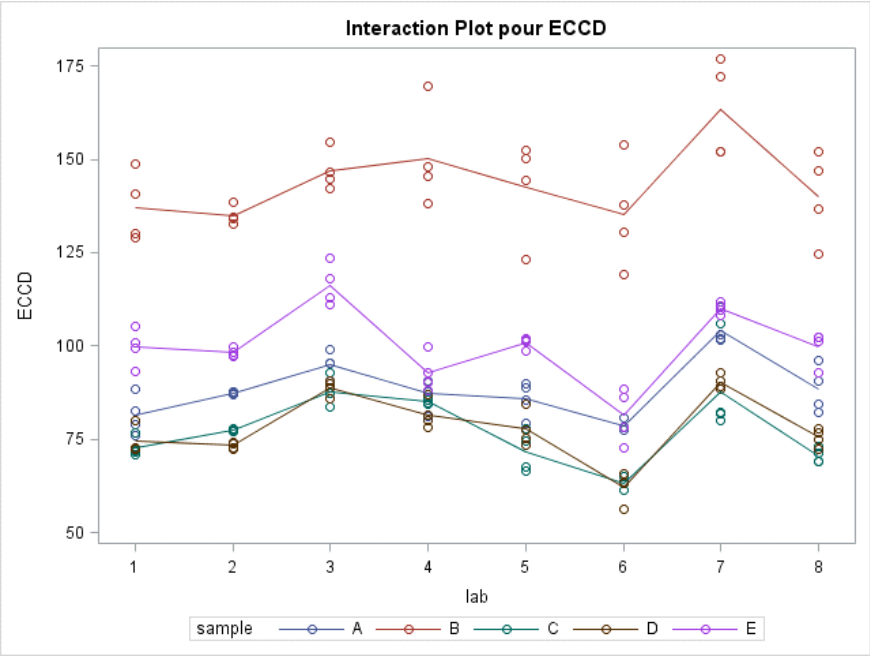

Figure 3: Conductivity results after controlled deterioration test for the eight labs according to the seed lot.

Correlation between EC and germination after CD test

Each laboratory showed a clear and significant correlation between their conductivity results and their germination results. High correlations (Pearson coefficient r from -0.816 to -0.979, with the exception of lab 3) were obtained between total germination and EC after CD (table 5). Lab 3 had the lowest correlation, probably due to its germination method which did not lead to full germination, as did lab 4 with a slighter effect on correlation. Similar high correlations were observed between EC after CD and normal germination (r=- 0.656 to -0.959, table 5)

Table 5: Correlation between conductivity and germination after controlled deterioration test (CD) estimated with Pearson coefficient (r) calculation. TG_CD= total germination after CD, EC_CD= electro-conductivity after CD; NG_CD= normal germination after CD.

Lab1	*TG_CD*	*EC_CD*	*NG_CD*
TG_CD	1,000		
EC_CD	**-0,979**	1,000	
NG_CD	0,845	-0,766	1,000

Lab5	*TG_CD*	*EC_CD*	*NG_CD*
TG_CD	1,000		
EC_CD	**-0,877**	1,000	
NG_CD	0,955	-0,698	1,000

Lab2	*TG_CD*	*EC_CD*	*NG_CD*
TG_CD	1,000		
EC_CD	**-0,960**	1,000	
NG_CD	0,962	-0,959	1,000

Lab6	*TG_CD*	*EC_CD*	*NG_CD*
TG_CD	1,000		
EC_CD	**-0,923**	1,000	
NG_CD	0,893	-0,815	1,000

Lab3	*TG_CD*	*EC_CD*	*NG_CD*
TG_CD	1,000		
EC_CD	**-0,556**	1,000	
NG_CD	0,991	-0,656	1,000

Lab7	*TG_CD*	*EC_CD*	*NG_CD*
TG_CD	1,000		
EC_CD	**-0,962**	1,000	
NG_CD	0,898	-0,796	1,000

Lab4	*TG_CD*	*EC_CD*	*NG_CD*
TG_CD	1,000		
EC_CD	**-0,816**	1,000	
NG_CD	0,996	-0,772	1,000

Lab8	*TG_CD*	*EC_CD*	*NG_CD*
TG_CD	1,000		
EC_CD	**-0,974**	1,000	
NG_CD	0,881	-0,954	1,000

Moreover, the eight laboratories had good repeatability and reproducibility in seed vigour of oilseed rape (table 6). The values for repeatability and reproducibility are within a similar range to those for other validated vigour tests (controlled deterioration, Powell, 2009; radicle emergence, Matthews *et al.* 2011; conductivity test applied to *Phaseolus vulgaris* and *Glycine max,* Powell, 2009a,b).

