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Document OGM17-07

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

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

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

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

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

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

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

Comparative test protocol

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

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

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

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

Statistical analysis

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

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

Data exploration with side-by-side boxplots

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

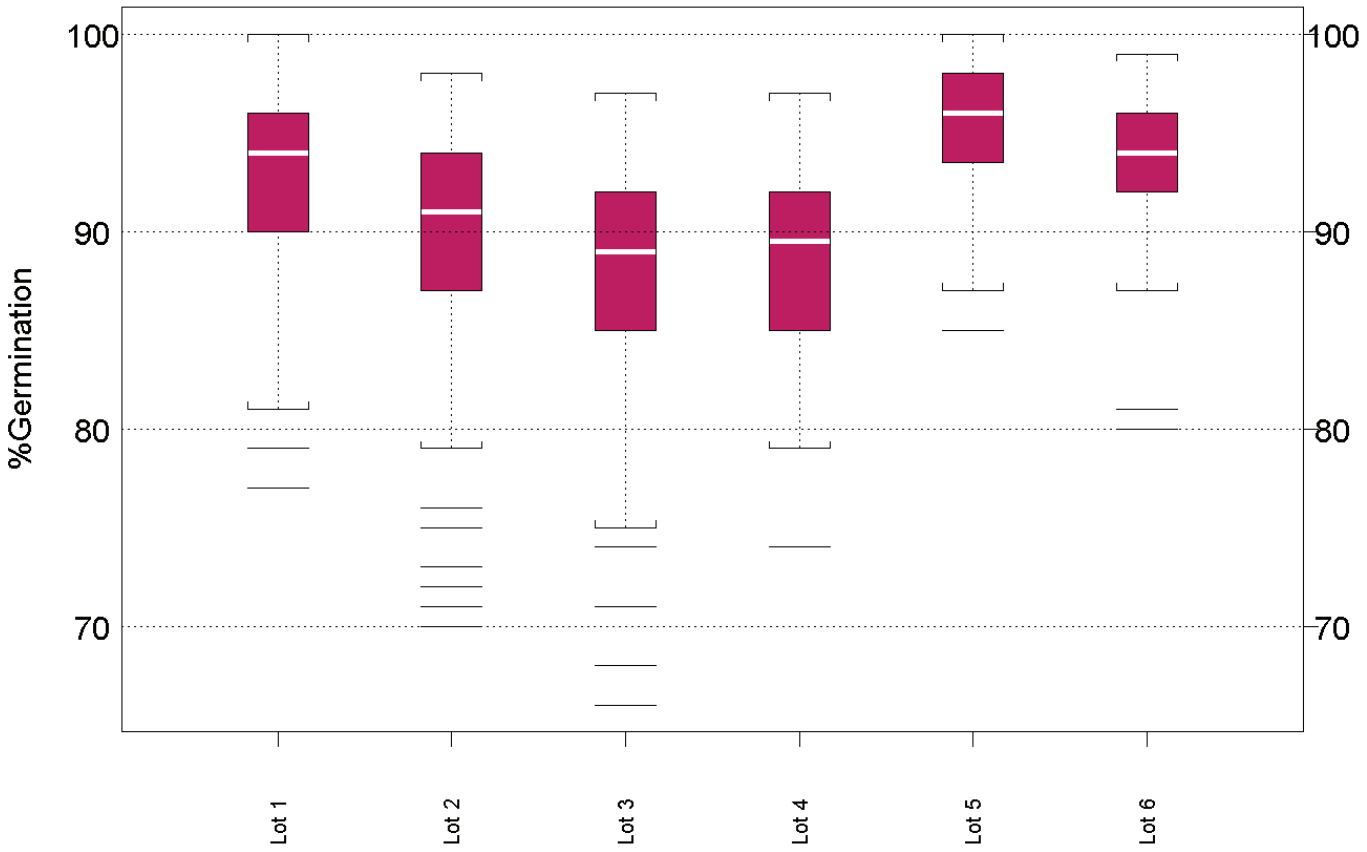


Figure 2: Boxplots for the six laboratories grouped across lots and methods.

