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Seed Quality Assurance

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Method Validation Reports on Rules Proposals for the International Rules for Seed Testing 2022 Edition

Investigations to harmonise pre-treatment variations within ISTA validated seed health methods

Aim

The ISTA Seed Health Committee wished to harmonise the pre-treatments given to seeds prior to plating for the validated seed health methods for fungal pathogens: 7-005, 7-006, 7-014, 7-016 and 7-022.

For one of the methods (7-016) samples were rinsed in sterile water after pre-treatment with sodium hypochlorite, and this may be beneficial to the other test methods.

Sodium hypochlorite at 1% available chlorine is used to reduce surface contamination on the seed coat by pathogens and saprophytes. Removal of fast-growing saprophytes and surface borne pathogens allows the deep-seated pathogens responsible for seedling loss to grow out and be detected without being concealed by faster growing colonies. It is possible that residual sodium hypochlorite left on the seeds after pre-treatment may restrict the appearance of seed borne pathogens on the test media. Sodium hypochlorite is also known to restrict the germination of seeds and seedling growth at high concentrations.

The methods investigated are described in Table 1 below.

Table 1: Validated methods and current pre-treatments

Method number	Title of method	Current pre-treatment method
7-005	Detection of *Ascochyta pisi* on *Pisum sativum* (pea) seed	10 mins in a 1% (available chlorine) sodium hypochlorite solution followed by draining
7-006	Detection of *Colletotrichum lindemuthium* in *Phaseolus vulgaris* (bean) seed	1% (available chlorine) sodium hypochlorite for 10 mins and allowed to drain
7-014	Detection of *Parastagonospora nodorum* in *Triticum aestivum* subsp. *aestivum* (wheat) seed	10 mins in a 1% (available chlorine) sodium hypochlorite
7-016	Detection of *Phomopsis* complex in *Glycine max* (soy bean, soyabean) seed	Gently rinse seeds in NaOCl solution (1% available chlorine) for 30 s, then rinse for 30 s in sterile water. Blot the seed dry on sterile paper towel
7-022	Detection of *Microdochium nivale* and *Microdochium majus* in *Triticum* spp (wheat) seed	Immerse seeds in NaOCl solution (1% available chlorine) for 10 mins, then drain

Method

To do this, small-scale investigations were carried out comparing results with and without rinsing after sodium hypochlorite (with 1% available chlorine) soak as follows in Table 2. Two laboratories tested method 7-006.

Table 2: Pre-treatments, sample and replicate numbers observed

Method number	Comparison treatments	Number of samples/replicates	Recorded
7-005	• Without disinfection • With 10 min disinfection	A total of 5 samples were examined, 3 with *D. pinodes* and *D. pinodella*, and 2	The occurrence of both pathogens and saprophytes were recorded

Method number	Comparison treatments	Number of samples/replicates	Recorded
	• With 10 min disinfection and rinsing	with *D. pinodes* and *D. pisi* infection. 3 replicates for each treatment.	
7-006 (Lab 1)	• Without disinfection • With 10 min disinfection • With 10 min disinfection and rinsing	A total of 5 samples were examined. 4 replicates for each treatment.	The occurrence of both pathogens and saprophytes were recorded
7-006 (Lab 2)	• Without disinfection • With 10 min disinfection • With 10 min disinfection and rinsing distilled water • With 10 min disinfection and rinsing deionised water • With 10 min disinfection and rinsing sterile water	A total of 5 samples were examined. 8 replicates of 50 seeds.	The occurrence of *C. lindemuthium* and other saprophytes were recorded.
7-014	• 10 min disinfection and draining • 5 min disinfection and draining • 10 min disinfection draining and rinsing • 5 min disinfection draining and rinsing	A total of 6 samples were examined, only 2 with high levels of *Parastagonospora nodorum*. 400 seeds examined for each treatment.	The target pathogen and other seed-borne pathogens and saprophytes were recorded.
7-016	• 30 sec pre-treatment with 30 sec rinsing and blot dry • 60 sec pre-treatment with 30 sec rinsing and blot dry	A total of 5 samples were examined for Phomopsis complex and saprophytes. 4 replicates for each treatment.	The target pathogen and other seed-borne pathogens and saprophytes were recorded.
7-022	• 10 min disinfection and draining • 5 min disinfection and draining • 10 min disinfection draining and rinsing	A total of 6 samples were examined, 2 with low levels of *Microdochium spp.* 400 seeds examined for each treatment.	The target pathogen and other seed-borne pathogens and saprophytes were recorded.

Method number	Comparison treatments	Number of samples/replicates	Recorded
	• 5 min disinfection draining and rinsing		

Results

Results showing number of colonies detected/percentage infection are presented in Excel spreadsheets and are given as Appendices I to V in this report. Summarised result outcomes are given in Table 3 below.

Table 3: Summary of observed results

Method number	Results observed
7-005	There was no difference between draining and draining and rinsing. It was observed that rinsing avoids the harmful effects of hypochlorite.
7-006 (Lab 1)	Only one colony of *C. lindemuthium* observed. For 4 samples saprophytes were reduced by pre-treatment but no statistical difference was observed between draining only and draining and rinsing. One sample had high levels of bacterial growth. Rinsing reduced the harmful effects of hypochlorite. In the between paper test it is important that the paper is not too wet as the resulting high humidity leads to an increase in saprophytes and bacteria.
7-006 (Lab 2)	Low levels of pathogen were detected and lower numbers of saprophytes. No more difference could be observed between treatments than could be accounted for in sampling variation.
7-014	Statistical analysis for the 2 samples with good *Parastagonospora nodorum* infection showed no significant differences with p-values being greater than 0.05.
7-016	No statistical differences were seen for pathogen detection between 30 sec and 60 sec disinfection. 60 sec disinfection reduced the saprophyte numbers. A short disinfection time is important as the seed testa can detach if soaked for any longer.
7-022	Statistical analysis for most samples showed no significant differences with p-values being greater than 0.05. For one sample the p-value for treatment time (5 min/10 min comparison) for *Microdochium* was less than 0.05 but differences were no more than could be accounted for in sampling variation. Slightly more saprophytes were observed at 5 min soak compared to a 10 min soak but levels too low to compare statistically.

Conclusion

Although no statistical difference in pathogen detection was observed between the two treatments: a) draining only after pre-treatment in sodium hypochlorite, and b) draining and rinsing after pre-treatment in sodium hypochlorite, the Seed Health Committee have decided that rinsing the seeds after pre-treatment is preferable to avoid any negative effects of the sodium hypochlorite.

For the validated seed health methods 7-005, 7-006, 7-014 and 7-022 (all except 7-016), the pre-treatment statement will be:

Immerse seeds in a solution of sodium hypochlorite (NaOCl) (1% available chlorine) for 10 mins, then drain and rinse well in sterile water and drain.

For method 7-016 the pre-treatment statement will remain the same as in the current method:

Gently rinse seeds in a solution of sodium hypochlorite (NaOCl) (1% available chlorine) for 30 to 60 s, then rinse for 30 s in sterile water. Blot the seed dry on sterile paper towel.

Appendix I

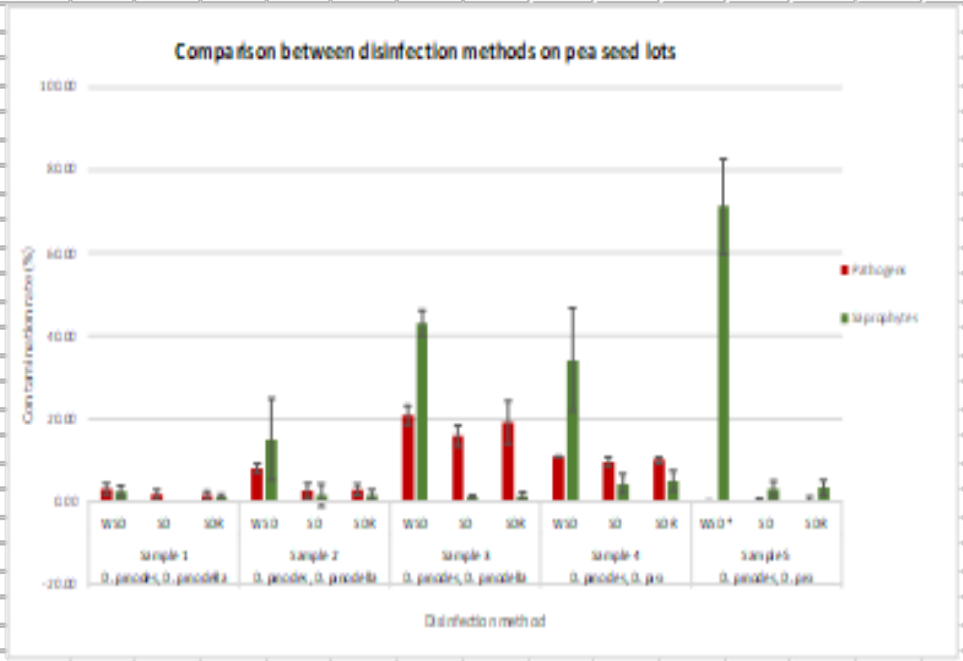
ISTA validated method 7-005

WSD	Without superficial disinfection
SD	With superficial disinfection, without rinsing — Sodium hypochlorite 1% during 10 min
SDR	With superficial disinfection, with rinsing — Sodium hypochlorite 1% during 10 min, rinsed under filtered water

Three replicates were analyzed for each condition.

	1021229			1019944			1002405			1024011			1019282		
Sample	Sample 1 *D. pinodes , D. pinodella*			Sample 2 *D. pinodes , D. pinodella*			Sample 3 *D. pinodes , D. pinodella*			Sample 4 *D. pinodes , D. pisi*			Sample 5 *D. pinodes , D. pisi*		
Method	WSD	SD	SDR	WSD	SD	SDR	WSD	SD	SDR	WSD	SD	SDR	WSD *	SD	SDR
Pathogens	3.17	1.75	1.50	8.17	2.75	3.00	20.88	15.92	19.00	10.92	9.67	10.00	0.13	0.33	0.58
Saprophytes	2.50	0.00	1.08	15.08	1.67	1.67	43.04	1.08	1.42	34.13	4.33	5.00	71.15	3.08	3.42
Standard deviation pathogens	1.38	1.25	1.09	1.04	1.80	1.32	2.17	2.55	5.11	0.13	1.18	0.87	0.18	0.38	0.52
Standard deviation saprophytes	1.30	0.00	0.80	9.91	2.67	1.23	3.22	0.52	0.95	12.47	2.32	2.46	11.45	1.88	1.77

* Only two replicates



Conclusion :

Superficial disinfection induced a decrease of the saprophytes without effect on the pathogens detection.

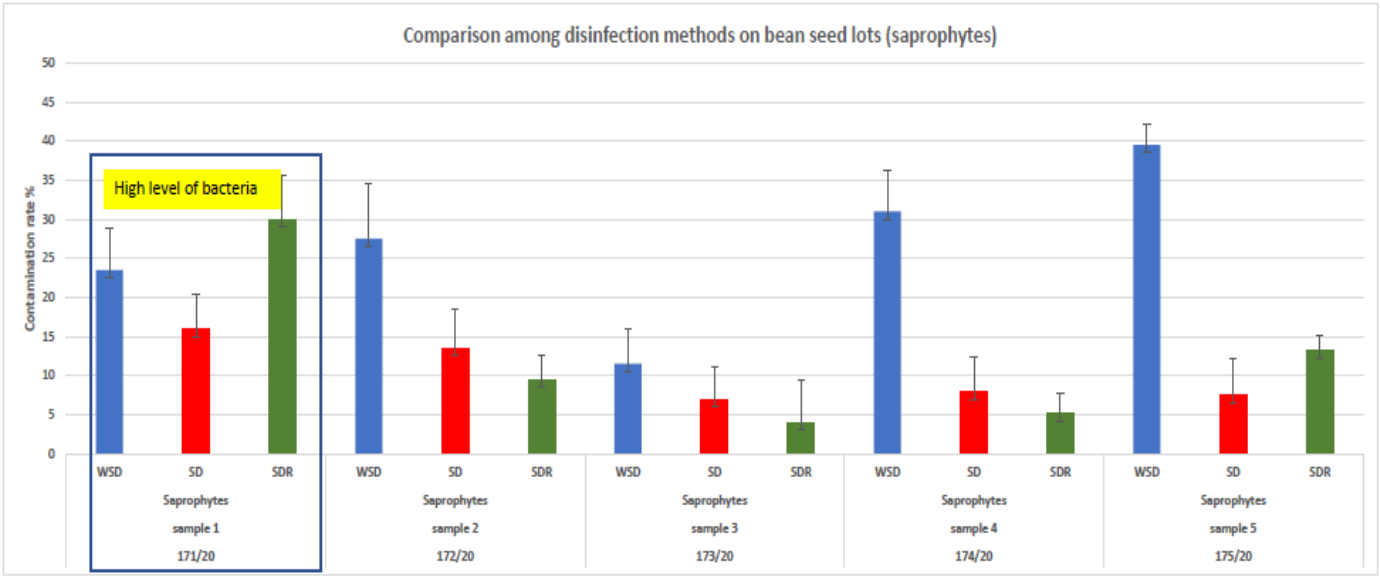
Sample 2 showed a decrease of the pathogens contamination rate with disinfection, this may be due to external contamination removed by disinfection. Rinsing did not show a negative effect on the detection of pathogens; moreover, rinsing avoids the harmful effects of hypochlorite.

Appendix II
ISTA validated method 7-006 (Lab 1)

Comparison among disinfection methods on bean seed lots (only saprophytes)

	171/20 sample 1			172/20 sample 2			173/20 sample 3			174/20 sample 4			175/20 sample 5		
	Saprophytes			Saprophytes			Saprophytes			Saprophytes			Saprophytes		
Method	WSD	SD	SDR	WSD	SD	SDR	WSD	SD	SDR	WSD	SD	SDR	WSD	SD	SDR
Saprophytes	23,5	16,0	30,0	27,5	13,5	9,5	11,5	7,0	4,0	31,0	8,0	5,3	39,5	7,5	13,3
Standard deviation saprophytes	5,3	4,3	5,7	7,0	5,0	3,0	4,4	4,2	5,4	5,2	4,3	2,5	2,6	4,8	1,9

4 replicates for every condition



OBSERVATIONS:
- only one colony of *Colletotrichum* was detected.
- Sample 1 had a very high level of bacteria: it has been very difficult elaborate the comparison among the method
- Superficial disinfection induced a decrease of the saprophytes (statistical differences between WSD and SD/SDR - except in sample 3)
- Rinsing seems to attenuate the harmful effects of hypochlorite

Appendix III

ISTA validated method 7-006 (Lab 2)

Hypochlorite Pretreatment Test - 7-006

Crop :	Bean ; 1 Japanese commercial cultivars ; 50 seeds x 8 replicates, contaminated seeds, in each treatment
Pathogen :	*C. lindemuthianum*

Method :	1. Trial #1	submerged in 1% Hypochlorite for 10 min.	→	drain, rinse with DW	→	Incubation	Check the spot caused by C. lin. or others
	2. Trial #2		→	drain, rinse with deionized water	→		
	3. Trial #3		→	drain, rinse with sterilized DW	→		
	4. 7-006		→	drain	→		
	5. not treated		→		→		

Result :

Method	1	2	3	4	5	
C. lin.	3	6	5	2	6	←Number of seed on which pathogen was observed.
others	1	4	0	1	1	←Number of seed on which other microbes were observed.

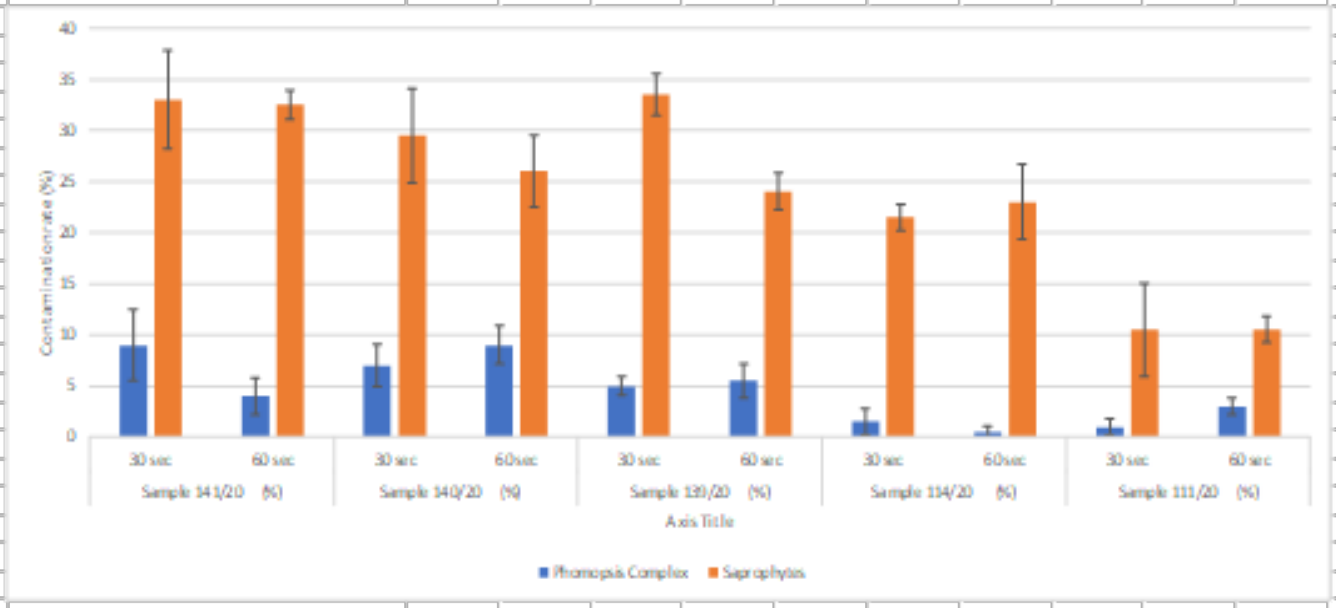
Appendix IV

ISTA validated method 7-016

DISINFECTION OF SOYBEAN WITH RINSING: 30 sec VS 60 sec.