Table 6: Values for repeatability and reproducibility of results from the conductivity and germination after controlled deterioration test on oilseed rape

	Conductivity		**Normal germination**	
Lot	**Repeatability**	**Reproducibility**	**Repeatability**	**Reproducibility**
A	4.219	8.764	3.280	16.901
B	11.267	13.676	3.210	15.694
C	5.093	10.058	4.412	11.045
D	3.233	9.471	4.452	18.863
E	4.464	11.202	3.192	15.057

Discussion

The results obtained for the conductivity of seed leachates after CD was significantly related to the germination after CD in eight laboratories. Furthermore, differences in vigour were consistently identified in all eight laboratories. These results therefore support the proposal that an EC test could provide an alternative to the germination test that follows the deterioration phase of the CD test.

The seed lot and the laboratory main effects were significant for both conductivity and germination results after controlled deterioration test. However the EC measurements after CD were more reproducible than germination after CD, possibly because all laboratories were experienced in conducting the EC test. In contrast the lack of experience in two laboratories in the germination test for this species may explain the significant differences in the CD test results

when the germination test was used.

Acknowledgements

We sincerely thank Laura Bowden, Frédéric Corre, Malavika Dadlani, Ibrahim Demir, Hulya Ilbi, Mohammad Khajeh Hosseini, Gillian McLaren, Carey Matthiessen, Enrico Noli and Yan Rong Wang for their contribution to this work. We are also grateful to Cathy Colombano (Syngenta SAS) for providing samples.

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Appendix 1

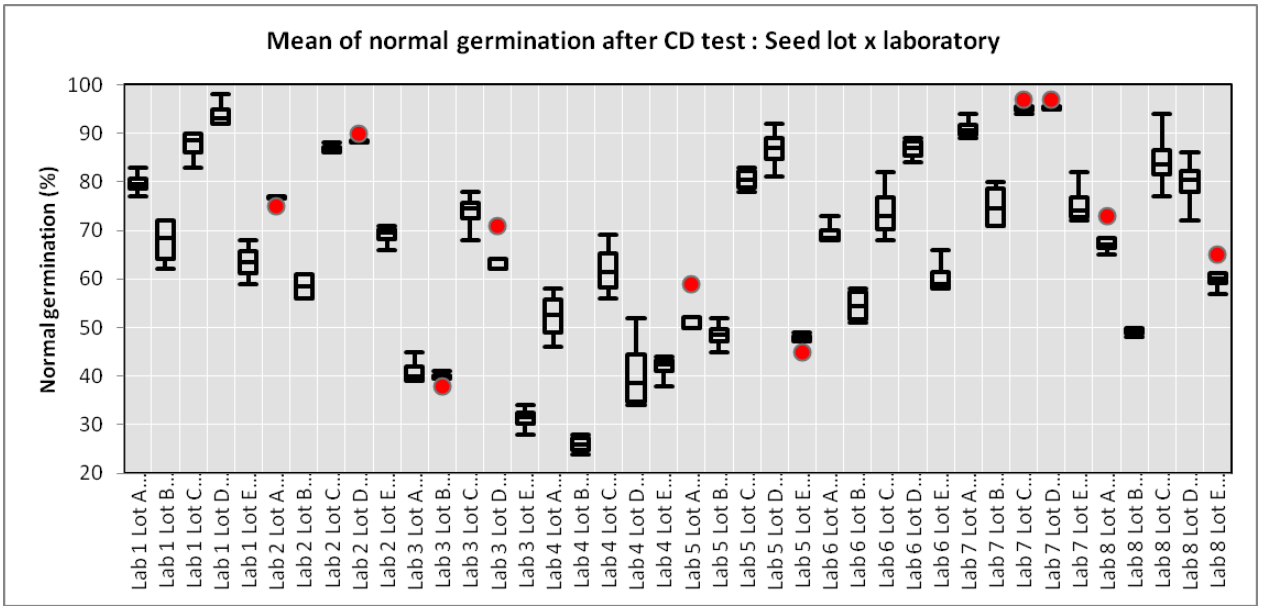
Mean germinations (NG = normal germination, TG = total germination) after controlled deterioration test and tolerance ranges (4 replicates x 100 seeds) for five lots of oilseed rape tested in eight laboratories.