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

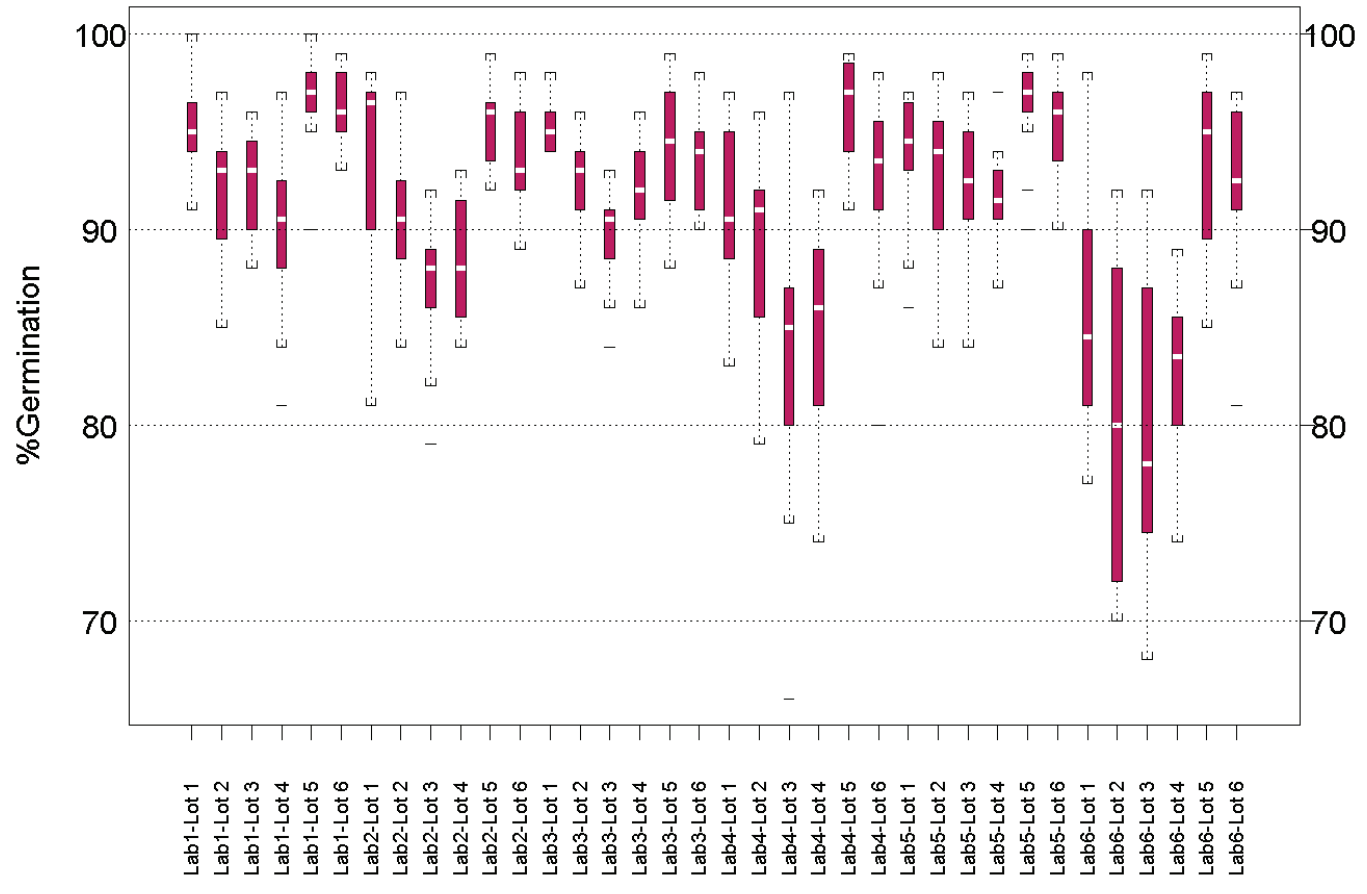


Figure 4: Boxplots for the methods grouped across lots and laboratories.