	Sample 141/20 (%)		Sample 140/20 (%)		Sample 139/20 (%)		Sample 114/20 (%)		Sample 111/20 (%)	
Methods	30 sec	60 sec	30 sec	60 sec	30 sec	60 sec	30 sec	60 sec	30 sec	60 sec
Phomopsis Complex	9	4	7	9	5	5.5	1.5	0.5	1	3
Saprophytes	33	32.5	29.5	26	33.5	24	21.5	23	10.5	10.5
Standard deviation *Phomopsis* Complex	3.5	1.82	2.06	1.82	0.91	1.66	1.29	0.57	0.81	0.81
Standard deviation saprophytes	4.79	1.4	4.65	3.5	2.06	1.82	1.29	3.65	4.57	1.29

4 replicates for both conditions



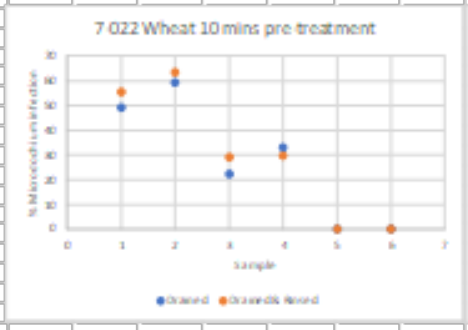
Observation: No statistical differences were detected between 30 sec or 60 sec of disinfection (except sample 139/20 for saprophytes), but it's possible to see a lower level of saprophytes with seeds disinfection for 60 sec. This is important because it's easier the "check" of Petri dishes for the quantification of *Phomps*is complex.

Appendix V

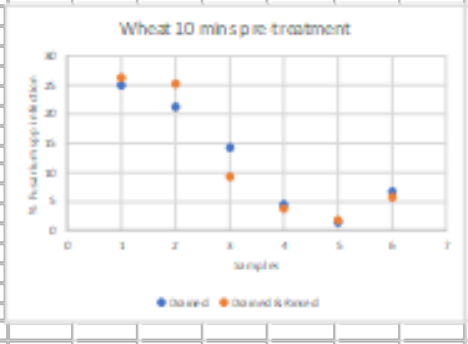
ISTA validated methods 7-014 and 7-022

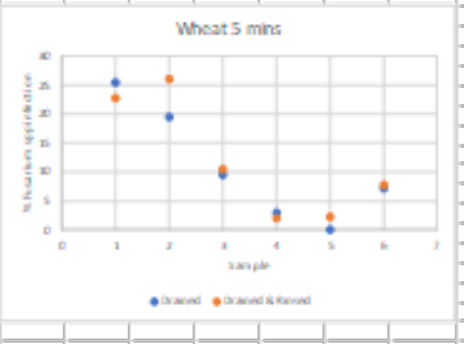
7-022 Microdochium species pre-treatment summary results

10 mins	Mean results presented from 4 replicates of 100 seeds examined.		
		% Microdochium spp	
Sample		Drained	Drained & Rinsed
65784	A	49.25	55.50
65786	B	59.25	63.50
66112	C	22.25	29.25
65526	D	33.00	29.75
60459	E	0.00	0.00
60455	F	0.00	0.00
5 mins	Mean results presented from 4 replicates of 100 seeds examined.		
		% Microdochium spp	
Sample		Drained	Drained & Rinsed
65784	A	52.75	55.50
65786	B	65.00	65.75
66112	C	28.75	25.25
65526	D	30.75	32.75
60459	E	0.50	0.00
60455	F	0.00	0.00



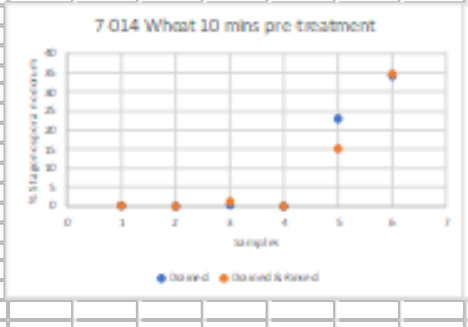
10 mins	Mean results presented from 4 replicates of 100 seeds examined.		
		% Fusarium spp	
Sample		Drained	Drained & Rinsed
65784	A	25.00	26.25
65786	B	21.25	25.25
66112	C	14.25	9.25
65526	D	4.50	3.75
60459	E	1.50	1.75
60455	F	6.75	5.75
5 mins	Mean results presented from 4 replicates of 100 seeds examined.		
		% Fusarium spp	
Sample		Drained	Drained & Rinsed
65784	A	25.50	22.75
65786	B	19.50	26.00
66112	C	9.50	10.50
65526	D	3.00	2.00
60459	E	0.00	2.25
60455	F	7.25	7.75

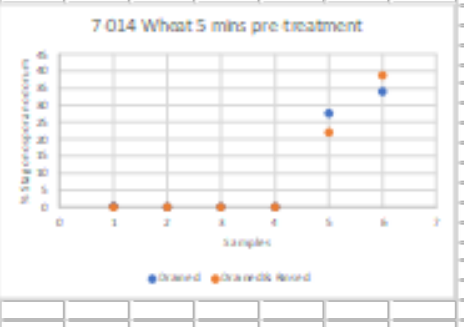




Method 7-014

10 mins	Mean results presented from 4 replicates of 100 seeds examined.		
		% Stagonosporanodorum	
Sample		Drained	Drained & Rinsed
65784	A	0.25	0.25
65786	B	0.00	0.00
66112	C	0.50	1.25
65526	D	0.00	0.00
60459	E	23.00	15.25
60455	F	34.25	34.75
5 mins	Mean results presented from 4 replicates of 100 seeds examined.		
		% Stagonosporanodorum	
Sample		Drained	Drained & Rinsed
65784	A	0.25	0.00
65786	B	0.00	0.00
66112	C	0.00	0.00
65526	D	0.00	0.00
60459	E	27.75	22.00
60455	F	34.00	38.75





Conclusion: Statistical analysis of results for all the pathogens recorded showed no significant difference between pre-treatments with p-values greater than 0.05.
More saprophytes observed visually at 5 min soak than 10 min soak but too low to compare statistically.

ISTA validation study for germination test of *Chenopodium quinoa* Willd.

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Summary

The objective of this validation study was to develop a germination procedure to test *Chenopodium quinoa* Willd. seeds to be included in the chapter five of the ISTA Rules. The experiment was carried out by seven ISTA-accredited laboratories using four seed lots. The quinoa seeds were germinated on between paper media (BP), and top of paper media (TP), using 20°C and the alternating temperature regime 20<=>30°C, light was supplied for 8 hours. The first and final count were made at fourth and seventh day respectively. All the methods tested gave comparable results in all the participating laboratories, and based on the statistical analysis and considering the results of repeatability and reproducibility, the germination method proposed to be included in the ISTA Rules to test *Chenopodium quinoa* seeds is: BP-TP; 20°C; 4 days (first count); 7 days (final count).

Introduction

Quinoa *(Chenopodium quinoa)* is an annual herbaceous plant which usually reaches a height of 1 to 2 m. This species is originated in the Andean zone of South America, specifically in the surroundings of Titicaca Lake, which is located between Peru and Bolivia. (Mujica *et al*., 2001) Over the years, quinoa was spread across the neighbor countries: Ecuador on the north and all the Chilean territory on the south and north of Argentina, and actually the crop is produced in more than seventy countries. In the last thirty years the interest in this crop has increased considerably due mainly to its potential as food for human consumption as well as its medicinal uses, especially for the quality of the protein that contains eight of the essential amino acids besides vitamins and minerals.

During the quinoa domestication, the original towns, selected different quinoa genotypes, considering the tolerance of the plants to different kinds of stresses and finally the current ecotypes were obtained with resistance to cold, salinity, earliness, and high protein value, among others.

These initiatives have been retaken with the purpose of recovering this crop and different seeds companies began a breeding programs with the aim of generating varieties with higher yields and higher harvest index besides a greater homogeneity and quality of the product, given the higher market demands. (von Baer *et al*., 2009).

As part of these work, several companies trade this species and need the opportunity of being able to issue an ISTA Certificate.

Material and methods

Seed material

Four seed lots of *Chenopodium quinoa* harvested during 2018 were selected representing different levels of germination ranging from 75 to 95% based on pre-tests provided data. The low germination level was about 75 to 80%, the medium level about 80 to 85%, and the high level was around 90-95%. All the material used for this validation study was supplied by the Canadian Company Northern Quinoa Production Corporation, and comes from the same variety.

Each selected seed lot was mixed and divided into required sample quantity according to ISTA procedures described in chapter 2. Each sample sent to the laboratories had approximately 4800 pure seeds (approximately 13 g), ensuring enough seeds to perform all the germination tests on 400 seeds and a retest of any of the samples.

Participant laboratories

The following seven ISTA accredited laboratories expressed their interests and met the criteria required (indicated in the "ISTA Method Validation for seed Testing") to conduct the tests of this validation study: GEVES-SNES Station Nationale d'Essais de Semences (France); SAG-Livestock and Agricultural Service (Chile); SGS Biovision (Canada); SGS Mid-West Seed Services (USA); Swedish Board of Agriculture (Sweden), Seed Science and Technology Section, Saskatoon Laboratory, Canadian Food Inspection Agency (Canada); and Science and Advice for Scottish Agriculture, SASA (United Kingdom).

Testing method.

Based on the experience of Chilean seed laboratory that usually tests this species and the preliminary studies, the proposed methodology was as follows:

Four seed lots were tested.

For each test, a total of 400 pure seeds were tested following the PSD 10 described in chapter 3 when preparing seed to be used in the germination test.

The proposed germination methods are between paper media (BP) and top of paper media (TP) using 20°C and 20<=>30°C temperature regimes. Light was supplied for 8 hours. No pretreatment was applied considering that this species doesn´t present dormancy.

The first count was made after 4 days and the final count after 7 days.

Seedlings were assessed according to Seedling Type E – Seedling Group A-2-1-1-1 described in the ISTA Handbook on seedling evaluation.

And the non-germinated seeds were checked following the ISTA working sheets on tetrazolium testing indicated for *Chenopodium* or determined by dissection or excised embryo.

The specific germination methods used in this study are listed as follows:

Table 1. Germination Testing methods for *Chenopodium quinoa* considered in this validation study

Test Code	Temperature Regime	Germination Media	Light	Intermediate count (days)	Final Count (days)
1	20°C	BP	8 hours	4	7
2	20<=>30°C	BP	8 hours	4	7
3	20°C	TP	8 hours	4	7
4	20<=>30°C	TP	8 hours	4	7

Statistical analysis of the results

Germination results obtained by the different laboratories

Figure 1 presents the distribution of germination percentage obtained in all the laboratories.

The results provided were comparable for all the lots and methods tested and therefore they were considered in the analysis of the data. Figure 2 shows the distributions of the percentage of normal seedlings per laboratory, per lot.

All the laboratories obtained the best results with lot 1, followed by lot 4, then the lot 2, and finally lot 3 which showed the lowest germination results in all the tests performed for the participant laboratories.

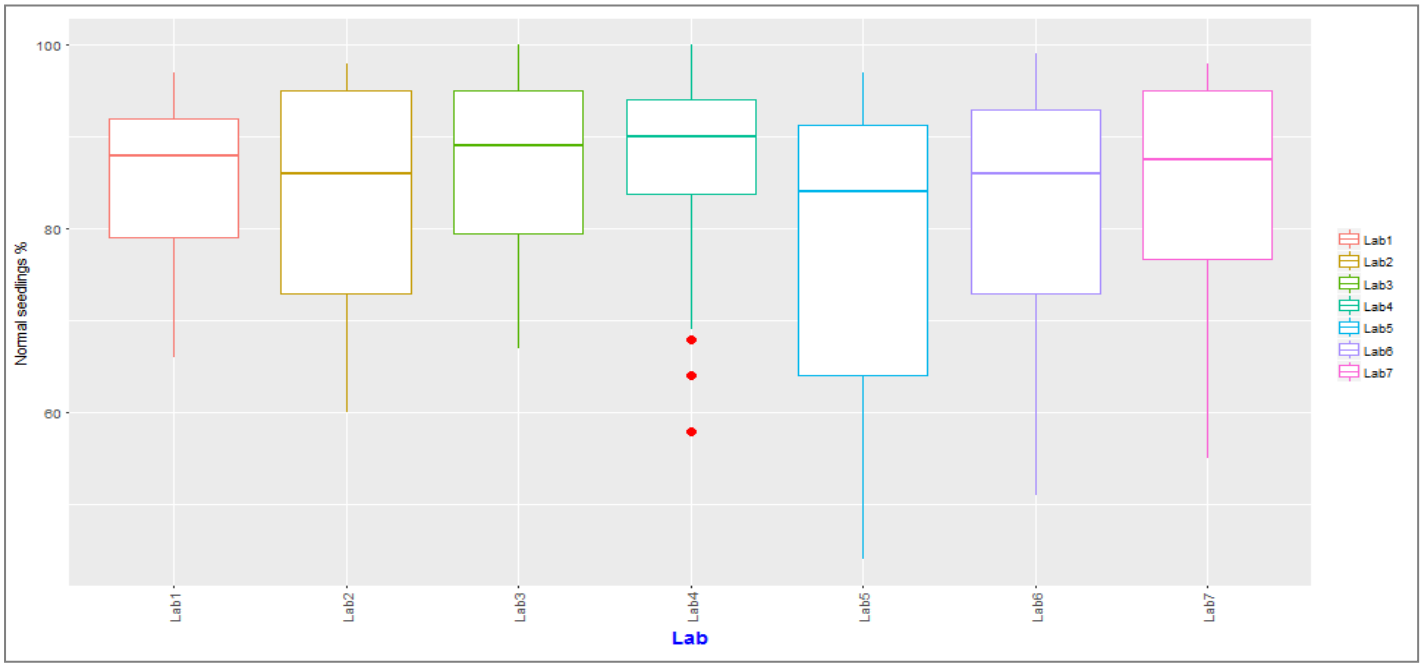

Figure 1. Box plots of the percentage of normal seedlings for all the samples and all the methods, per laboratory.

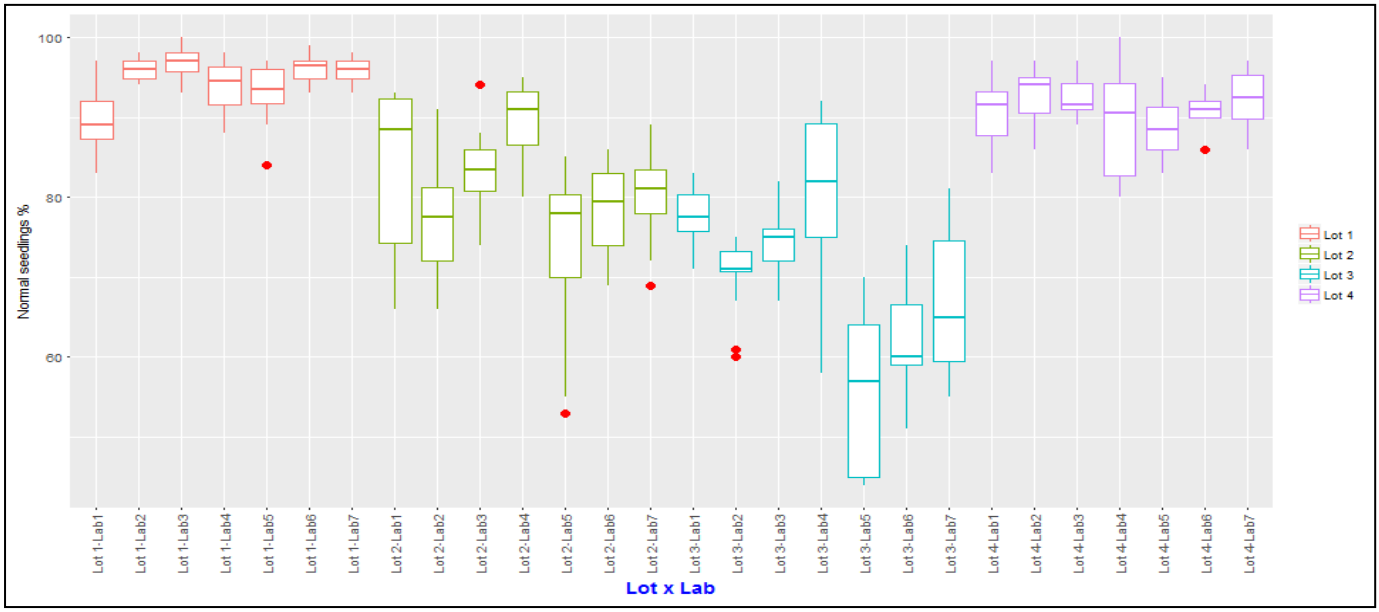

Figure 2. Box plots of the percentage of normal seedlings per laboratory, per lot.

Germination results of the four seed lots

Figure 3 shows the distributions of the germination percentage per lot. It can be observed that the best results were obtained with lot 1 for all the methods and all the laboratories, and the lowest performance was obtained with lot 3.