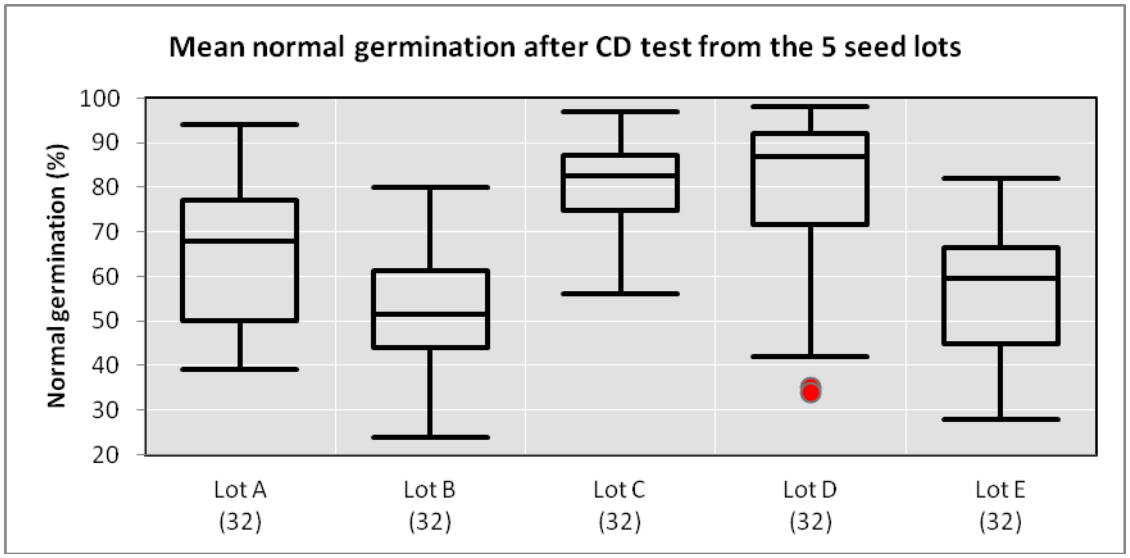
	Seed lot	**A**		**B**		**C**		**D**		**E**	
		NG	**TG**	**NG**	**TG**	**NG**	**TG**	**NG**	**TG**	**NG**	**TG**
Lab1	Maximum tolerance range	16	10	18	15	13	5	10	6	19	12
	Observed range	6	5	10	11	7	2	6	4	9	8
	Mean	**80**	**93**	**68**	**82**	**88**	**99**	**94**	**98**	**64**	**90**
Lab2	Maximum tolerance range	17	7	19	13	13	5	13	6	18	10
	Observed range	2	1	5	7	2	0	2	1	5	5
	Mean	**77**	**97**	**59**	**89**	**87**	**100**	**89**	**98**	**69**	**93**
Lab3	Maximum tolerance range	19	19	19	20	17	16	19	18	18	19
	Observed range	6	8	3	6	10	7	9	12	6	7
	Mean	**41**	**45**	**40**	**50**	**74**	**79**	**64**	**71**	**31**	**37**
Lab4	Maximum tolerance range	20	19	17	19	19	18	19	20	19	20
	Observed range	12	15	4	9	13	15	18	16	6	8
	Mean	**52**	**65**	**26**	**39**	**62**	**71**	**41**	**54**	**41**	**55**
Lab5	Maximum tolerance range	20	15	20	18	16	7	13	6	20	17
	Observed range	9	5	7	10	5	3	11	6	4	4
	Mean	**52**	**83**	**49**	**71**	**81**	**97**	**87**	**98**	**48**	**77**
Lab6	Maximum tolerance range	18	10	20	16	17	6	13	7	19	13
	Observed range	5	10	7	11	14	4	5	3	8	9
	Mean	**70**	**95**	**55**	**80**	**74**	**98**	**87**	**98**	**61**	**87**
Lab7	Maximum tolerance range	11	5	17	13	9	5	9	5	17	10
	Observed range	5	3	9	8	3	1	2	2	10	8
	Mean	**91**	**99**	**75**	**88**	**95**	**100**	**96**	**100**	**76**	**94**
Lab8	Maximum tolerance range	18	10	20	16	14	6	16	5	19	9
	Observed range	8	4	2	7	17*	3	14	2	8	5
	Mean	**68**	**94**	**49**	**80**	**85**	**98**	**80**	**99**	**61**	**95**