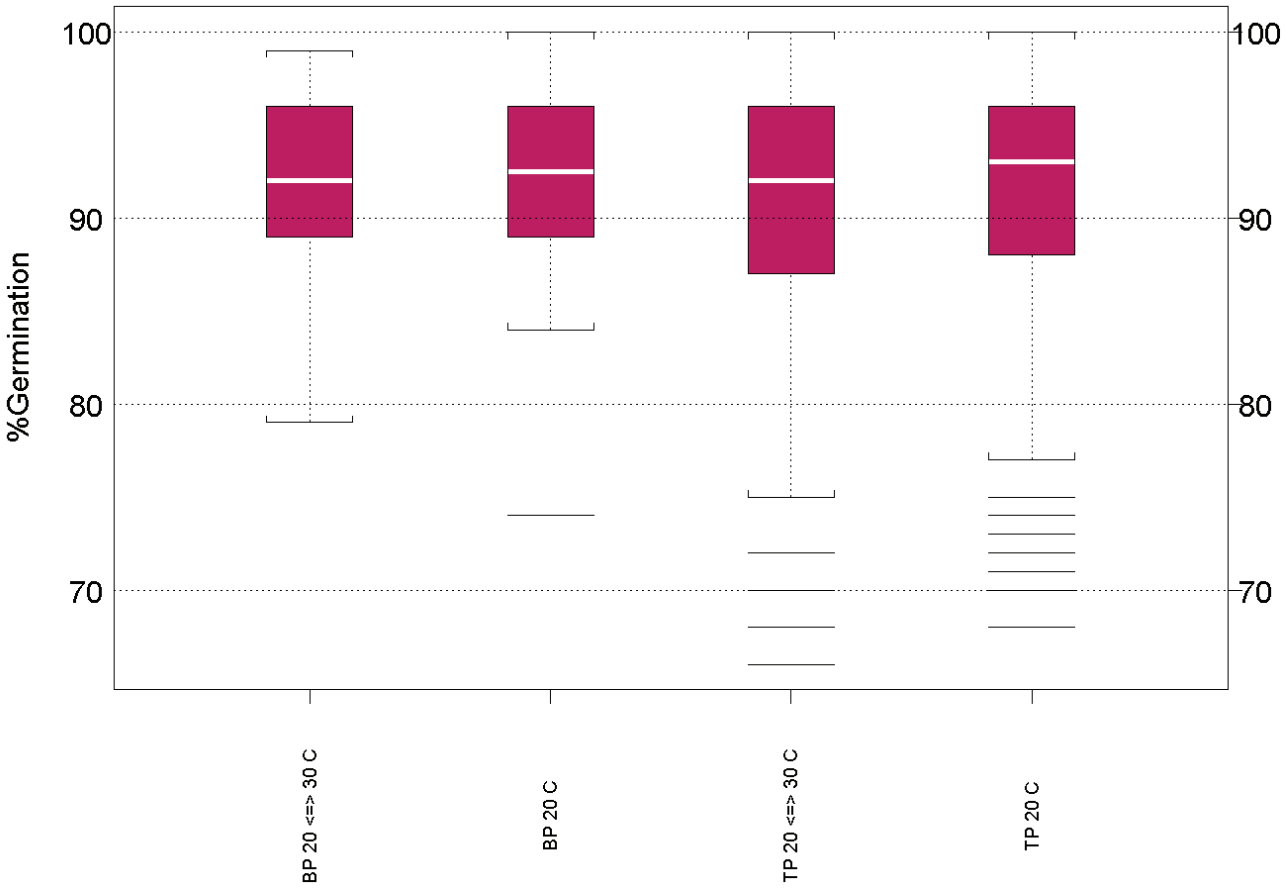
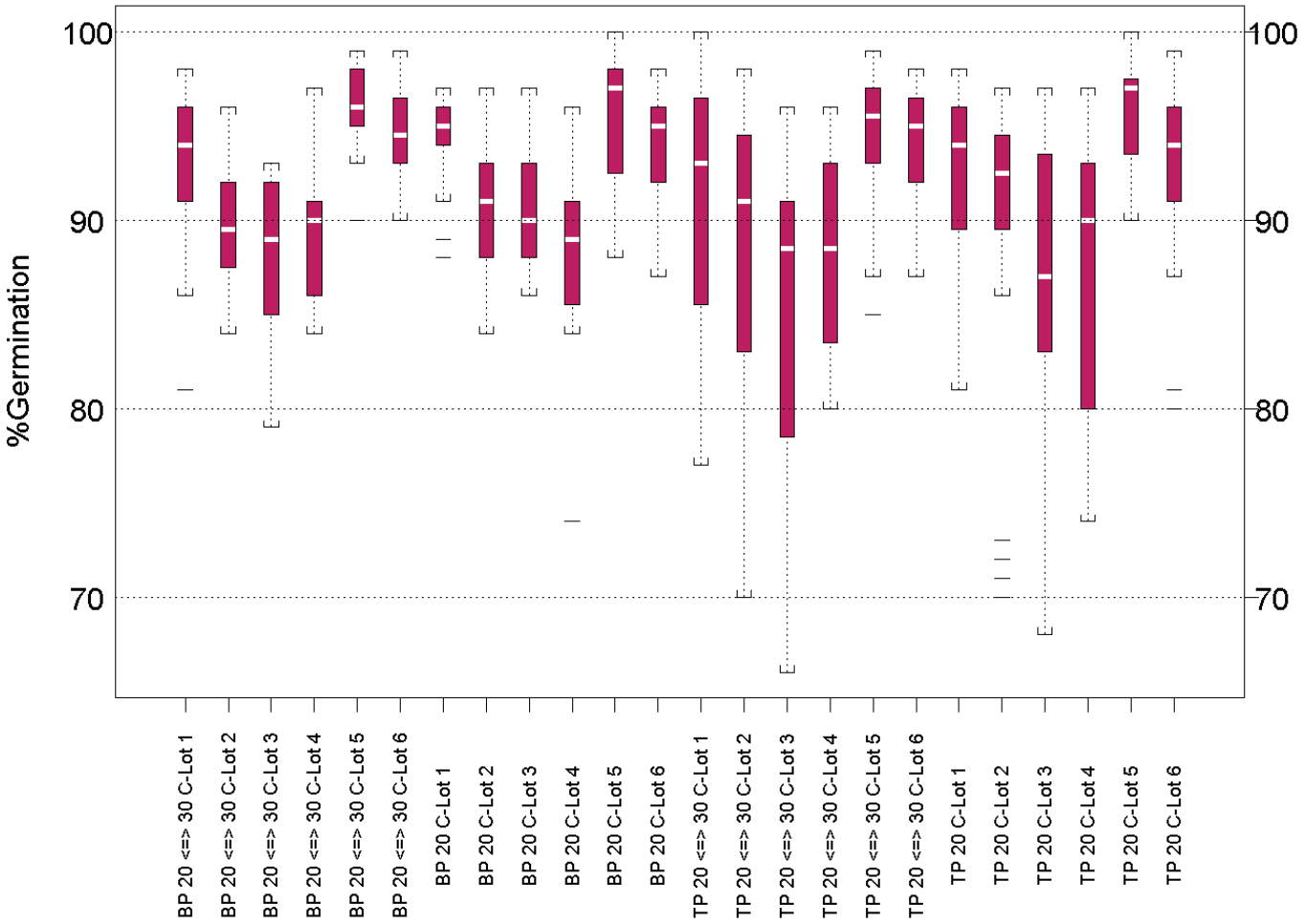


Figure 5: Boxplots for the methods x seedlots grouped across laboratories.



Results of data checking

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

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

Repeatability/Reproducibility

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

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

in which:

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

. μ is the intercept.

. α_i is the fixed effect of lot *i*.

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

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

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

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

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

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

Repeatability (S_r) results:

BP 20<=>30°C

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

BP 20°C

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

TP 20<=>30°C

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

TP 20°C

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

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

Reproducibility (S_R) results

BP 20<=>30°C

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

BP 20°C

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

TP 20<=>30°C

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

TP 20°C

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

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

Conclusions

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

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

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

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