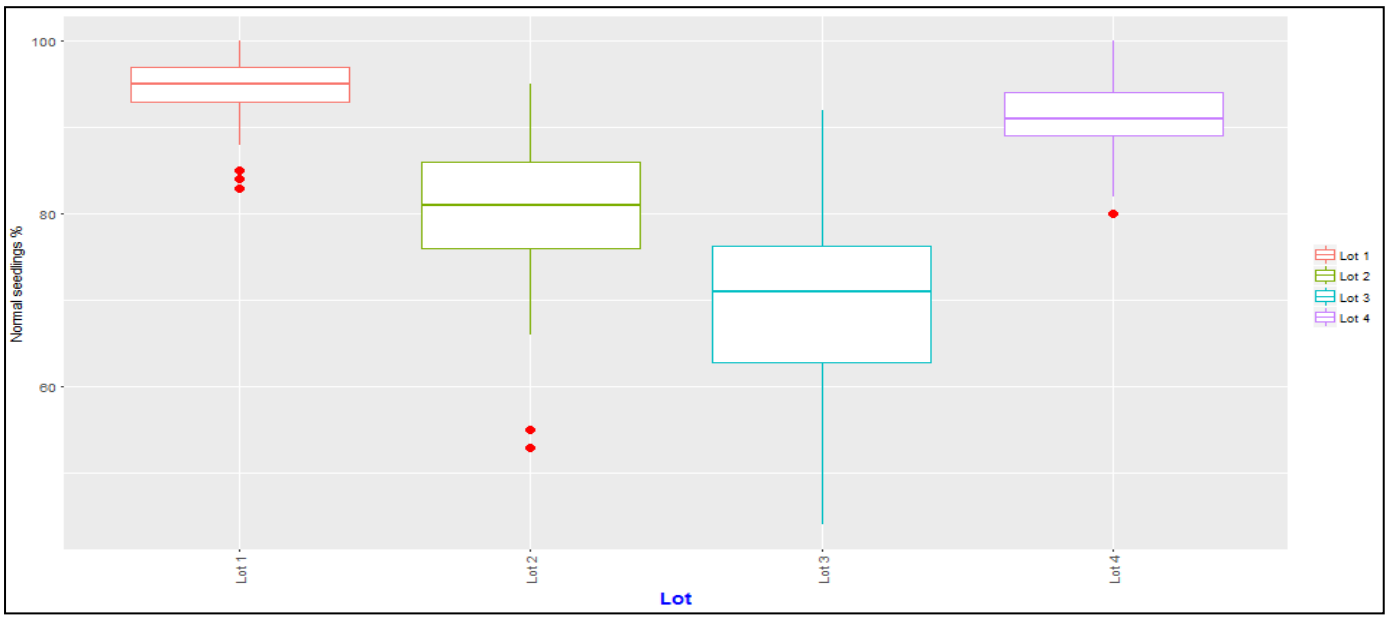

Figure 3. Box plots of the percentage of normal seedlings for all the laboratories and all the methods, per lot.

Germination results obtained with different testing methods

Effect of the testing method

Figure 4 presents the distributions of the percentage of normal seedlings obtained for all the samples and all the laboratories, depending on the seed testing method and it was observed that the best germination results were obtained with the method BP 20 °C, with an average of 85%, while the lowest germination results were obtained with TP 20<=>30°C, with an average of 82%.

Figure 5 presents the distributions of the percentage of normal seedlings depending on the seed testing method, per lot and it was observed that all the lots tested showed the same distribution of the results independently of the method being tested. Consequently, the best germination was obtained with lot 1, followed by lot 4, and lot 2, and finally lot 3 which showed the lowest germination results regardless of the method being tested. Figure 6 shows the same data of normal seedlings depending on the seed testing method, per lot and laboratory.

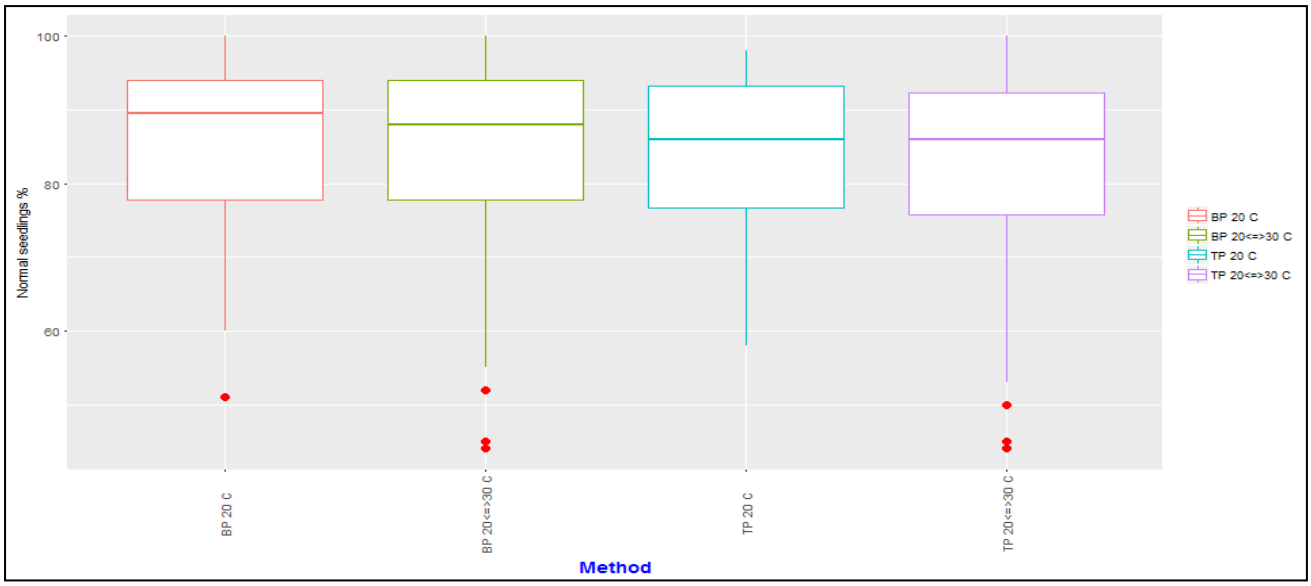

Figure 4. Box plots of the percentage of normal seedlings results per method.

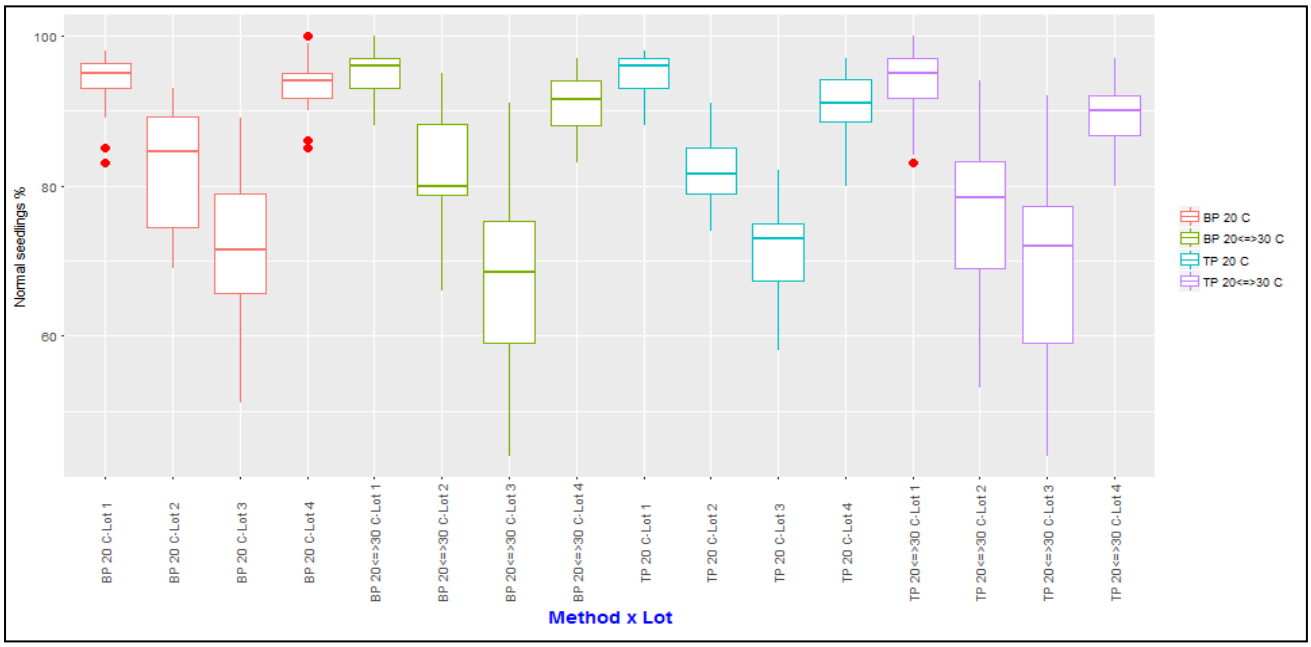

Figure 5. Box plots of the percentage of normal seedlings, per method and per lot.

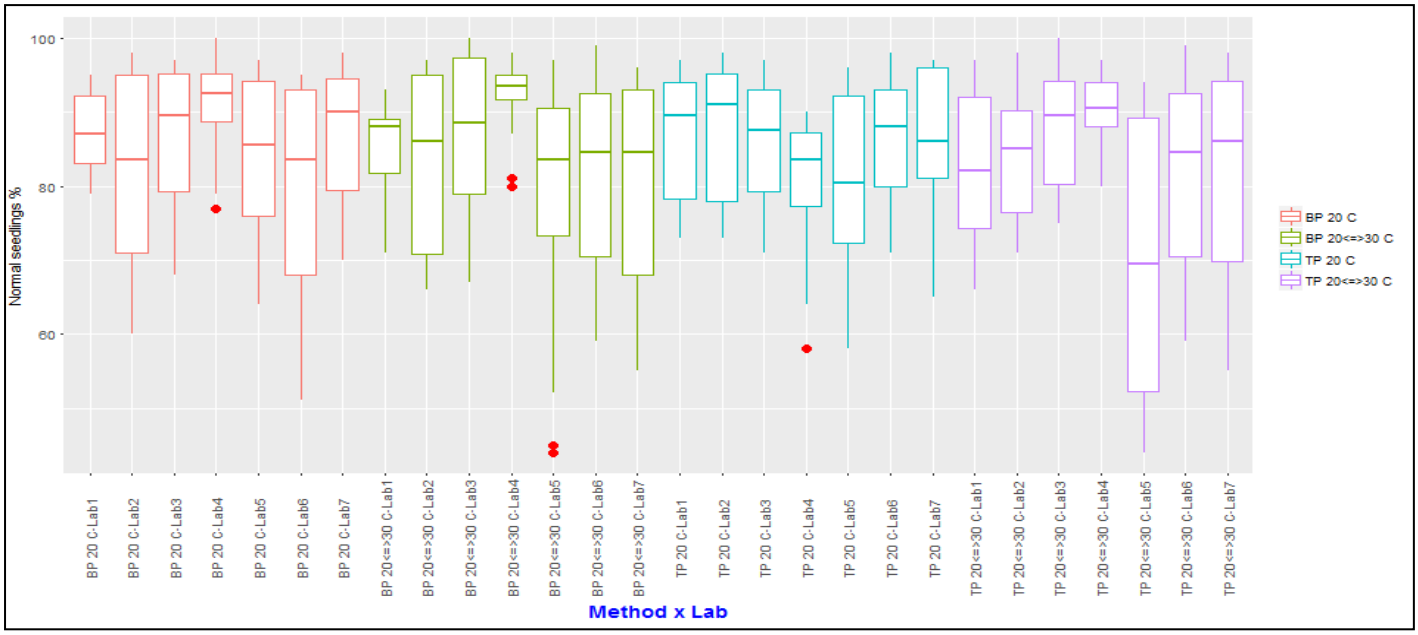

Figure 6. Box plots of the percentage of normal seedlings per method, per lot and per laboratory.

Mixed model for comparing Method and Lot Means

Results from fitting the mixed model are displayed in Table 2 indicating that only the lot main effect was significant.

Table 2. Tests of fixed effects for "lot", "method" and "lot*method".

Source of variation	Sum of Squares	Mean Square	NumDF	Den DF	F value	Pr(>F)
Method	28,56	9,52	3	18	0,99	0,42
Lot	1181,27	393,76	3	18	40,85	0,00
Method:lot	64,57	7,17	9	54	0,74	0,67

Data checking

Data checking has been performed according to ISTA rules by computing tolerances for germination test replicates. Only one result of 112 results was out of tolerance, corresponding to Lot 4, method TP 20° C.

Repeatability/Reproducibility

In order to evaluate the quality of the testing methods used for the germination of the *Chenopodium quinoa* seed samples, data of normal seedlings were analyzed using *ISTAgermMV* R package following

statistical tools 'inter-laboratory tests', developed by the ISTA Statistics Committee for repeatability and reproducibility, where repeatability quantifies the average variability of results within a laboratory and reproducibility quantifies the average variability among laboratories.

The results of Repeatability and Reproducibility of the germination methods are presented in Table 3. All the f_r values were close to 1, which indicates that all the methods did not show problems regarding repeatability.

The standard deviation values of reproducibility were higher in all the tests performed at alternating temperatures, obtaining the highest value in TP 20<=>30°C (S_R= 10.31), followed by BP 20<=>30°C (S_R= 7.92), indicating that the tests performed under this temperature regime were less reproducible than the tests conducted at 20°C.

Table 3. Repeatability and Reproducibility of the germination methods.

Method	$\overline{p}_{...}$	S_r	f_r	S_R	$\sqrt{\hat{\sigma}^2_{Lab}}$	$\sqrt{\hat{\sigma}^2_{Lot\times Lab}}$
BP 20 C	85	3.1	0.88	6.76	2.58	5.42
BP 20<=>30 C	84	3.07	0.83	7.92	3.39	6.47
TP 20 C	85	3.34	0.92	4.82	2.74	2.14
TP 20<=>30 C	82	2.89	0.75	10.31	4.99	8.54

The means for all the tested methods were compared following the mixed model analysis and the results are indicated in Table 4. The results showed no significant differences between BP and TP substrates for the germination test of this species.

Table 4. Comparison of the BP and TP means as a germination growing media for quinoa seeds.

Method	Media	n	S.E.	
BP 20 C	85,54	28	2,27	A
TP 20 C	84,46	28	2,27	A
BP 20<=>30 C	83,79	28	2,27	A
TP 20<=>30 C	82,11	28	2,27	A
(p > 0,05)				

Conclusion

After analyzing the results, it was observed that regardless of the tested method or the lot tested, the higher germination percentages were obtained using 20 ° C instead of 20<=>30°C.

In the same way, the standard deviation values of reproducibility were higher in all the tests performed at 20<=>30°C, obtaining the highest value in TP 20<=>30°C, meanings that the analyzes performed under this temperature regime were less reproducible than the tests performed at 20°C.

For all of the reasons mentioned above and according to the results obtained and with the objective to report reliable results, it was decided to only include 20°C as a temperature regime.

The analysis of variance performed to compare both substrates, BP and TP, showed no significant differences between them in the germination results, suggesting that both could be recommended as a growing media for quinoa seeds.
The germination method proposed to be included in ISTA Rules for *Chenopodium quinoa* seeds is: BP or TP; 20°C; 4 – 7 days.

References

ISTAgermMV R package following statistical tools 'inter-laboratory tests, developed by the ISTA Statistics Committee.

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Von Baer, I., Bazile D., Martínez E. (2009) "Cuarenta años de mejoramiento de quínoa (*Chenopodium quinoa* Willd.) en la Araucanía: Origen de La Regalona-B".

Validation study for an additional seed germination method for *Eustoma exaltatum*

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Summary

Eustoma exaltatum is a species belonging to the family Gentianaceae. The introduction of this species in to the ISTA Rules was approved by the ISTA membership in 2018 and has been effective since 1st January 2019. The validation study to support this introduction was carried out in 2016. Six laboratories participated and three germination methods differing only in temperature (20°C, 25°C and 20°C<=>30°C) were included in the study (Zecchinelli R. and Grim A., 2018). Statistical analysis showed only one method 20°C<=>30°C had acceptable repeatability and reproducibility. This is now the prescribed ISTA method for seed germination of *Eustoma exaltatum*. The object of this second validation study is to introduce an additional germination method for *Eustoma exaltatum*, to the ISTA Rules, Chapter 5, Table 5A, part 3. Detailed methods for germination tests: Flower, spice, herb and medicinal species. This study re-evaluates the temperature 20°C given its importance to laboratories testing seed for cut flowers of *Eustoma exaltatum* in Japan and the inclusion of this temperature in the AOSA Rules for Testing Seed. The method TP, 20°C (first count 4-7 days; final count: 21 days) with the additional treatment of light was compared with the ISTA prescribed method TP, 20°C <=>30°C (first count 4-7 days; final count: 21 days) with additional treatment of light. Six experienced ISTA laboratories were chosen from five different countries and three seed samples with germination in the range 80-95% were distributed for testing. The laboratories were also advised to extend the test by 7 days in case the evaluation of seedlings was difficult, due to the very small size of the seedlings. Statistical analysis of the returned data showed overall percentage germination for both methods was 88%. The dispersion factors for repeatability were very close to one for both methods, 1.02 and 0.97 for 20°C <=>30°C and 20°C respectively. The reproducibility standard deviation values were also very similar at 3.83 for 20°C <=>30°C and 4.11 for the 20°C method. Based on the results of the analysis, the additional germination method with temperature regime 20°C is proposed to be included in the ISTA Rules for *Eustoma exaltatum.*

Introduction

Native to the warm areas of the Americas *Eustoma exaltatum* is an herbaceous species belonging to the family Gentianaceae. Commercially, it is an annual plant, known by the common names lisianthus

and prairie gentian. Lisianthus is cultivated mainly as a cut flower but can be used as potted and bedding plants as well. Seed represent the preferred propagating material for the species.