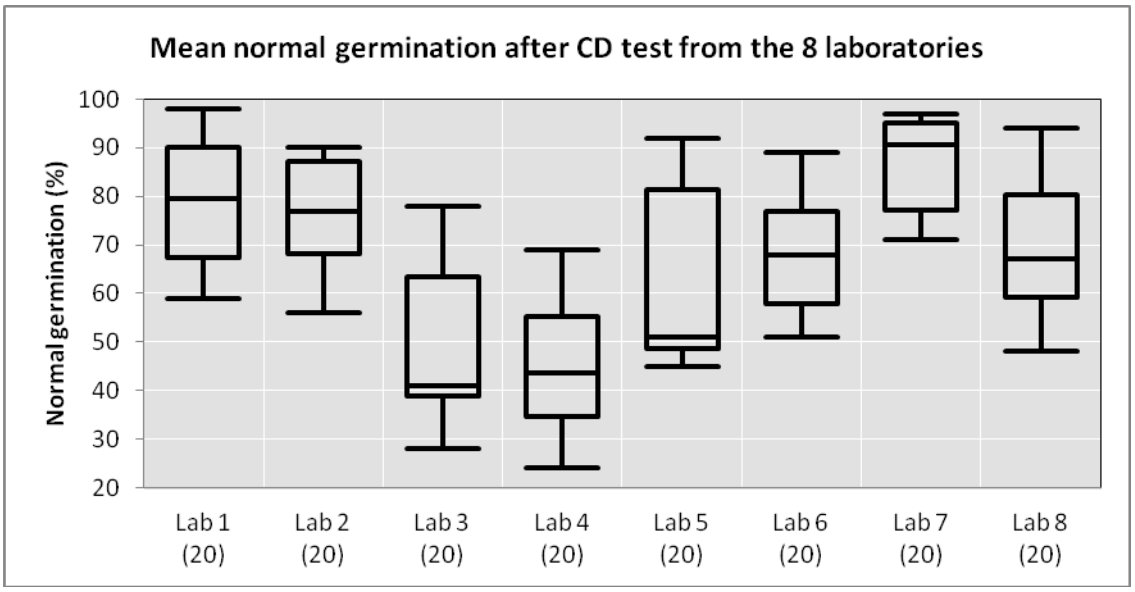
*out of tolerance

Appendix 2

Comparisons of the mean normal germination assessments after CD test with side-by -side boxplots from the interaction seed lot x laboratory: 10 outliers were detected mainly on lot D (second graph).







Appendix 3

Results of the generalised linear model for CD test (normal germination)

Source	DDL	Type III SS	Quadratic mean	F value	Pr > F
lab	7	28064.97500	4009.28214	284.18	<.0001
sample	4	21456.97500	5364.24375	380.22	<.0001
lab*sample	28	5075.02500	181.25089	12.85	<.0001

Appendix 4 Data for each replicate conductivity after CD test (µS.cm^{-1}.g^{-1}) for each of five lots taken in each of eight laboratories

Lot	Rep	Lab1	Lab2	Lab3	Lab4	Lab5	Lab6	Lab7	Lab8
A	1	88.3	87.2	95.3	90.3	79.2	78.3	101.9	84.4
	2	75.9	87.8	95.3	81.0	85.6	80.9	110.4	90.6
	3	78.9	87.7	98.9	90.5	88.8	78.3	103.1	96.1
	4	82.6	87.0	90.6	87.8	89.9	77.5	101.6	82.1
	Mean	**81.4**	**87.4**	**95.0**	**87.4**	**85.9**	**78.8**	**104.3**	**88.3**
B	1	148.8	132.5	144.7	138.3	144.3	137.7	151.9	152.1
	2	140.8	134.1	154.5	145.3	150.3	119.1	152.2	147.0
	3	130.0	138.5	146.7	169.5	123.0	130.3	172.0	136.7
	4	129.0	134.4	142.2	147.9	152.4	153.8	176.9	124.5
	Mean	**137.2**	**134.9**	**147.0**	**150.3**	**142.5**	**135.2**	**163.2**	**140.1**
C	1	76.7	77.9	83.8	84.9	66.6	64.9	82.3	69.2
	2	70.8	77.0	87.4	86.1	67.7	63.2	105.9	71.3
	3	71.5	77.4	93.0	85.5	77.7	61.5	80.0	73.0
	4	71.9	77.2	87.2	84.5	74.4	63.7	81.9	69.0
	Mean	**72.7**	**77.4**	**87.9**	**85.3**	**71.6**	**63.2**	**87.5**	**70.6**
D	1	72.3	72.5	85.9	78.1	75.3	63.1	90.5	72.4
	2	72.3	72.7	90.0	81.5	77.5	56.3	88.3	76.6
	3	72.7	74.0	90.6	86.9	73.6	63.5	92.9	75.0
	4	80.2	74.2	88.8	80.1	84.5	65.6	89.3	78.0
	Mean	**74.3**	**73.3**	**88.8**	**81.7**	**77.7**	**62.1**	**90.2**	**75.5**
E	1	105.2	97.5	112.9	90.3	101.5	72.6	109.5	102.2
	2	99.4	99.6	111.1	92.7	101.9	88.3	108.2	93.0
	3	100.9	97.3	123.4	99.9	98.8	86.2	110.9	101.1
	4	93.1	98.2	117.9	88.2	101.1	78.3	111.7	102.4
	Mean	**99.6**	**98.1**	**116.4**	**92.8**	**100.8**	**81.3**	**110.1**	**99.7**

Application of the electrical conductivity test to radish seed (*Raphanus sativus*)

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Summary

Six seed lots of *Raphanus sativus*, all having a laboratory germination of >80%, were tested by four laboratories using the electrical conductivity test, with four replicates of 100 seeds each soaked in 40ml deionised water for 17 hours at 20°C. All laboratories consistently identified the same significant differences in the seed lot conductivity and the data was repeatable within laboratories and reproducible between laboratories. The results of all tests gave a z-score between +2.00 and -2.00. This provides evidence in support of the inclusion of *Raphanus sativus* as a species to which the conductivity test can be applied and can be included in the ISTA Rules.

Introduction

The electrical conductivity (EC) test is a well-established vigour test for grain legumes and is validated in the ISTA Rules for *Pisum sativum*, *Glycine max*, *Phaseolus sativum* and *Cicer arietinum* (Kabuli type) (ISTA 2014). For these, and other, grain legumes, the results of the test reveal vigour differences as they are expressed in their emergence in the field, particularly when field conditions are not ideal. Thus seed lots giving high EC values show poor emergence in the field, that is, they are low vigour lots, when compared to seed lots giving low conductivity which emerge well and have high vigour. Recently Mavi *et al.* (2014) have shown that the results of a bulk conductivity test also reveal differences in the seed vigour of radish seeds. In this case the EC values related to both the field emergence of the lots and their storage potential over a period of one year. The objective of this comparative test was to determine whether the bulk EC test is both repeatable and reproducible when applied to radish and therefore this species could be added to those to which the EC test can be applied.

Materials and Methods

The leaders of the Vigour Committee Working Group for this comparative test were Kazim Mavi, Mustafa Kemal University, Turkey and Stan Matthews, University of Aberdeen, UK.

Seeds of 10 seed lots of radish (*Raphanus sativus*) were obtained from different seed suppliers in Turkey; all lots were chemically treated. The influence of the chemical treatment on EC was assessed by washing the dry seed for a few seconds with 100 ml deionised water, followed by measuring the EC of the washing. The EC values obtained were low (20 - 40 µS cm^{-1} g^{-1}). The 10 seed lots were also tested for electrical conductivity after 17 h at 20°C (4 replicates of 100 seeds each in 40ml water). The EC of the washings was not related to the EC after 17 h ($r = 0.442$, $P \leq 0.201$). Subsequent EC measurements were therefore not adjusted to allow for the chemical treatment.

Six lots showing clear differences in conductivity after 17 h at 20°C were selected for the comparative test. The six seed lots selected had standard germinations above 80% (table 1). Samples of the six lots were sealed in foil packets and coded before sending to the participating laboratories (table 2). On receipt of the seed, they were kept <10°C before use.

Electrical conductivity test: The general guidelines for completing the conductivity test (ISTA Rules 2015) were followed, with modifications as described below.

Four replicate 4cm diameter tubes, 7-8 cm high, were prepared for each seed lot and 40 ml deionised or distilled water with a reading of ≤5 µS added to each tube. Two control tubes were used for the comparative test of six lots. The water was checked for cleanliness as described in 15.8.1.5.

Four replicates of 100 seeds were weighed to 0.0001g, and one replicate added to each tube. The top of the tube was covered e.g. with a screw cap (after making sure the inside of this was clean), cling-film or aluminium foil. The tubes were set up to ensure that the conductivity readings could be taken within 15 minutes of the end of the 17 hour soaking period.

The seeds in the tubes were placed at 20° ± 2°C for 17 hours ± 15 minutes. A conductivity reading of the control and each seed lot replicate was then taken.

The reading of the control tube was subtracted from each reading, which was then expressed as the conductivity per gram of the initial seed weight. The mean EC for the four replicates of each lot was calculated.

The data was analysed by ANOVA and possible outliers were assessed using side by side boxplots (figure 1) and by computing tolerances for germination test replicates. Z-scores were calculated excluding the outliers. Repeatability and reproducibility were analysed with the statistical tool developed by S. Grégoire, based on ISO 5725-2.

Results

All laboratories returned data for the conductivity of six seed lots of radish. However, one laboratory had problems maintaining the temperature during the test and therefore the data from this laboratory has not been included in this validation report. In addition, laboratory B completed the test on only three replicates

Data exploration with side-by-side boxplots

Box plot analysis revealed differences between the median EC readings for the six seed lots (figure 1A) with outliers in lots 1, 4 and 5. There were small differences in the median values obtained by the four laboratories (figure 1B). No seed lot x lab interaction was exhibited in the side-by-side box plots (figure 1C).