The first validation study (Zecchinelli and Grim, 2018) was carried out in 2016, with six participating laboratories and three germination methods evaluated. The methods only differed by the temperature regime, 20°C, 25°C or 20<=>30°C using TP with first count 4-7 days; final count: 21 days. Additional treatment light recommended. The statistical analysis showed that only one method (20 <=>30°C) had acceptable repeatability and reproducibility. This method was included in the ISTA Rules in 2019. The other two methods (TP; 20°C and TP; 25°C) did not provide acceptable results.
After the conclusion of the first study, a member of the ISTA Flower Seed Testing Committee (FSC) from Japan pointed out that germination at no more than 25 °C is used by the growers to prevent the plant rosetting where the production of cut flowers is intended. Germination testing by Japanese laboratories is also performed at no more than 25 °C. It was also noted that 20°C is already included in the AOSA Rules for *Eustoma exaltatum*. Taking this into account, the FSC, supported by the ISTA Germination Committee (GER), approved the proposal to repeat the experiment for the germination method at 20°C.
This second validation study was organised in 2019 by Shizka Takeuchi, Kumiko Jinno and Chihiro Igarashi from Sakata Seed Corporation (Japan), in cooperation with the ISTA Committees FSC and GER. The second validation study was undertaken to determine if TP; 20°C (first count 4-7 days; final count: 21 days) could be added as an additional germination method for *Eustoma exaltatum* in the ISTA Rules. The test plan (consistent with the 2016 test plan) included direction for both methods to use light as an additional treatment by all participant laboratories. The laboratories were also requested to extend the test by 7 days if evaluation of seedlings was difficult, due to the very small size.

The validation study was performed through a multi-laboratory comparative test, (ISTA,2007). Results of the laboratories are reported anonymously.

Material and Methods

Seed material

Three lots of untreated seed of *Eustoma exaltatum* were used in the study, all originated from Japan. The three lots were all intended for marketing, having a different germination potential, but all at commercial level. The lots distributed to the laboratories were coded 1, 2, 3 and the original test results were not disclosed to the laboratories.
The delivery of the seed lots took time, due to the geographical distribution of the laboratories which led to delays in customs formalities (Japan, Israel, Italy, Netherlands, USA).

Participant laboratories

Six laboratories participated in the study: Sakata Seed Corporation (Japan), Takii Seed (Japan), CREA-DC (Italy), Vulcani Center (Israel), Naktuinbouw (The Netherlands), SGS (USA). All laboratories had previous experience with testing *Eustoma exaltatum*

Additional treatments

No additional treatments were used, but the laboratories were asked to germinate the seed in the light.

Germination method

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

Following the agreed test plan, germination counts were made after 4 or 7 days (1st count) and 21 days (final count). The seeds of the species are very small and therefore seedling evaluation can be challenging. The test plan thus recommended laboratories extend the duration of the test by 7 days where necessary.

Laboratories were asked to evaluate the seedlings based on seedling group A-2-1-1-1 (primary root required without consideration of secondary roots).

Results and Analysis

Results were received from all laboratories. The comparative test involved a total of six germination tests completed by each laboratory. The data received was checked for completeness and accuracy and then submitted for statistical analysis.

Checks confirmed that the sum of the percentages always equaled 100%. Tolerance checks on germination test replicates according to ISTA Rules 2018, Chapter 5, Table 5b showed no test results were found to be out of tolerance.

The laboratories also provided a description of the abnormal seedlings. The laboratories agreed that the most frequent abnormalities were the following: 00/01 (seedling deformed), 11/01 (primary root stunted), 11/04 (primary root missing), 31/02 (cotyledons deformed).

Some laboratories wanted to highlight the opinion that the germination of Eustoma is slower at 20 °C than at 20 <=> 30 °C. Some laboratories therefore extended the duration of the test to 28 days, for lot 3 and for the 20 °C method. One laboratory suggested a preference for the first count to be performed after 14 days and not before.

Statistical analysis of the results

Germination results by seed lots

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

The median values for normal seedlings, figure 1, is, lot 1, 90%, lot 2, 93% and lot 3, 83%.

The overall average percentage of normal seedlings was calculated as 89.7%, 91.8% and 83.0% respectively.

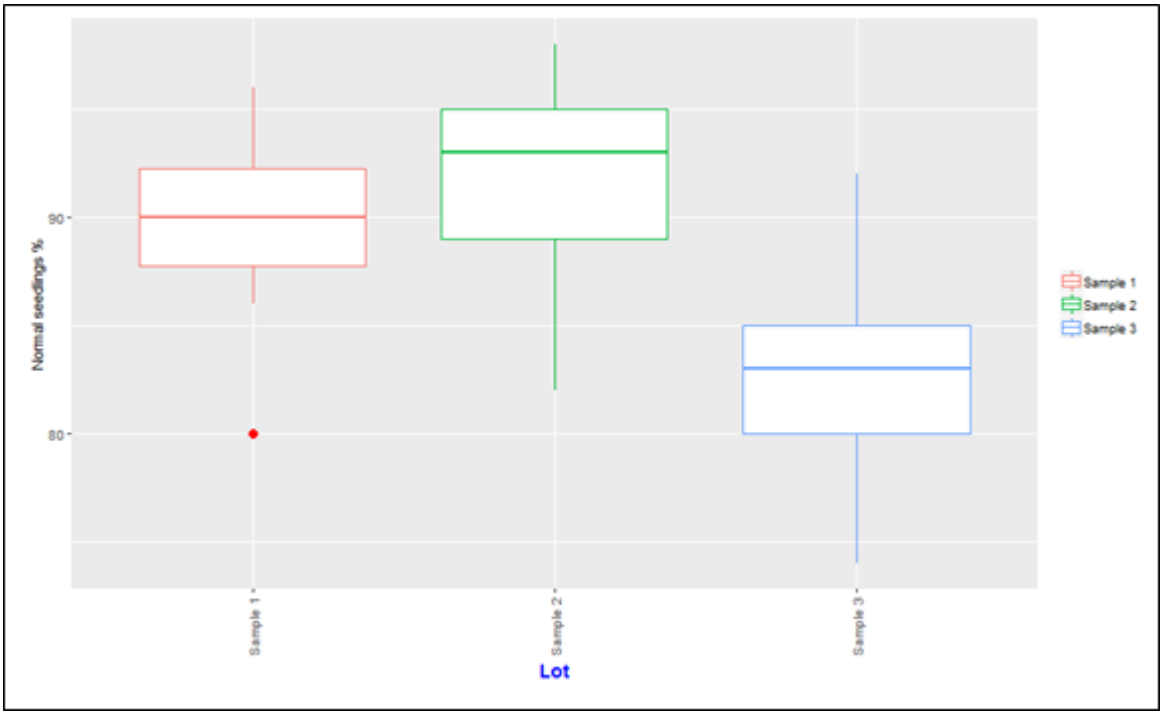

Figure 1. Percentage of normal seedlings for all methods and all laboratories, per lot.

Germination results by laboratory and by method and lot

Figure 2 shows the data of the percentage of normal seedlings for all lots and all methods, by each laboratory. Figure 3 shows the data for percentage of normal seedlings for all laboratories, by method and lot. One can observe the good quality of the data: although laboratory 4 shows some outliers (figure 2), the differences are not sufficient to exclude the laboratory from the analysis.

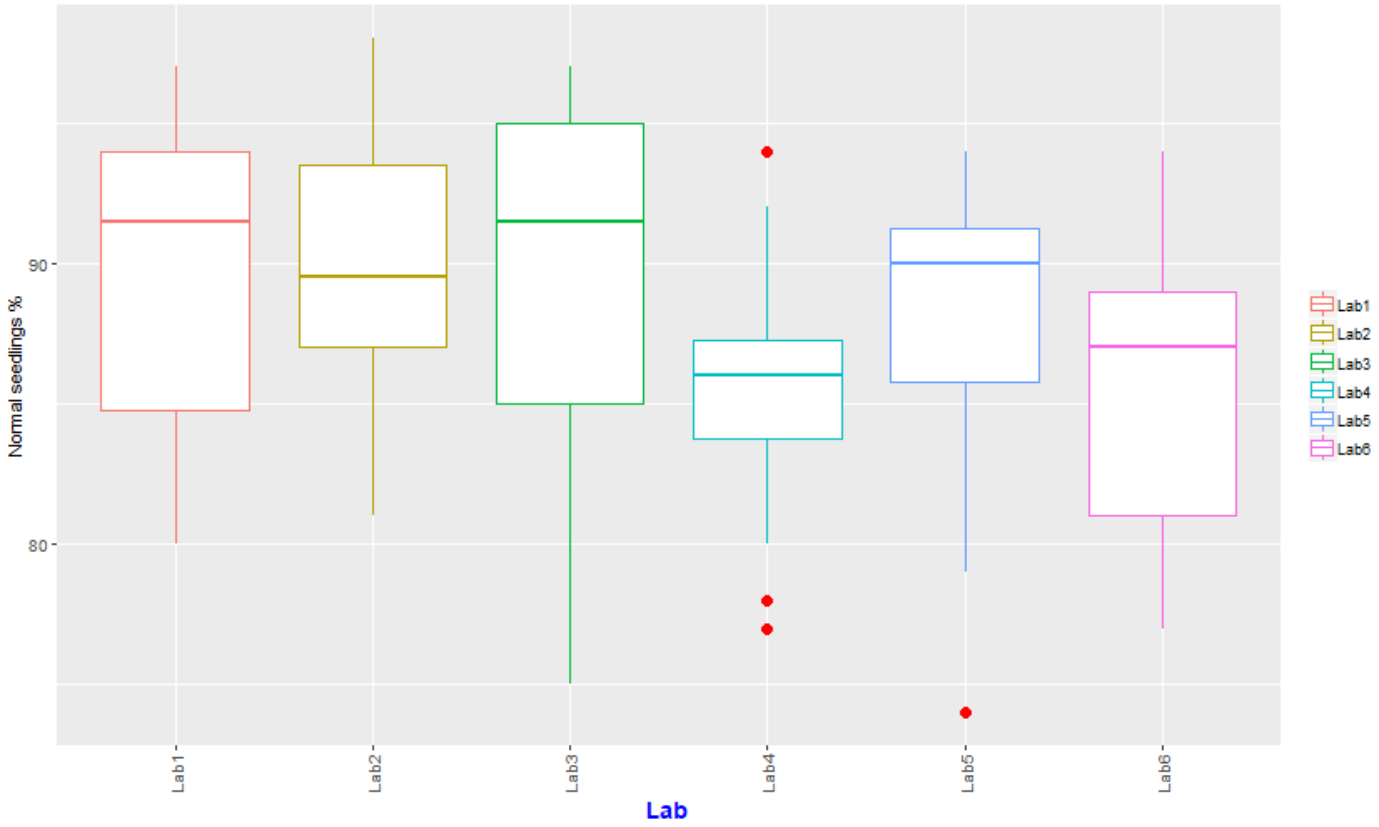

Figure 2. Percentage of normal seedlings for all lots and all methods, per each laboratory.

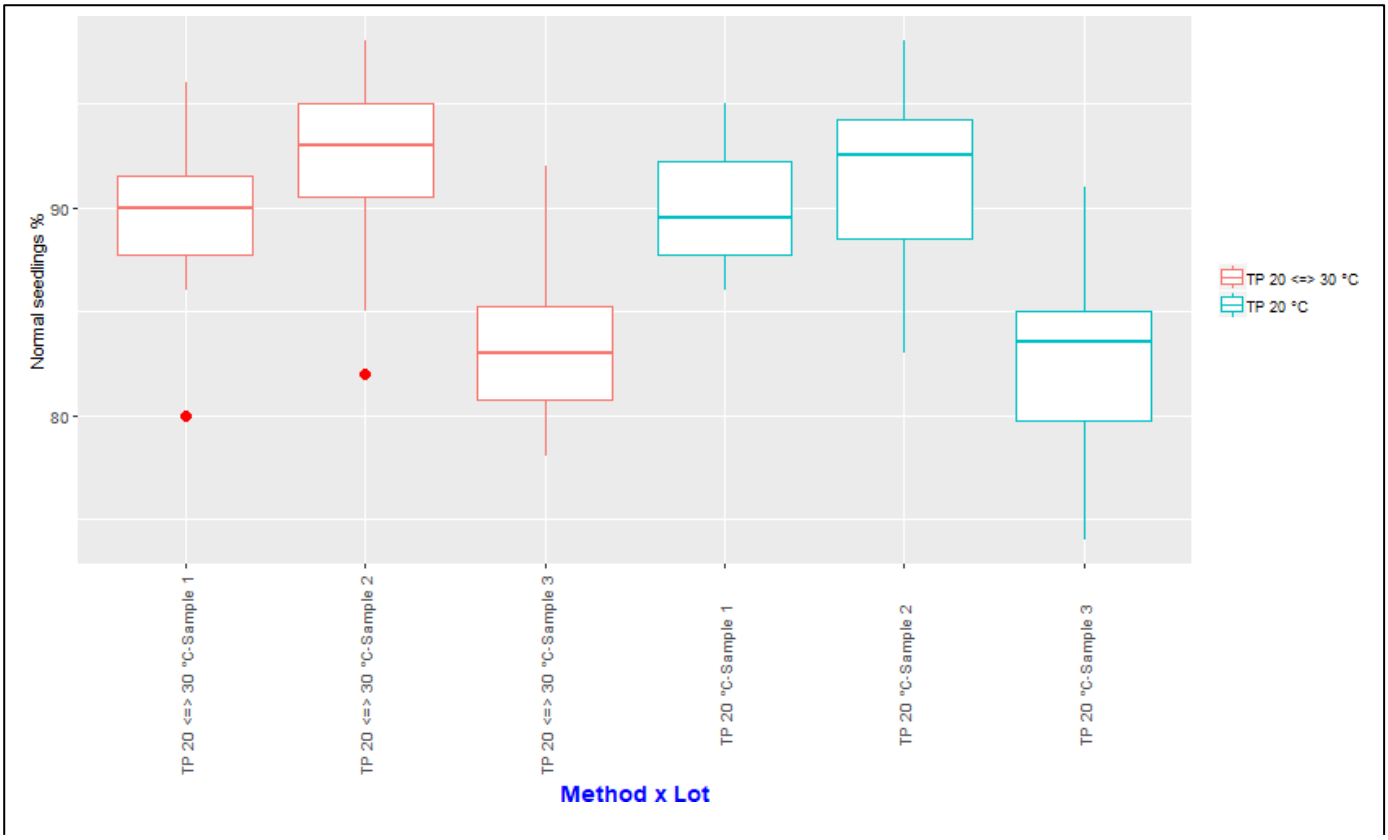

Figure 3. Percentage of normal seedlings for all laboratory, per each method and lot.

Germination results by method

Figure 4 shows the data of the percentage of normal seedlings for all lots and all laboratories, by each method. The figure clearly shows that the variation seen in figures 2 and 3 is not driven by method.

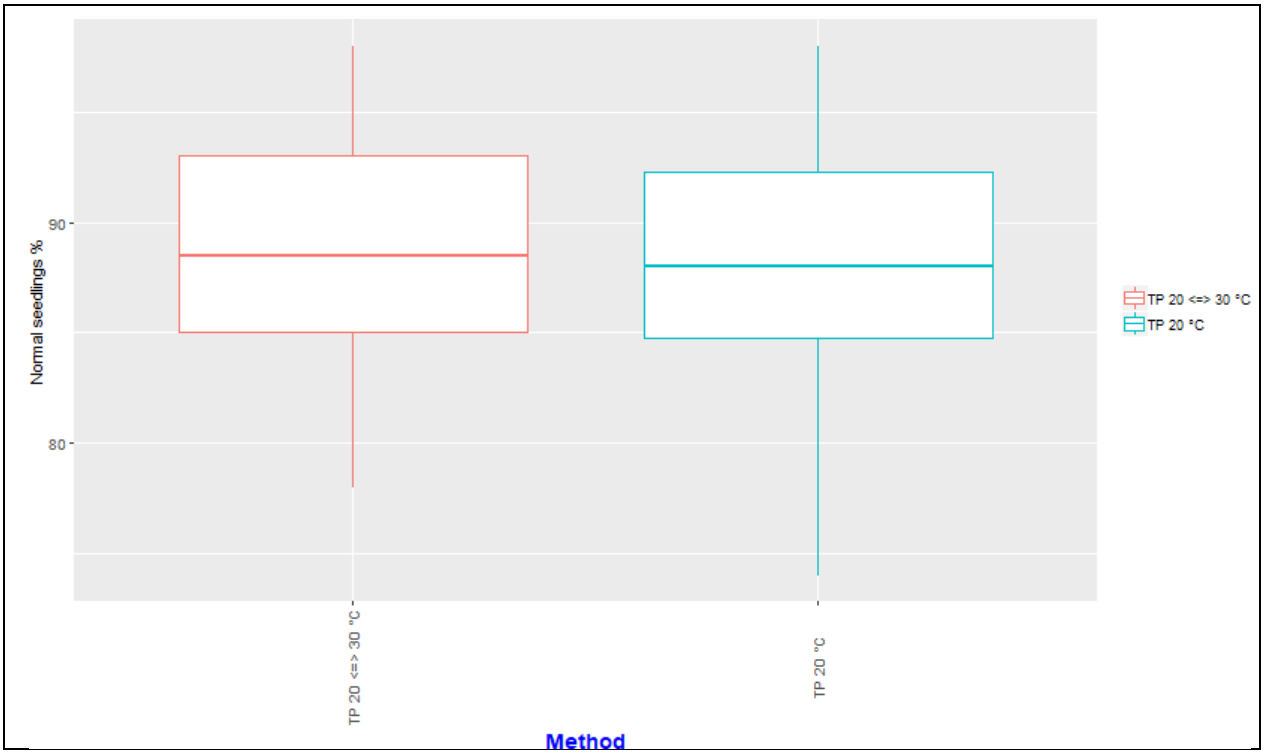

Figure 4. Percentage of normal seedlings for all lots and all laboratories, per method.