Comparison of lots and laboratories

There were clear and significant differences between the mean EC values of the seed lots (table 3), ranging from 102.8 µS cm^{-1} g^{-1} for lot 1 to 339.9 µS cm^{-1} g^{-1} for lot 4. There were small, although significant, differences in the overall mean values for the laboratories.

Z-scores (table 3), calculated excluding the outliers identified by box-plots, fell within the range -1.17 to 1.49 i.e. within the acceptable range of -2.00 to 2.00.

Repeatability and Reproducibility

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

There were only two significant h-values at $p< 0.05$ for lots 1 and 5 in lab 3 (figure 2A). There were no significant k-values (figure 2B).

Values for repeatability and reproducibility (table 4) were higher than previously observed in the validation of the conductivity test on grain legumes (*Phaseolus vulgaris* and *Glycine max;* Powell, 2009, 2010). However, values for both repeatability and reproducibility depend on the scale and unit of measurement. In the current work, the conductivity values for radish were much higher than those recorded for grain legumes, with a range from 103.9 to 339.9 µS cm^{-1} g^{-1}, whereas those for *P. vulgaris* and *G. max* were all below 40 µS cm^{-1} g^{-1}.

Discussion

Clear and consistent differences in EC values were identified between the seed lots. Such differences have previously been shown to be related to the vigour of radish seed lots as seen

in both field emergence and storage potential (Mavi *et al*., 2014). The replication of the data (Appendix 1), and the means and z-scores from the laboratories (table 3) all indicated that the test is repeatable and reproducible, as did the h- and k-values. The values for repeatability and reproducibility (table 4) could not be compared to previous EC validations since the scale of the values obtained for radish was much higher than for *P. vulgaris* and *G. max*.

This data therefore supports the proposal that the EC test could be applied as a vigour test for radish seed lots.

Acknowledgements

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Table 1: Seed lots of radish used in the EC comparative test

Seed lot	Standard germination (normal seedlings %)
1	100
2	98
3	91
4	80
5	93
6	93

Table 2: Participants in conductivity comparative test for radish

Laboratory	**Participant**
Department of Horticulture, Ege Unversity, Izmir, Turkey	Hulya Ilbi
Department of Horticulture, Ankara University, Turkey	Ibrahim Demir
SNES, GEVES, Angers, France	Marie-Hélène Wagner
Mustafa Kemal University, Hatay, Turkey	Kazim Mavi
OSTS, SASA, Edinburgh, UK	Gillian McLaren

Table 3: Mean conductivity and z-score data obtained by four laboratories for six seed lots of radish. The seed lots are ranked from the lowest conductivity (high vigour) at the top, to the highest conductivity (low vigour) at the bottom.

Lot	Laboratory					
	A	B	C	D	Mean	SD
EC data						
1	98.2	95.0	116.1	101.9	103.5^{A}	10.69
2	176.3	154.1	177.6	163.0	172.3^{B}	18.09
6	158.6	167.5	198.5	172.1	174.2^{B}	13.87
5	187.8	206.0	228.7	220.9	210.9^{C}	14.07
3	249.9	263.4	235.7	251.5	250.1^{D}	17.87
4	351.1	338.8	330.2	339.5	339.9^{E}	22.00
Mean	203.7a	204.0a	214.5b	208.2a		
Z scores						
1	-0.50	-0.80	1.45	-0.15		
2	0.22	-1.00	1.30	-0.51		
6	-1.17	-0.34	1.18	0.34		
5	-0.58	-0.58	1.49	-0.32		
3	-0.99	0.50	1.15	-0.66		
4	0.45	-0.42	1.13	-0.37		

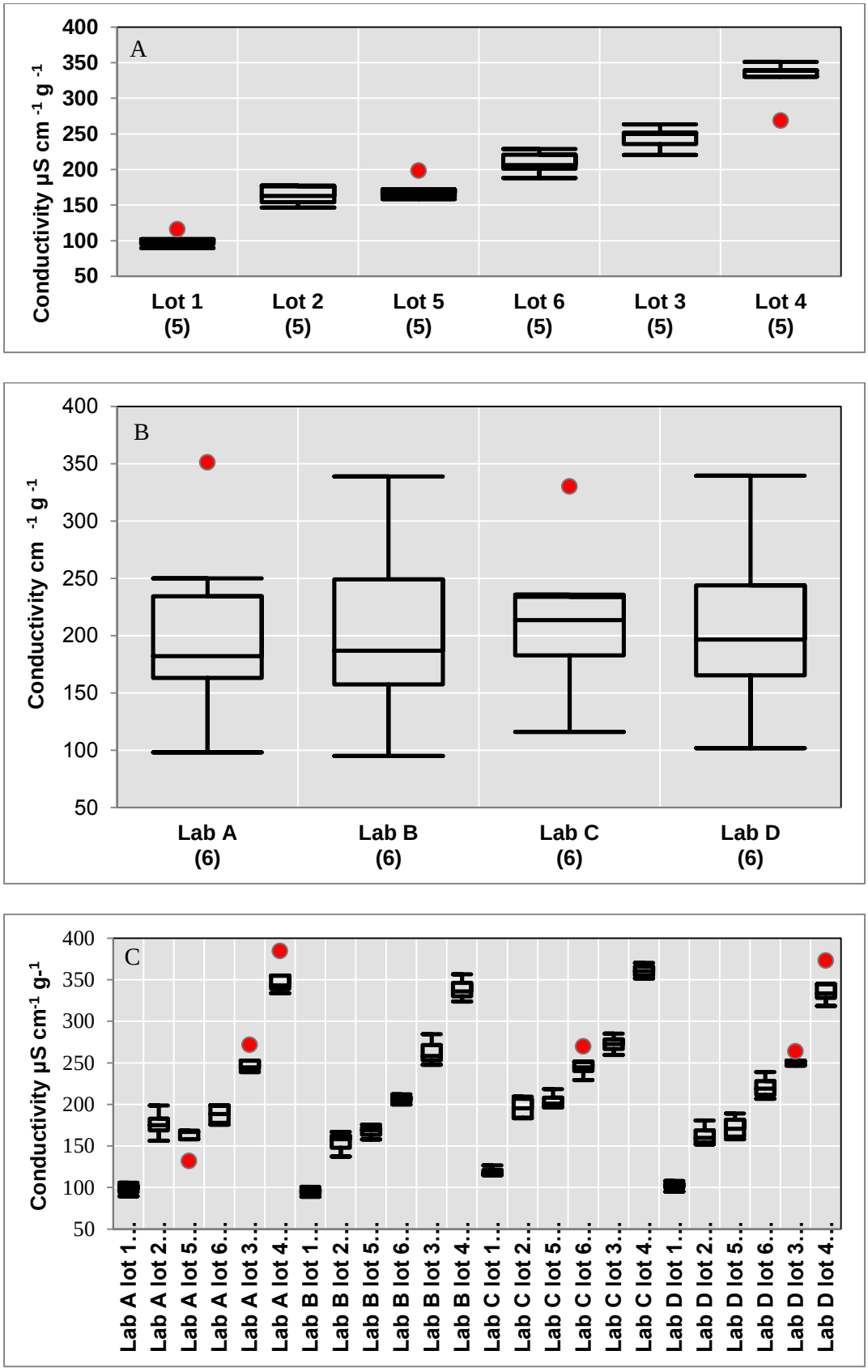
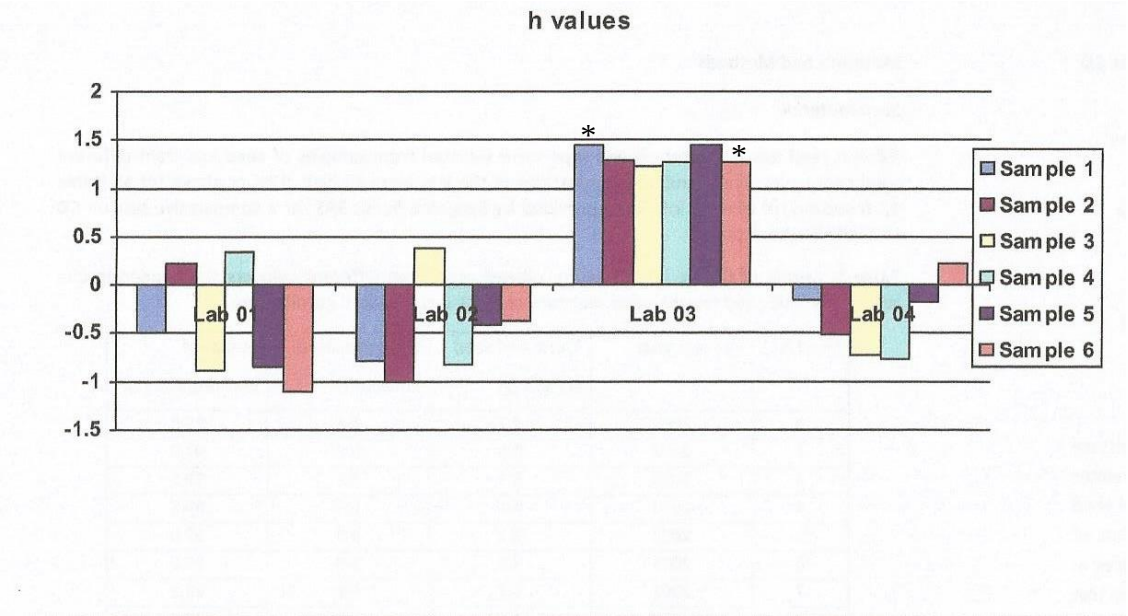