Repeatability/Reproducibility

Repeatability and Reproducibility were estimated using a Linear Mixed model, through the R package ISTA germ MV. The results are shown in table 1 below.

Table 1. Estimate of repeatability and reproducibility of methods.

Method	Mean	s_repeatability	dispersion	s_Reproducibility	s_Lab	s_LotxLab
TP 20 <=> 30 °C	88.00	3.25	1.02	3.83	1.81	0.94
TP 20 °C	88.00	3.15	0.97	4.11	1.78	1.93

The overall average percentage (mean) of the normal seedlings is the same for the two methods, 88%. Looking at the repeatability standard deviation, the dispersion factor is close to 1 for both methods The reproducibility standard deviation (3.83 and 4.11) is similar for both methods.

Conclusions

While in the first study, Eustoma seed germination at 20 °C showed to be poorly reproducible between the laboratories (in particular for the results obtained from one of them), with the current study all laboratories have provided comparable results, as illustrated above. The results of the study have successfully shown that the 20°C method has in fact provided results in accordance with those

produced with the standard method. The results in terms of average percentage of normal seedlings, repeatability and reproducibility are acceptable.
The recommendation from the ISTA Flower Seed Testing Committee is to add in the ISTA Rules the following germination method for *Eustoma exaltatum:*

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

Acknowledgments

Thanks to Sakata Seed Corporation (Japan) for providing the seed lots used in the validation study. Thanks to the technical reviewers of the test plan, Sylvie Ducournau and Valerie Cockerell. And thank you to Jean-Louis Laffont, statistical reviewer of the test plan appointed by the ISTA Secretariat. Thanks to the staff of the participating ISTA laboratories: Sakata Seed Corporation (Japan), Takii Seed (Japan), CREA-DC (Italy), Vulcani Center (Israel), Naktuinbouw (The Netherlands), SGS (USA). Thanks, in particular to the reference persons of those laboratories: Shizka Takeuchi, Takayuki Okuda, Rita Zecchinelli, Meriam Dekalo-Keren, Erik van Egmond, Sarah Dammen.

Thanks to Sylvie Ducournau who performed the statistical analysis of the results and to Jean-Louis Laffont who performed the review of that analysis.
Thanks to Nadine Ettel and Andreea Militaru, TCOM coordinators at the ISTA Secretariat for the support.

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Germination Committee Technical Report: Validation of germination method utilizing crepe cellulose paper (CCP) for the top of paper (TP) method for *Glycine max*.

Sarah Dammen, SGS, USA and David M. Johnston, Louisiana Dept. of Agriculture and Forestry, USA.

Summary

This study was conducted to determine the suitability of utilizing crepe cellulose paper (CCP) as a primary media for the top of paper (TP) method for *Glycine max* and adding this media to the ISTA Rules Table 5A Part 1. The different combinations of media and temperatures were studied. The temperatures of 25° C and 20<=>30° C were compared and were used in combination with the methods of sand (S), between paper (BP), top of paper using crepe cellulose paper (CCP) with a sand covering (TPS) and top of paper (TP) utilizing CCP with no form of covering. Six laboratories analyzed three seed lots of *Glycine max* of varying germination and vigor levels. Statistical analyses of the data showed that the methods utilizing CCP for the TP method produced acceptable results of repeatability when compared to the other methods used for this study. It is therefore requested to add the option of CCP for the TP method to the ISTA Rules for *Glycine max.*

Introduction

The current ISTA Rules germination media options for *Glycine max* top of paper (TP) do not include CCP. In part, the purpose of this study is to harmonize the ISTA Rules with the Association of Official Seed Analysts (AOSA) Rules. The use of CCP for the TP method was adopted into the AOSA Rules in 1980. Since then, this highly efficient test method has been successfully utilized for accurately assessing the germination of seed lots by both governmental and non-governmental laboratories across multiple decades.

Although the use of CCP is not widespread around the world, it is widely utilized in laboratories in North America. Several laboratories, including company laboratories that produce and test *Glycine max* seed outside of the United States, desire that the ISTA Rules also adopt the TP CCP method. These laboratories tend to have an extremely large volume of *Glycine max* samples and require an extremely fast through-put of samples and are seeking to adopt automation to meet this requirement. The TP CCP method greatly supports the implementation of technology (e.g. automated sowing) to increase the efficiency of laboratory testing.

Materials and Methods

Seed material

Three *Glycine max* commercial seed lots of varying germination and vigor levels were supplied by the Bayer Seed Physiology Laboratory located in Waterman, IL USA. None of the three seed lots had been

commercially treated prior to being used for the study. None of the seed lots exhibited any dormancy during germination testing.

Participating laboratories

A total of six laboratories participated in this ISTA validation study, two of which are ISTA accredited for this species. All laboratories were required to be familiar with the use of the TP method utilizing CCP for this species. To facilitate the timely movement of samples and the avoidance of phytosanitary certificate requirements as well as the importation concerns of biotechnology (i.e. GMO) seeds, all laboratories were located within the U.S.A.

Germination methods

All participating laboratories compared the TP CCP method with each of the current ISTA methods using S, BP, TPS media and using the prescribed test temperatures of 20°C and 20<=>30°C. All participants used 8 hours of light in every 24-hour cycle. For the alternating temperature testing portion, light was used during the highest temperature setting. All laboratories were encouraged to conduct only one seedling evaluation count at 7 days. Evaluations were conducted using ISTA Seedling Type E Seedling Group A-1-2-3-2.

Statistical analyses

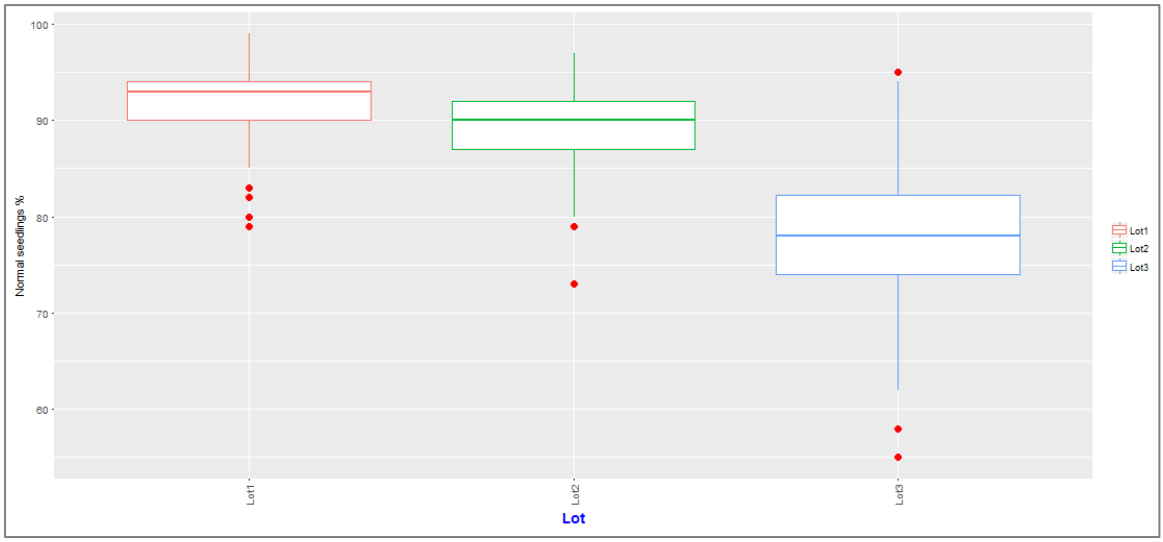
Statistical analyses were performed by Jean-Louis Laffont, PhD and Chair of the ISTA Statistics Committee and Riad Baalbaki, PhD and member of the AOSA/SCST Statistics Committee. Analysis was performed utilizing the R package developed by the ISTA Statistics Committee 'ISTAgermMV'.

Results and Discussion

1. Data exploration with side-by-side boxplots

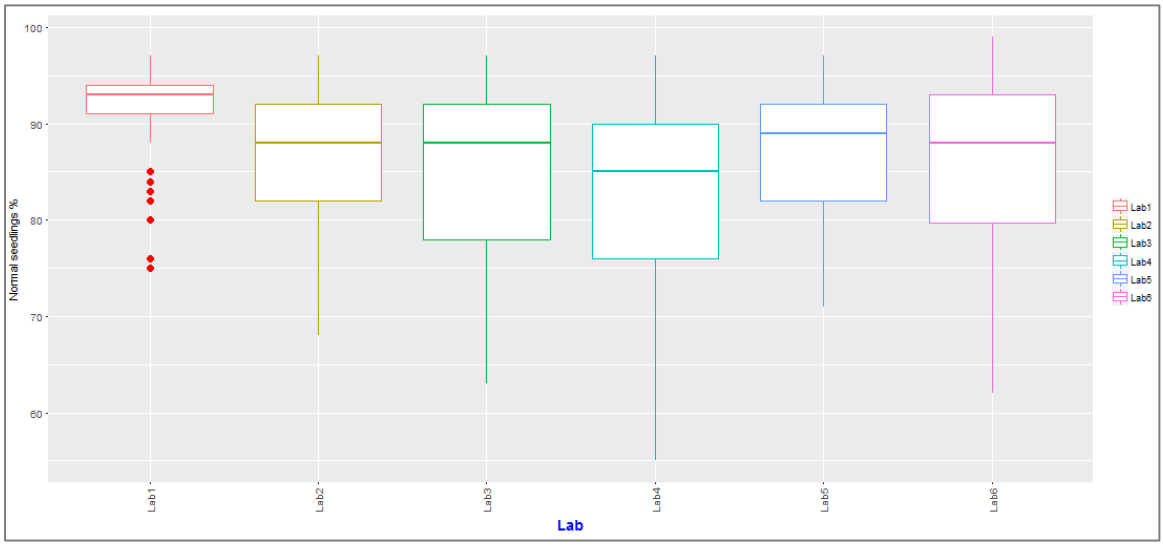
a. Lots: Results averaged over all methods and laboratories verified that lots covered a wide range of quality, expressed as germination (Fig. 1, Lot). This was also verified through analysis of variance (Table 2), which indicated that lot effect on germination results was highly significant. The same trend was observed for method x lot (Figs. 4 and 7) and lab x lot (Fig. 5) results, ranking lot 1 as highest and lot 3 as lowest, respectively.

Fig. 1



Strong lot effect.

Fig. 2



b. Methods: In contrast to difference in lots, germination results of all methods, averaged over lots and labs, were similar (Fig. 3), and narrowly ranged between 86 and 87% (Table 3). Analysis of variance (Table 2) also indicated that the effect of method on final percentage germination was not significant.

Fig. 3

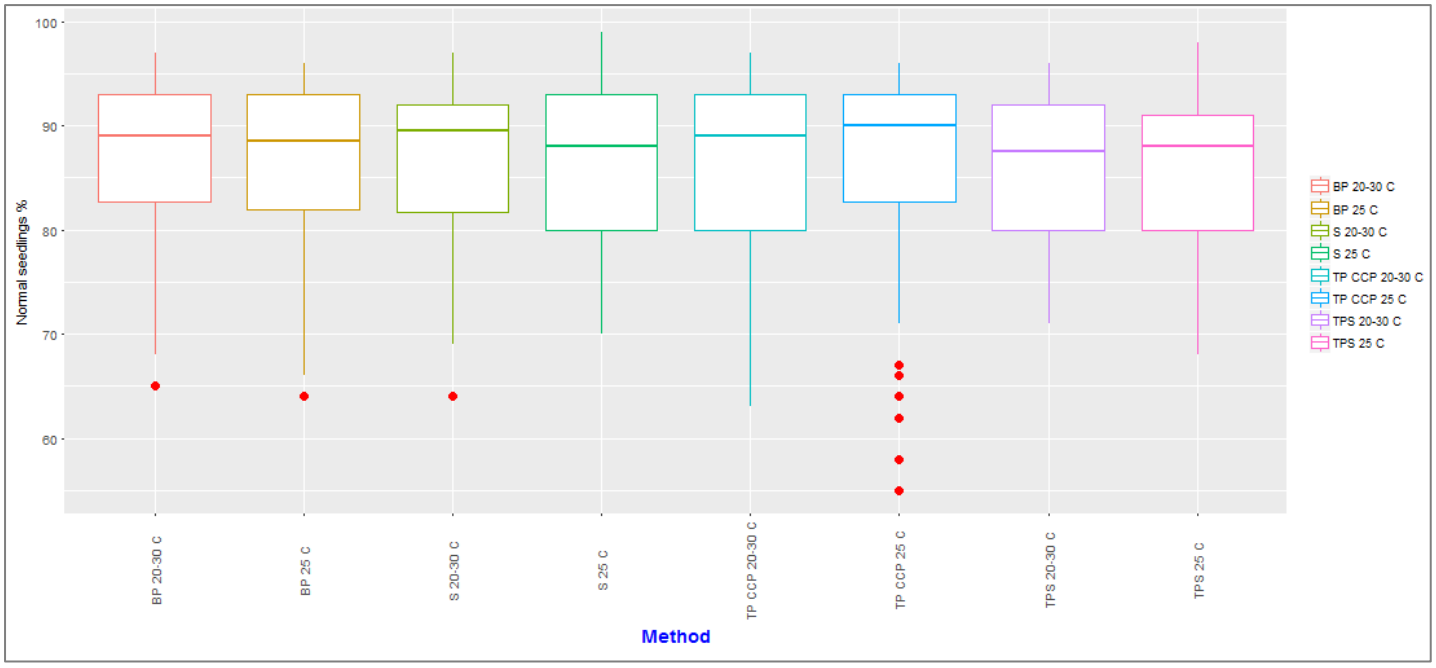

Normal seedlings %
100
90
80
70
60
BP 20-30 C
BP 25 C
S 20-30 C
S 25 C
TP CCP 20-30 C
TP CCP 25 C
TPS 20-30 C
TPS 25 C
Method
BP 20-30 C
BP 25 C
S 20-30 C
S 25 C
TP CCP 20-30 C
TP CCP 25 C
TPS 20-30 C
TPS 25 C

Fig. 4

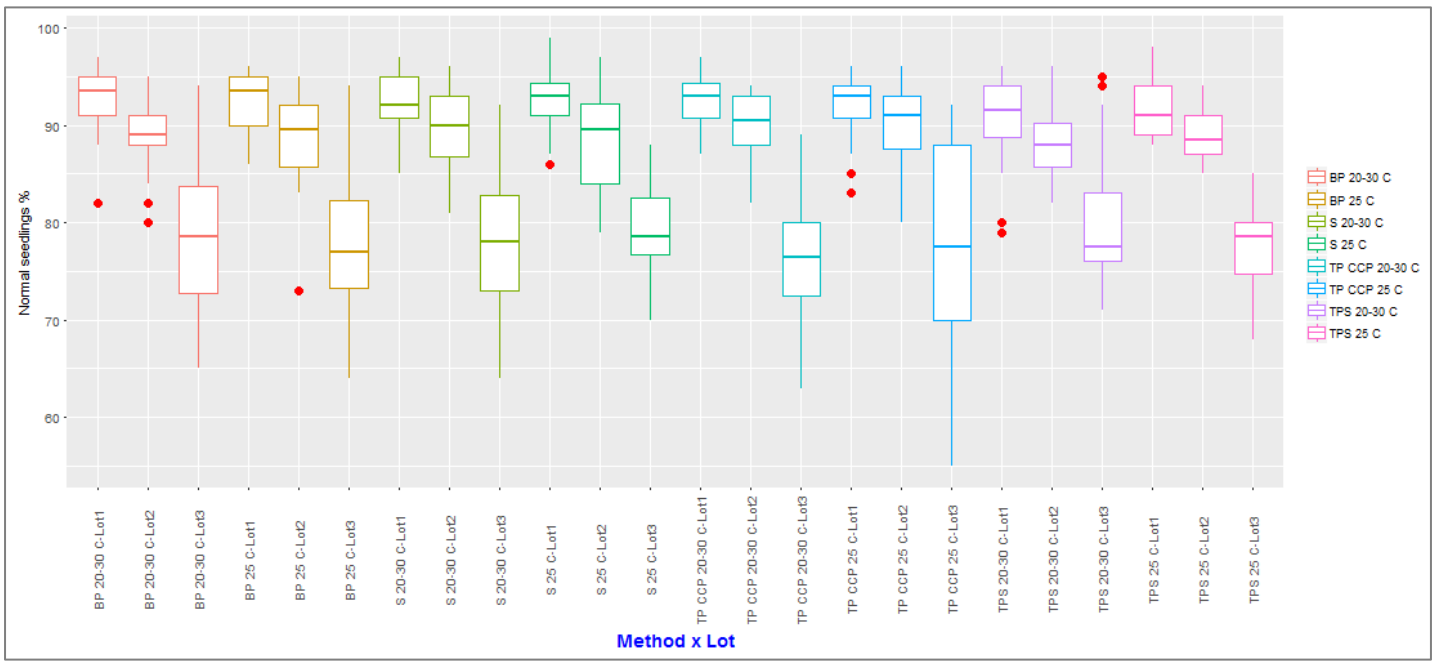
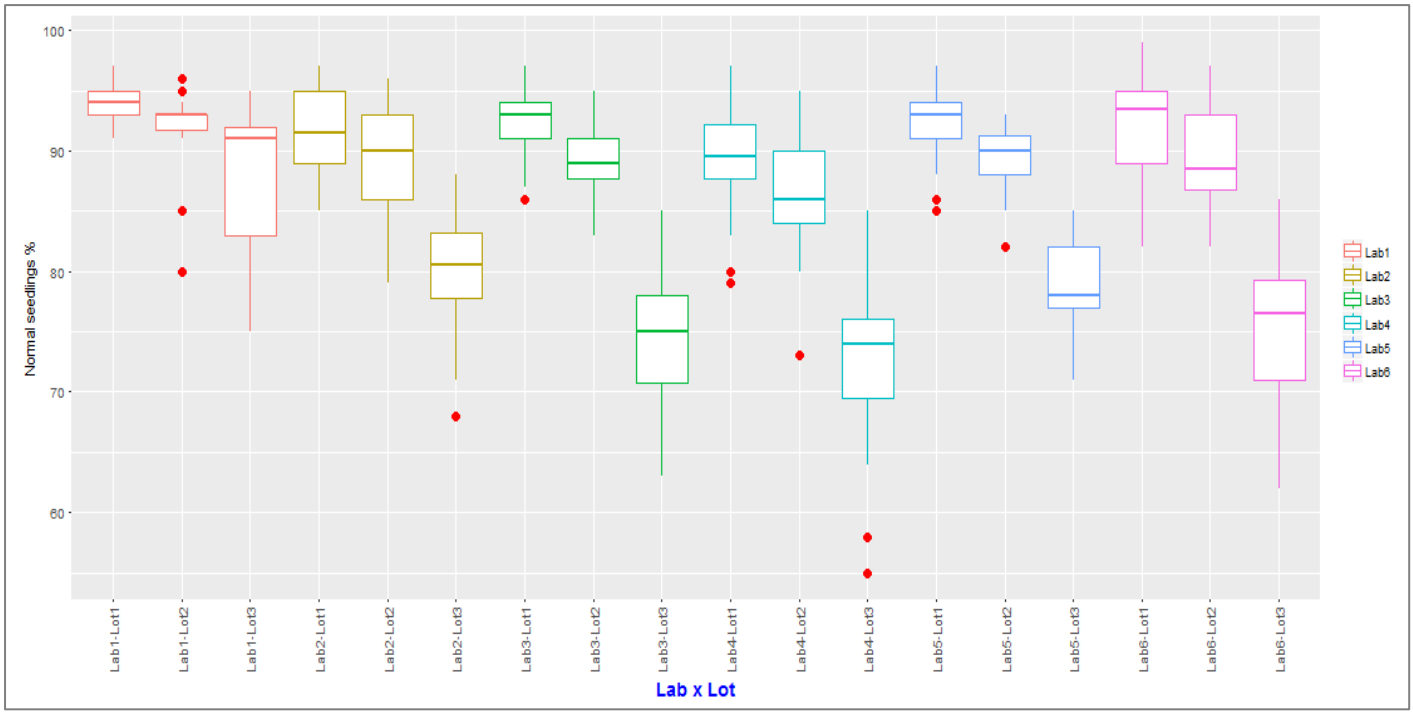