Figure 1: Box plot comparisons of the EC data from seed lots (A), laboratories (B) and seed lot x laboratory (C

A)



B)

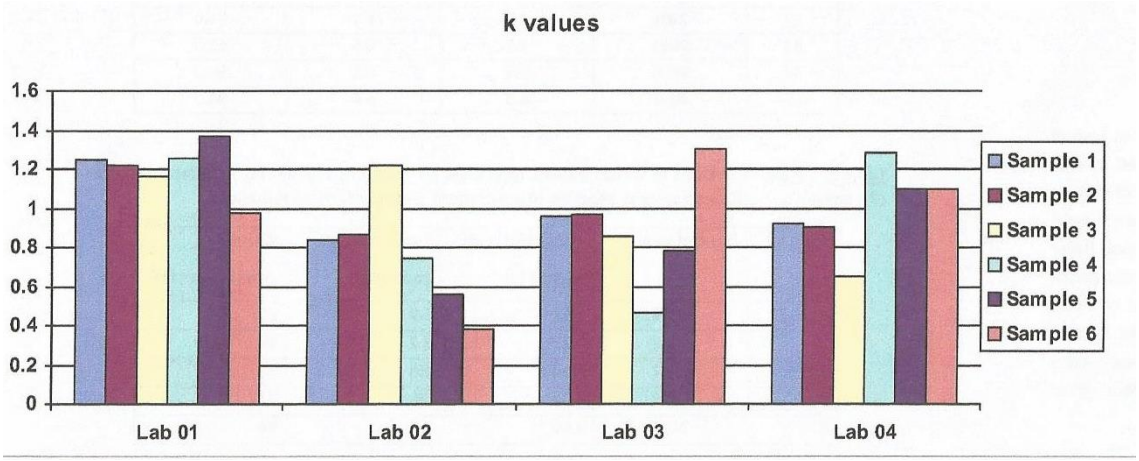


Figure 2: h-values (A) and k-values (B) for six seed lots of *Raphanus vulgaris* following the conductivity test in four laboratories. * indicates a significant difference at $p < 0.05$

Table 4 Values for repeatability and reproducibility of results from the conductivity test on *Raphanus sativus*

Seed lot	Repeatability	Reproducibility
1	5.7769	11.8044
2	14.2776	21.9260
3	12.6539	15.2036
4	18.1548	18.8435
5	13.0498	22.8583
6	12.8005	27.3938

Appendix 1: Summary of raw data obtained for the conductivity test conducted by four laboratories on four replicates of 100 seeds of radish at $20^{o} \pm 2^{o}$C for 17 hours ± 15 minutes.

Lot	Rep	Laboratory			
		A	B	C	D
1	1	89.4	95.1	119.9	101.1
	2	105.8	89.0	120.3	95.1
	3	101.8	100.8	111.2	103.6
	4	95.6	-	112.9	107.8
	Mean	98.2	95.0	116.1	101.9
2	1	172.9	137.4	170.7	180.5
	2	156.3	166.9	185.8	154.9
	3	198.7	158.0	163.7	151.8
	4	177.4	-	190.0	164.8
	Mean	176.3	154.1	177.6	163.0
3	1	271.6	258.2	236.4	246.8
	2	246.4	247.6	234.4	246.6
	3	238.8	284.3	226.4	248.9
	4	242.9	-	245.4	263.7
	Mean	249.9	263.4	235.7	251.5
4	1	384.6	323.8	345.4	318.5
	2	333.9	356.7	340.4	331.3
	3	345.1	336.0	316.4	372.8
	4	340.9	-	318.4	335.4
	Mean	351.1	338.8	330.2	339.5
5	1	175.5	206.0	213.4	206.9
	2	178.5	200.0	226.4	224.6
	3	199.0	212.1	225.4	238.8
	4	198.1	-	249.4	213.1
	Mean	187.8	206.0	228.7	220.9
6	1	168.6	157.9	198.1	178.9
	2	166.7	175.5	210.4	158.3
	3	131.9	169.0	197.4	189.0
	4	167.1	-	188.4	162.3
	Mean	158.6	167.5	195.5	172.1