Normal seedlings %
100
90
80
70
60
BP 20-30 C-Lot1
BP 20-30 C-Lot2
BP 20-30 C-Lot3
BP 25 C-Lot1
BP 25 C-Lot2
BP 25 C-Lot3
S 20-30 C-Lot1
S 20-30 C-Lot2
S 20-30 C-Lot3
S 25 C-Lot1
S 25 C-Lot2
S 25 C-Lot3
TP CCP 20-30 C-Lot1
TP CCP 20-30 C-Lot2
TP CCP 20-30 C-Lot3
TP CCP 25 C-Lot1
TP CCP 25 C-Lot2
TP CCP 25 C-Lot3
TPS 20-30 C-Lot1
TPS 20-30 C-Lot2
TPS 20-30 C-Lot3
TPS 25 C-Lot1
TPS 25 C-Lot2
TPS 25 C-Lot3
Method x Lot
BP 20-30 C
BP 25 C
S 20-30 C
S 25 C
TP CCP 20-30 C
TP CCP 25 C
TPS 20-30 C
TPS 25 C

Fig. 5


Normal seedlings %
100
90
80
70
60
Lab1-Lot1
Lab1-Lot2
Lab1-Lot3
Lab2-Lot1
Lab2-Lot2
Lab2-Lot3
Lab3-Lot1
Lab3-Lot2
Lab3-Lot3
Lab4-Lot1
Lab4-Lot2
Lab4-Lot3
Lab5-Lot1
Lab5-Lot2
Lab5-Lot3
Lab6-Lot1
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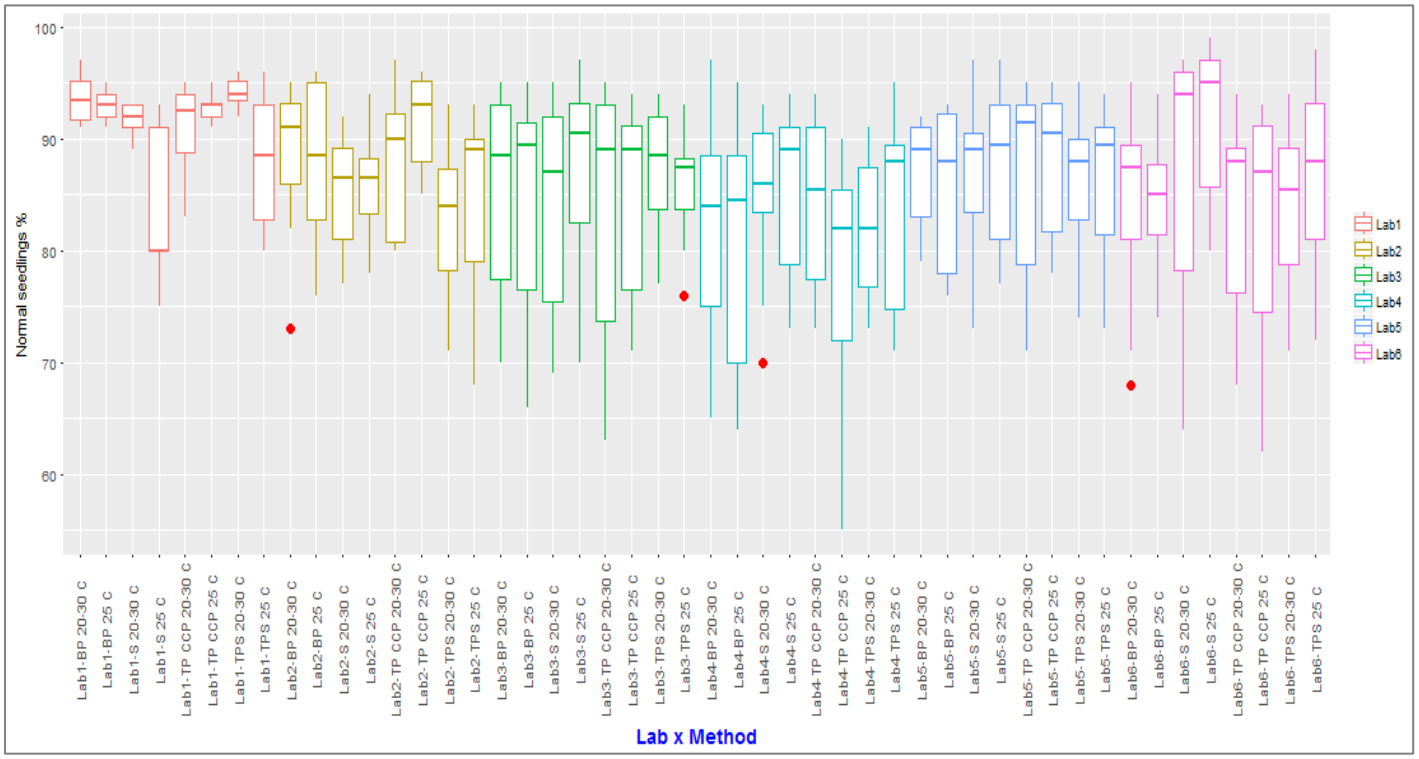
Lab6-Lot3
Lab x Lot
Lab1
Lab2
Lab3
Lab4
Lab5
Lab6

Fig. 6


Normal seedlings %
100
90
80
70
60
Lab1-BP 20-30 C
Lab1-BP 25 C
Lab1-S 20-30 C
Lab1-S 25 C
Lab1-TP CCP 20-30 C
Lab1-TP CCP 25 C
Lab1-TPS 20-30 C
Lab1-TPS 25 C
Lab2-BP 20-30 C
Lab2-BP 25 C
Lab2-S 20-30 C
Lab2-S 25 C
Lab2-TP CCP 20-30 C
Lab2-TP CCP 25 C
Lab2-TPS 20-30 C
Lab2-TPS 25 C
Lab3-BP 20-30 C
Lab3-BP 25 C
Lab3-S 20-30 C
Lab3-S 25 C
Lab3-TP CCP 20-30 C
Lab3-TP CCP 25 C
Lab3-TPS 20-30 C
Lab3-TPS 25 C
Lab4-BP 20-30 C
Lab4-BP 25 C
Lab4-S 20-30 C
Lab4-S 25 C
Lab4-TP CCP 20-30 C
Lab4-TP CCP 25 C
Lab4-TPS 20-30 C
Lab4-TPS 25 C
Lab5-BP 20-30 C
Lab5-BP 25 C
Lab5-S 20-30 C
Lab5-S 25 C
Lab5-TP CCP 20-30 C
Lab5-TP CCP 25 C
Lab5-TPS 20-30 C
Lab5-TPS 25 C
Lab6-BP 20-30 C
Lab6-BP 25 C
Lab6-S 20-30 C
Lab6-S 25 C
Lab6-TP CCP 20-30 C
Lab6-TP CCP 25 C
Lab6-TPS 20-30 C
Lab6-TPS 25 C
Lab x Method
Lab1
Lab2
Lab3
Lab4
Lab5
Lab6

2. Data checking

Data checking has been performed according to ISTA Rules by computing tolerances for germination test replicates. Four test results out of 144 were out of tolerance for replication, but were included in the analysis (Table 1). None of the out of tolerance results were observed using the proposed new method (TP CCP).

Results: see worksheet *Data checking* from the file *Stat analysis for David Johnston - 091820.xlsx*.

Table 1: Out of Tolerance Replicates

Method	Lot#	Lab #	Mean	# Reps	# seeds/rep	Range	Tol	Out of Tol
BP 20-30 C	Lot1	Lab6	90	4	100	13	12	OUT
BP 20-30 C	Lot3	Lab6	76	4	100	18	17	OUT
BP 25 C	Lot2	Lab4	84	4	100	22	14	OUT
S 20-30 C	Lot1	Lab5	92	4	100	12	11	OUT

3. Analysis of variance

Analysis of variance (Table 2), as noted earlier, indicated that lots had strong effect on germination results ($F = 42.68$; $p < 0.001$), while choice of method did not significantly affect germination. The method x lot interaction was not significant, implying that method results were not differentially affected by lot quality.

Table 2: ANOVA Results

Source of variation	Sum of Squares	Mean Square	Num DF	Den DF	F value	Pr(>F)
Method	7.30	1.04	7	35.00	0.10	1.00
Lot	925.29	462.64	2	10.00	42.68	0.00
Method:Lot	140.54	10.04	14	70.00	0.93	0.54

4. Repeatability/Reproducibility

Table 3: Comparison

Method	$\overline{p}_{...}$	S_r	f_r	S_R	$\sqrt{\hat{\sigma}^2_{Lab}}$	$\sqrt{\hat{\sigma}^2_{Lot\times Lab}}$
BP 20-30 C	87	3.78	1.12	5.95	3.13	3.37
BP 25 C	86	3.48	1.01	6.39	3.64	3.94
S 20-30 C	87	3.51	1.03	5.51	1.13	4.09
S 25 C	87	3.14	0.93	4.87	2.27	2.95
TP CCP 20-30 C	86	2.86	0.83	4.50	2.33	2.58
TP CCP 25 C	86	3.31	0.96	7.67	5.31	4.43
TPS 20-30 C	86	3.08	0.89	5.62	3.98	2.52
TPS 25 C	86	3.09	0.89	3.36	0.59	1.19

At the two tested temperature regimes, TP CCP repeatability tended to be the lowest among all methods, in contrast to BP, especially at 20<=>30° C, which had the highest repeatability (Table3).

Reproducibility, a measure of variation among labs, was highest (7.67) for TP CCP 25° C compared with all other methods. This was likely due to the low germination results obtained by lab 4 and lab 6 for lot 3 (Fig. 9). This lot exhibited a significant number of seedlings with mechanical damage, thus making these seedlings more difficult to evaluate. In contrast, TP CCP 20⇔30° C had a relatively low reproducibility variance, and the lowest dispersion (0.83) of any method.

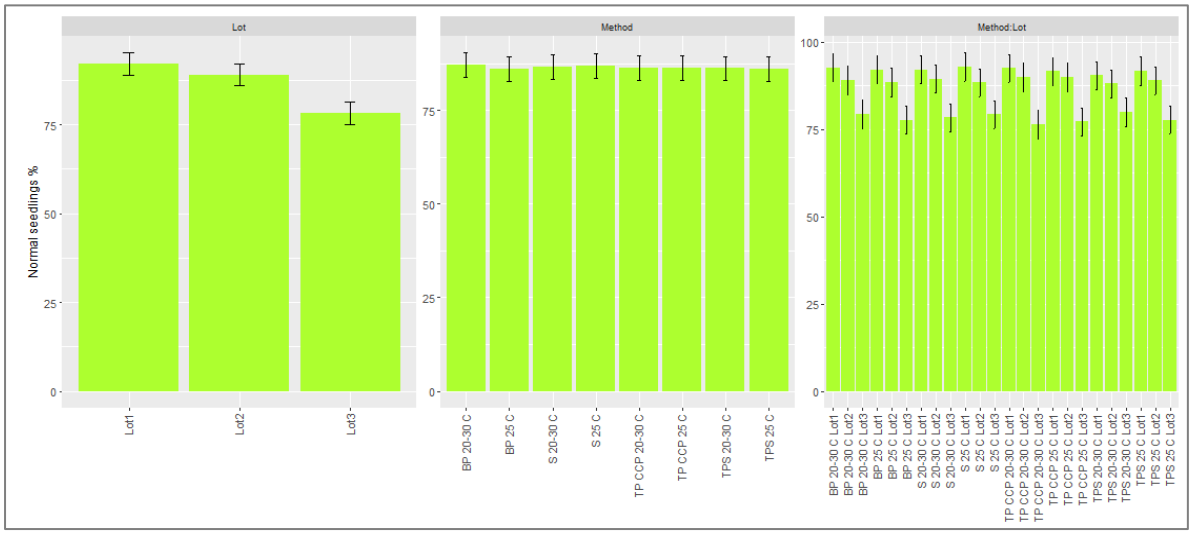
5. Mixed model for comparing Method and Lot means

Results from fitting the mixed model are in the worksheets *ANOVA table*, *LS Means* and *Diff of LS Means* from the file *Stat analysis for David Johnston - 091820.xlsx*.

The lot main effect is highly significant. The Method and the Method x Lot effects are not significant (Fig. 7).

The Least Squares Means are displayed in the following bar plot (Fig. 7) with error bars added corresponding to their standard errors:

Fig. 7



The differences of Least Squares Means table with p-values, standard-errors, and 95% confidence intervals are displayed in the following bar plot (Fig. 8).

Fig. 8

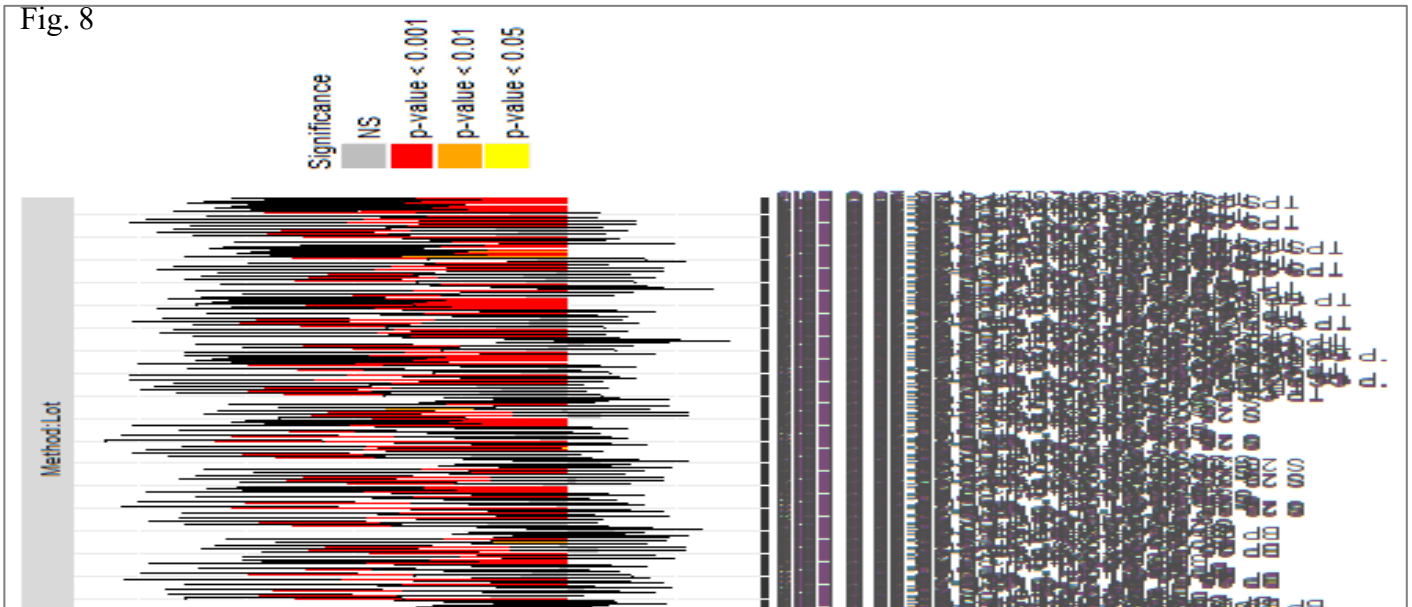
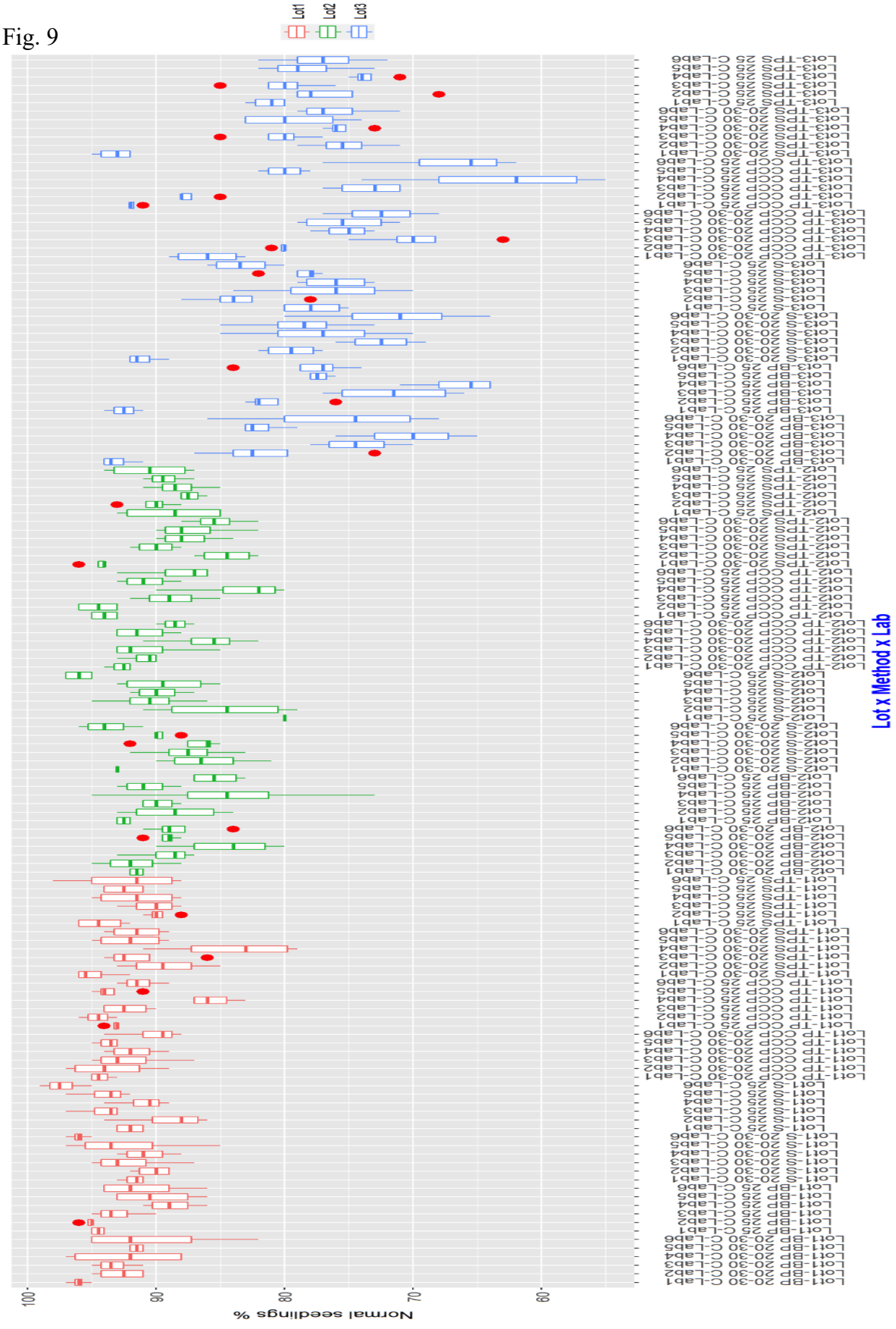


Fig. 9


Lot1
Lot2
Lot3
Normal seedlings %
100
90
80
70
60
Lot x Method x Lab

General Conclusions

1. Analysis of variance, as well as comparisons of LS, indicated that percentage germination did not significantly differ among methods, i.e. the proposed TP CCP methods produced germination results similar to current official ISTA methods.
2. Analysis of variance showed that results from all methods were consistent, regardless of lot germination capacity.
3. Repeatability standard deviation *(S_r)*, i.e. replicate variation, of TP CCP 25° C was lower than both BP methods and slightly higher than other methods, while S_r of TP CCP 20<=>30° C was lower than any other method.
4. In addition to acceptable S_r values, dispersion factors for both CCP methods were below 1 and for CCP 20<=>30° C, lower than any other method.
5. Reproducibility standard deviation, a measure of lab to lab variation plus repeatability, for TP CCP 20<=>30° C was among the lowest of all methods.
6. It is recommended to add TP CCP to the list of approved methods for *Glycine max* in the ISTA Rules.

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Thank you to the participating laboratories for their efforts and donated resources required to complete this study: Illinois Crop Improvement, Champaign, IL USA; Iowa State University, Ames, IA USA; Louisiana Dept. of Agriculture and Forestry, Baton Rouge, LA, USA; Bayer Seed Physiology Laboratory, Waterman, IL USA; SGS North America Brookings, SD, USA; SoDak Labs, Brookings, SD USA.

Thank you to Nadine Ettel, ISTA TCOM Coordinator; Germination TCOM Technical Reviewers Erik van Egmond, Naktuinbouw, Netherlands and Meriam Dekalo-Keren of ARO Volcani Center Official Seed Testing Laboratory, Israel; Gillian Musgrove, the very supportive ISTA Germ TCOM Chair and the enthusiastic Germ TCOM members.

Thank you to Jean-Louis Laffont, PhD ISTA Statistical Committee Chair and Riad Baalbaki, PhD AOSA/SCST Statistical Committee member for their much needed and appreciated statistical support.

References

1. International Seed Testing Association. 2019. ISTA International Rules for Seed Testing. Bassersdorf, Switzerland

Germination Committee Technical Report: Validation of germination method utilizing agar (A) for method for *Pinus sylvestris*.

David M. Johnston Louisiana, Dept. of Agriculture and Forestry, USA.

Summary

This study was conducted to determine the suitability of utilizing agar (A) as a primary media for *Pinus sylvestris* and adding this media to the ISTA Rules Table 5A Part 2. Different combinations of media and temperatures were studied. The temperatures of 20° C, and 20<=>30° C were compared and were used in combination with the following methods of top of paper (TP) and agar (A). Six laboratories analyzed three seed lots of *Pinus sylvestris* of varying germination and vigor levels. Statistical analyses of the data showed that the methods utilizing agar produced acceptable results of repeatability when compared to the other methods used for this study. It is therefore requested to add the option of agar as a media to the ISTA Rules for *Pinus sylvestris.*

Introduction

According to the USDA Forest Service and other knowledgeable sources, *Pinus sylvestris* is recognized as one of the most widely distributed and cultivated *Pinus sp.* in the world. Thus, this species is of significant global economic importance for the various products created from this crop.

Seed testing laboratories around the world are involved with determining the quality and value of numerous seed lots produced annually of this crop. For decades, the majority of laboratory germination tests conducted according to the ISTA Rules utilized paper as the primary germination test media of this species. Currently, the only germination test media option for *Pinus sylvestris* is top of paper (TP).

More recently, agar has been used as a germination test media in a few laboratories, mainly for in-house germination quality assessments. The ISTA Forest Tree and Shrub (FTS) TCOM was interested in validating agar as a germination test media for *Pinus sylvestris* and asked the ISTA Germination TCOM to collaborate with them in this study.

Materials and Methods

Seed Material

For this test plan, there were three commercial quality *Pinus sylvestris* seed lots of varying germination and vigor levels used. A total of 12 tests were conducted using all combinations of testing temperatures (i.e. 20° C and 20<=>30° C) and germination media (i.e. TP and agar). None of the three seed lots had been commercially treated prior to being used for the study. No dormancy breaking methods were used for this study.

Participating Laboratories

Six labs were selected to participate in this validation study, based upon their testing knowledge and experience with this species. These labs were located in Poland, Czech Republic, and the United States of America and most are ISTA accredited.

Germination Methods

All participating laboratories compared the proposed agar method with the current ISTA TP method. Test temperatures of 20° C and 20<=>30° C were used. All participants used 8 hours of light in every 24-hour cycle. For the alternating temperature testing portion, light was used during the highest temperature setting. All laboratories were encouraged to conduct seedling evaluation counts at 7-day intervals during the 21-day testing period. Evaluations were conducted using ISTA Seedling Type E Seedling Group A-1-2-3-2.

Participating labs were instructed to use agar powder with Chemical Assessment Number (CAS) 9002-18-0. The inclusion or additions of anti-microbial or nutritional additives to the agar were prohibited. The agar was prepared to a concentration between 0.7 and 1.0% and required to have a depth of no less than 3 mm in the container used to conduct germination tests. As with the TP method, participants were informed to conduct agar tests in a moisture proof sealable container to help mitigate moisture loss from the agar. The pure seed units were sown on top of paper (TP) as well as on top of agar.

Statistical Analyses

Statistical analyses was performed by Jean-Louis Laffont, PhD Chair of the ISTA Statistic Committee and Riad Baalbaki, PhD member of the AOSA/SCST Statistics Committee. Analysis was performed utilizing the R package ‘ISTAgermMV’ developed by the ISTA Statistics Committee.

Results and Discussion

The following data were suppressed from the data analysis. The 2 labs responsible for this data have documented that there was a test condition issue involving these germination tests. The details of this suppressed data are: Lab2 Lot3 Agar 20<=>30° C; Lab6 Lot2 TP 20° C; Lab6 Lot3 TP 20° C.

1. Data exploration with side-by-side boxplots

Results by lot verified that lots covered a wide range of quality, expressed as germination (Figure 1), and were also confirmed through analysis of variance results (Table 2), with a highly significant lot effect. The same trend was observed for the three lots over laboratories (Figure 2).

Fig. 1

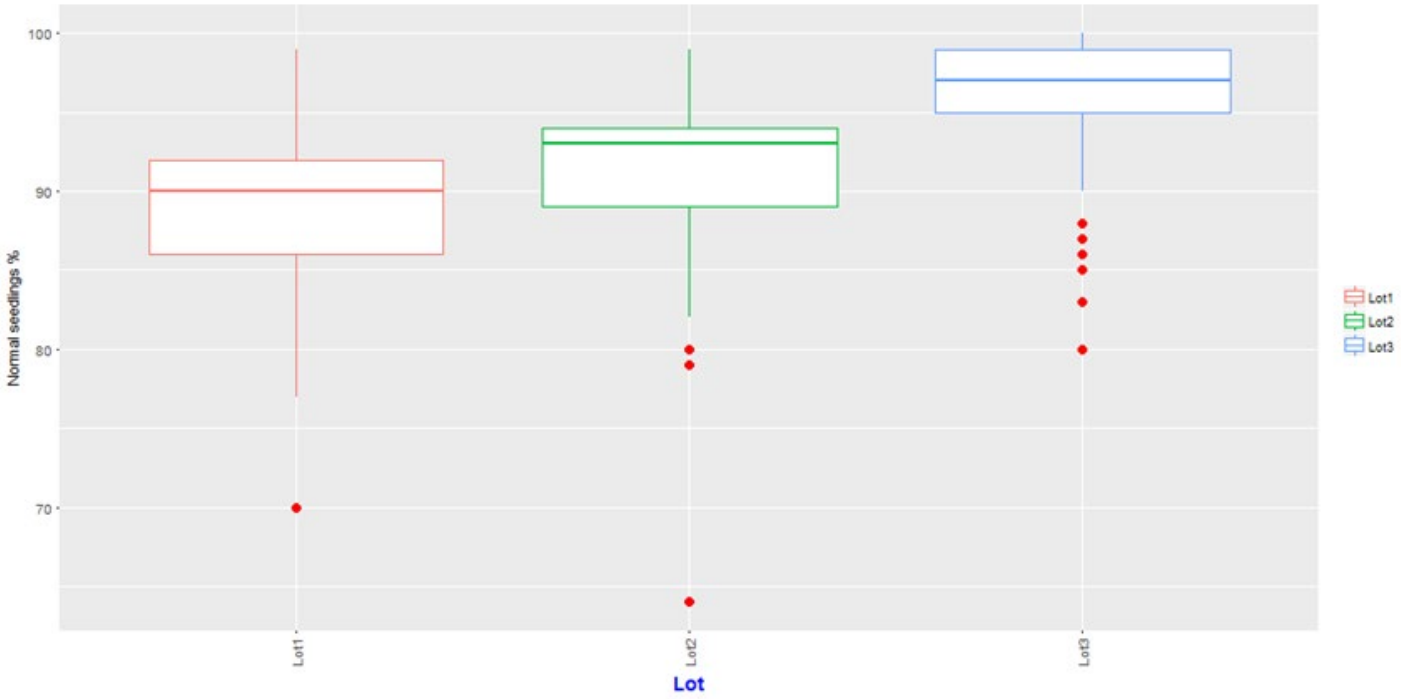
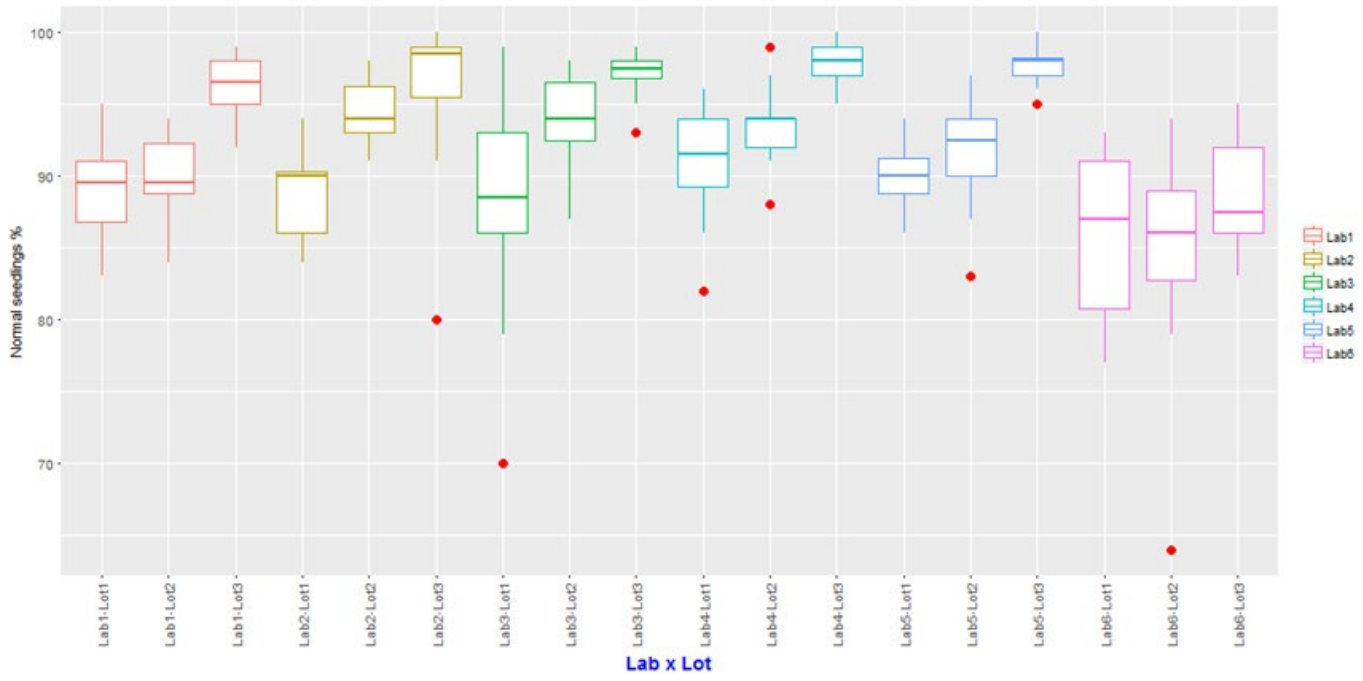


Fig. 2



2. Data Checking

Data checking has been performed according to ISTA Rules by computing tolerances for germination test replicates. Three test results out of 70 were out of tolerance for replications, but were included in the analysis (Table 1).

Table 1: Out of Tolerance Replicates

Method	Lot#	Lab#	Mean	# Reps	# seeds/rep	Range	Tol	Out of Tol
A 20-30 C	Lot3	Lab2	91	4	100	20	11	OUT
A 20 C	Lot1	Lab3	83	4	100	19	15	OUT
TP 20 C	Lot2	Lab6	79	4	100	24	16	OUT

3. Analysis of Variance

The lot main effect was highly significant ($F = 33.46$; $p < 0.001$) while the Method and the Method x Lot interaction effects were not significant (Table 2).Table 2: ANOVA

Source of variation	Sum of Squares	Mean Square	Num DF	Den DF	F value	Pr(>F)
Method	17.53	5.84	3	14.11	0.53	0.67
Lot	734.25	367.13	2	9.28	33.46	0.00
Method:Lot	66.50	11.08	6	25.97	1.01	0.44

4. Repeatability/Reproducibility

Table 3: Comparison

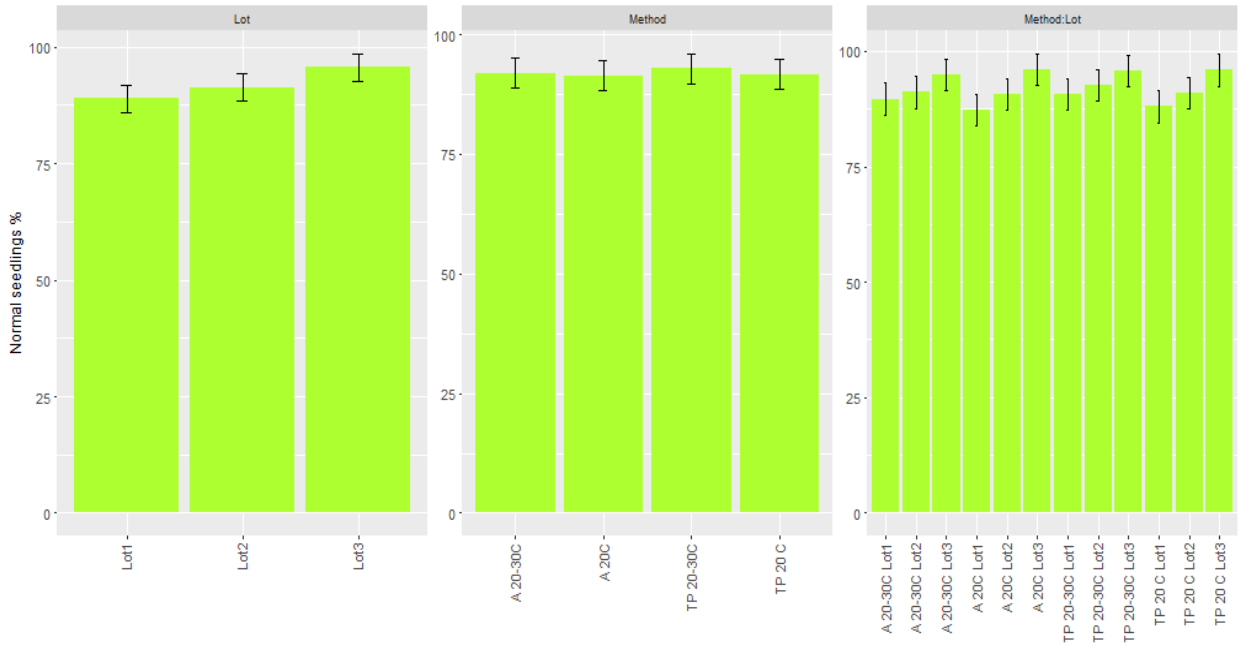
Method	Mean	s_repeatability	disp	s_Reproducibility	s_Lab	s_LotxLab
A 20-30C	92.00	3.46	1.28	4.29	0.00	2.53
A 20C	91.00	3.44	1.23	4.26	1.89	1.64
TP 20-30C	93.00	2.36	0.92	5.35	4.59	1.41
TP 20 C	92.00	3.81	1.39	5.49	3.95	0.00

At both temperature regimes, repeatability of agar methods was higher than TP 20-30 C, but lower than TP 20 C (Table 3). In contrast, reproducibility, i.e., variation among labs plus repeatability, of both agar methods was better (lower) than that of TP methods. Dispersion of almost all methods was higher than would be expected (Table 3) but still acceptable.

5. Method and Lot Means

A mixed model, considering Method and Lot as fixed effects and the Laboratory as a random effect, has been fitted to the data. The Least Squares Means are displayed in the following bar plot (Figure 3) with error bars added corresponding to their standard errors:

Fig. 3



Comparisons of LS mean for Lot and for Method x Lot showed that Lot 3, as a main affect or by method, had significantly lower germination than the other two lots (Fig. 3).

General Conclusions

1. ANOVA indicated that germination did not significantly differ among methods.
2. ANOVA indicated that the results from all methods were consistent, regardless of the lot germination capacity.
3. The repeatability standard-deviation, i.e. replicate variation, is the lowest for TP 20<=>30° C. The repeatability standard-deviations are lower for agar 20<=>30° C than for TP 20° C.
4. The reproducibility standard-deviations, i.e. lab to lab variation plus repeatability, are the lowest for agar.
5. It is recommended to add agar to the list of approved methods for *Pinus sylvestris* in the ISTA Rules.

Acknowledgements

Thank you to the staff of the Kostrzyca Forest Gene Bank in Mikow, Poland for providing the seed, obtaining the required phytosanitary certificates, and for packaging and shipping the study samples to the participating laboratories across the globe.

Thank you to the participating laboratories: Kostrzyca Forest Gene Bank Mikow, Poland; Forest and Game Management Research Institute, Czech Republic; Louisiana Dept. of Agriculture and Forestry,

Baton Rouge, LA, USA; Bayer CropScience Seed Technology Center, Waterman, IL, USA; SGS North America Brookings, SD, USA: USDA Forest

Service National Seed Laboratory, GA, USA. Their combined donated testing services exceeded a total of $2,200 USD.

Thank you to Nadine Ettel, ISTA TCOM Coordinator; ISTA Technical Reviewers Sylvie Ducournau GEVES-SNES France; Victor Vankus USDA Forest Service National Seed Laboratory, GA, USA; Elisa Serra Negra Vieira FTS TCOM Chair, Gillian Musgrove ISTA Germination TCOM Chair and the enthusiastic and supportive FTS TCOM and Germination TCOM members.

Thank you to Jean-Louis Laffont, PhD ISTA Statistics Committee Chair and Riad Baalbaki, PhD AOSA/SCST Statistical Committee member for their much needed and appreciated statistical support.

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1. International Seed Testing Association. 2019. ISTA International Rules for Seed Testing. Bassersdorf, Switzerland

Using methyl blue to stain *Ustilago nuda* hyphae in ISTA Methods 7-013a and 7-013b

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Abstract

To improve the visualisation of *Ustilago nuda* (C.N. Jensen) Kellerm. & Swingle (1890) hyphae within embryos of barley (*Hordeum vulgare* L.) extracted using ISTA seed health methods 7-013a and 7-013b by some laboratories, an optional stain using methyl blue was investigated. This was made as part of a proficiency test (PT) for these methods and a comparison of the PT no stain results with stain results was made. The inclusion of methyl blue stain in the test method of *U. nuda* in barley did not affect detection of this hyphae and this could help laboratories that have difficulty identifying hyphae in the scutellum. Keywords: *Hordeum vulgare*, Methyl blue stain, *Ustilago nuda*

Introduction

Results of the 2008 PT for *U. nuda* detection in barley (method 7-013) showed several laboratories were underestimating the number of infected embryos present in the samples (Cockerell & Roberts, 2011). Possibilities for underestimation of infected embryos include lack of training, experience and improper use of the method. Two laboratories commented on their difficulty in discriminating mycelium of *U. nuda* within the scutellum. Both laboratories suggested that staining of the mycelium might be an aid to identification. One laboratory went further and added a solution of lactic acid plus methyl blue to the extracted embryos from their PT samples and after re-examination found additional infected embryos. One of the conclusions from the 2008 PT was for the Seed Health Committee to consider optional staining to be included in Method 7-013. This was given further emphasis in comments from laboratories in the 2011 Seed Health Method Review (Seed Testing International, No. 144, 2012) with laboratories again requesting the inclusion of a staining step to improve detection.

Staining fungal hyphae has been used for many years and is necessary to detect *Ustilago tritici* (C. Bauhin) in wheat embryos, however this has not been necessary in barley due to the golden-brown mycelium being more visible.

The Seed Health Committee decided that the addition of an optional stain could be considered as a minor change, as use of a stain was unlikely to change the detection levels of *U. nuda* in barley. To support this consideration, it was decided to invite laboratories to take part in a small study. The study's aim was to provide data as proof that the addition of an optional staining method does not affect the detection rate and might improve detection for some laboratories. It was also decided to

include 7-013b in the comparative study. The results of the staining will be compared with results from PT15 SH *U. nuda*.

Materials and Method

Seed Lot and Samples

Samples were provided from the ISTA PT15 SH *U. nuda* (Cockerell & Roberts, 2016). Nine samples were provided in total for this staining comparative test. Three samples from each lot healthy (Nil), medium (0.5 to <1%) and high (>1%) infection were sent to each participating laboratory. For each sample the number of infected embryos found in 1000 embryos examined was recorded on the report sheet. In comparison, for PT15 participating laboratories were asked to test only one sample from each lot.

Laboratories

Thirteen laboratories took part in the study, eight of which are accredited for either method 7-013a or 7-013b.

Procedure

Method 7-013a

Laboratories were asked to carry out the analysis as prescribed in the current ISTA rules up to step 2.4. The embryos were then transferred to a beaker containing 50 ml of lactic acid solution with methyl blue (lactic acid 333ml + glycerol 333ml + tap water 333ml + 0. 16g methyl blue) and cleared by maintaining the lactic acid solution at boiling point for approximately 5 minutes in a fume cupboard. After this step the prescribed protocol was continued, and embryos examined as normal.

Method 7-013b

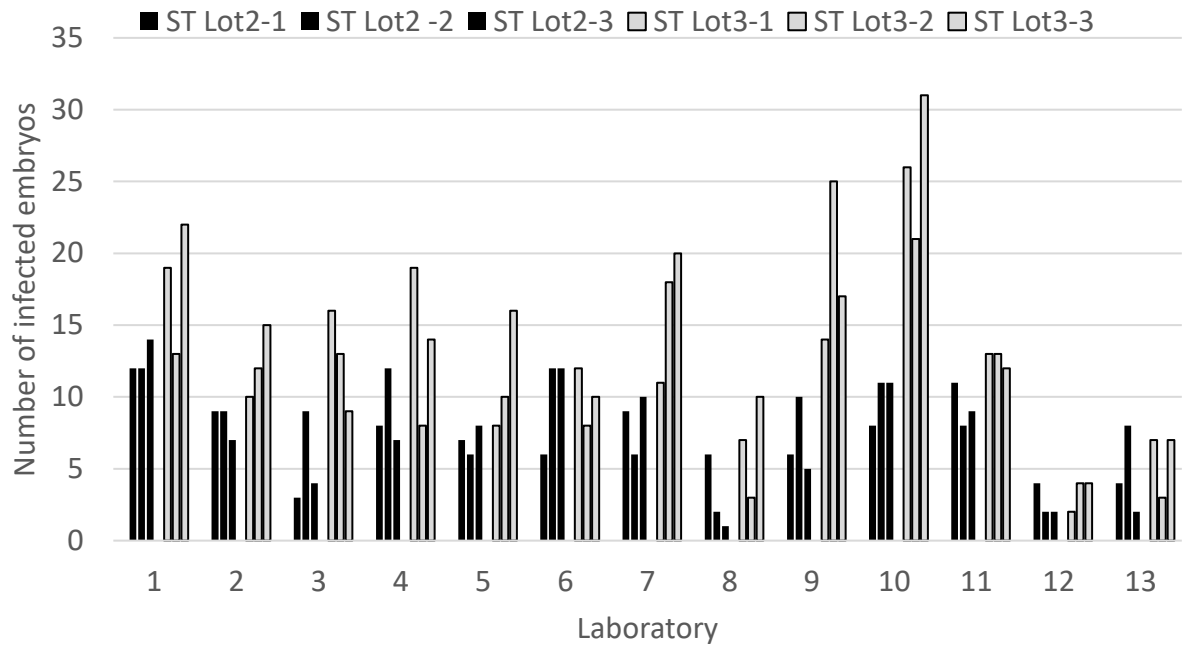
For this method laboratories were asked to carry out the analysis as prescribed in the current ISTA rules up to step 10, then replace normal lactic acid with a solution of lactic acid with 0.16g/L of methyl blue (lactic acid 1000ml + 0.16g methyl blue) and after this continue with the prescribed protocol and examine embryos as normal.

Results

Laboratories 8 and 10 recorded infected embryos in the healthy lot. Laboratory 8 recorded infected embryos in two of the three samples (1, 0, 2) and laboratory 10 in one of the samples tested (0, 1, 0) These are considered as false positives. Both laboratories recorded false positives in the healthy lot for ISTA PT15 SH (3 and 1 respectively). A summary of the results from the thirteen laboratories are given in figure 1 for lot 2 and lot 3. Overall results clearly showed that laboratories recorded a higher

infection in lot 3 (high infection) compared to lot 2 (medium infection), except for laboratories 6, 12 and 13.

Figure 1. Number of infected embryos found in 1000 embryos examined per sample for Lot 2 and Lot 3



The mean results of the three stained samples tested per lot for each laboratory were compared with the non-stained results from PT15 SH *U. nuda* using a t-Test. (Two-sample assuming equal variances, excel). One laboratory was excluded from the calculation as the laboratory had not followed the instructions for PT15 SH *U. nuda* (lab. 6). The t-test for both lot 2 and lot 3 showed no statistical significance ($p<0.05$) between the number of infected embryos found in the PT test and the stain test Table 1 and Table 2.

Table 1. Lot 2 t-Test: Two-Sample Assuming Equal Variances

	Lot 2 PT No stain	*Lot2Stain*
Mean	7.04	7.28
Variance	14.60	8.72
Observations	26	12
Pooled Variance	12.80	
Hypothesized Mean Difference	0	
df	36	

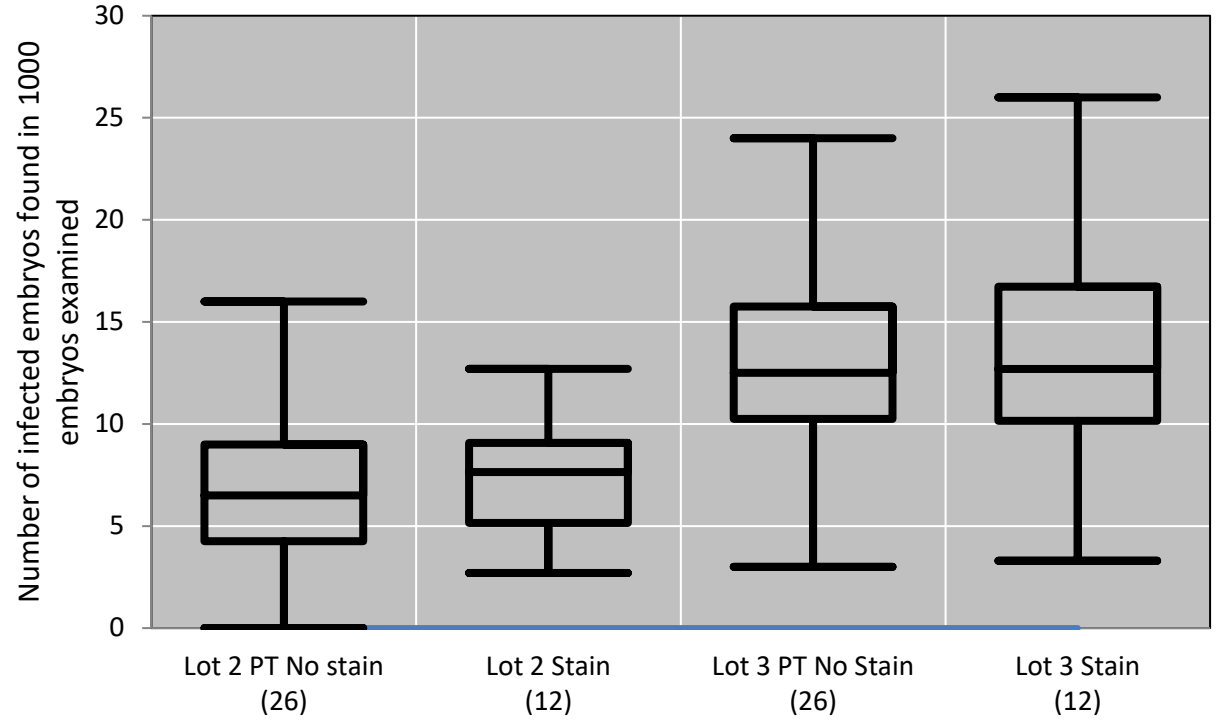
t Stat	-0.19
P(T<=t) two-tail	0.85
t Critical two-tail	2.03

Table 2. Lot 3 t-Test: Two-Sample Assuming Equal Variances

	Lot 3 PT No Stain	*Lot 3 Stain*
Mean	12.73	13.12
Variance	30.76	38.94
Observations	26	12
Pooled Variance	33.26	
Hypothesized Mean Difference	0	
df	36	
t Stat	-0.19	
P(T<=t) two-tail	0.85	
t Critical two-tail	2.03	

Figure 2 provides a comparison of the PT15 no stain results with stain results this study. Results for stained and unstained in both lots are similar. In lot 2 the range of upper and lower adjacent values is less for the stained and the median value slightly higher, 7.65 compared to 6.5 for the PT no stain. With the stain no laboratory recorded no infected embryos in lot 2. Laboratory 3 recorded no infected embryos in lot 2 during the PT but found infected embryos in all three samples tested during this study (Figure 1).

Figure 2. Comparison of No Stain (PT) and Stain methods for Lot 2 and Lot 3



Discussion

The inclusion of methyl blue stain for testing of *U. nuda* in barley does not affect the detection. From the limited data it does appear that it might help laboratories that have difficulty identifying hyphae in the scutellum.

It is recommended that the option of using methyl blue stain as described in 'Materials and Method' be included in Method 7-013a and 7-013b and considered as a minor change to the current method. It is also recommended that during future proficiency tests for these methods that data on the use of staining is requested to monitor any affect.

Acknowledgements

Thank you to the 13 laboratories that took the time and effort to help with this study and to the staff of the OSTS Scotland for preparing the samples.

References

Cockerell V. & Roberts A. 2011, Interlaboratory Proficiency Test for the detection of *Ustilago nuda* on *Hordeum vulgare. Seed Testing International*, International Seed Testing Association News Bulletin **No. 151** April 2016, Bassersdorf, Switzerland

Rennie W.J. 1982, Working sheet **No. 48**, Wheat, *Triticum aestivum* – Loose smut, *Ustilago nuda*. *ISTA Handbook on Seed Health Testing*, Zurich, Switzerland