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Proposal for a confirmation method of seed transmitted *Squash mosaic virus* (SqMV) on DAS-ELISA positive *Cucurbit* seeds

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Summary

A method for the confirmation of seed-transmitted SqMV on Cucurbit seed was developed by ISHI-Veg, ISF and validated in an international study between four laboratories. The method includes a greenhouse grow-out test - with seed of a lot having screened SqMV-positive in ISTA Rule 7-026 - and a DAS-ELISA confirmation of symptomatic plants at 3-4 true leaves stage. All participants detected and confirmed the seed transmitted SqMV in the positive seed samples supplied. SqMV infection was shown that it can be symptomless as pools of symptomless plants were SqMV-positive in the DAS-ELISA test. In case of negative confirmation or no symptoms' appearance the symptomless plants are DAS-ELISA confirmed as SqMV can be transmitted without symptoms expression. This method is an extension of the ISTA Rule 7-026 that verifies whether a SqMV-positive Cucurbit lot in ISTA Rule 7-026 will result in diseased seedlings. In this study, melon seeds were used. However, all Cucurbits could be evaluated in a greenhouse grow-out. Yet, the ELISA confirmation test on plant tissue samples should be evaluated and verified for the various cucurbits being evaluated in the grow-out test before accepting results.

Introduction

Squash mosaic virus (SqMV) belongs to the *Comovirus* genus (Bruening, 1978). It is pathogenic on several species of *Cucurbitaceae* family with melon being the principal host (Bruening, 1978; Freitag, 1956). It is a seed-borne virus, transmitted by seed, some species of beetles and mechanical inoculation (Freitag, 1956; Campbell, 1971; Alvarez and Campbell 1978; Nolan and Campbell, 1984). Seed transmission of SqMV and its influencing factors have been studied on seed of various Cucurbit species (Powell and Schegel, 1970; Alvarez and Campbell, 1978).

SqMV is located in the seed coat, in the papery layer and in the embryo of a seed. However, only the embryonic infection leads to virus transmission from seed to seedling (Alvarez and Campbell, 1978; Nolan and Campbell, 1984).

The actual reference method for the detection of SqMV in Cucurbit seed is described in ISTA Rule 7-026 which demonstrates the possibility of the simultaneous detection of CGMMV, MNSV and SqMV from a single extract of a ground seed sample (Koenraadt and Remeeus, 2009). The principle of this detection method lies on a ELISA test developed for plant viruses (Clark and Adams, 1977). The precision of a DAS-ELISA test in detecting SqMV melon infected seed in a given sample size has been demonstrated by Franken *et al.* (1990).

However, it is known that the DAS-ELISA test detects both infectious and non-infectious virus particles in a test sample and so it can lead to a false positive result (Nolan and Campbell 1984) and to an overestimation of the actual seed transmition level of the seed lot. In addition, using ground seeds as a tested sample, the DAS-ELISA test cannot give any information on the location of the virus in the seed, i.e. embryonic versus the seed coat. Thus, it cannot be known if an ELISA positive seed sample would result in diseased plants (Maury *et al.*, 1987; Koenraadt and Remeeus, 2009) and subsequently the virus transmission rate in the sample can be overestimated.

Nevertheless, DAS-ELISA tests can serve as a virus prescreening step (Hamilton and Nichols, 1978) of melon seed lots and its positive results can be further confirmed by a grow-out test with melon seeds from the same lot. Results of the grow-out will show whether the virus that is present in the lot, is alive/infectious and able to cause seed transmission (virus location in the embryo of the infected seeds) or not.

In the grow-out test, melon seeds are sown in a suitable substrate and are incubated in greenhouse conditions until emerged plants reach the growth stage of 3-4 true leaves when they are visually evaluated for SqMV symptoms (Powell and Shlegel 1970; Alvarez and Campbell, 1978; Nolan and Campbell, 1984). Symptoms are compared to symptoms developed on mechanically inoculated control plants following the inoculation method described by Alvarez and Campbell (1978). Tissue of plants showing typical and atypical SqMV symptoms is collected individually and tested by DAS-ELISA for confirmation of visual findings. The seed lot would be considered SqMV-positive as long as there is at least one positive result in the DAS-ELISA test. Preliminary tests have shown that SqMV can be

transmitted to plants without expressing any symptoms (H. Lybeert, HM-Clause SA, France, personal communication). Therefore, if no SqMV symptoms are observed on the plants in the grow-out, the tissue of symptomless plants is confirmed by DAS-ELISA test in pools of a maximum of 20 plant-tissue samples. The seed lot in this case would be considered SqMV-positive as long as there is at least one positive result in the DAS-ELISA test. The summarized procedure of the grow-out and DAS-ELISA confirmation tests application is presented in Appendix I.

Sensitivity of the grow-out method

The ability to detect SqMV could be influenced by variations in environmental conditions in the greenhouse. Therefore the recommended temperature and light should be respected and the grow-out test should not be performed during the winter period unless artificial light and heating can compensate for the lack of natural light and temperatures. If plants are damped off or other disease symptoms are present then the SqMV test should be considered invalid and redone.

The grow-out test method is suitable for untreated seed. It is also considered suitable for seed that has been treated using chemicals or physical processes with the aim of disinfestation/disinfection, as well as seed treated with protective chemicals or biological substances.

Aim and objective of the peer validation study

The aim of this ISHI-Veg peer validation study was to determine if the grow-out method is suitable and can be used by other laboratories to estimate the level of seed transmitted SqMV in the given seed lot.

The objective of this ISHI-Veg peer validation study is to provide an extension of the ISTA Rule 7-026 to verify whether a seed lot that has been found positive with the prescreening ELISA test on ground seed samples will result in diseased seedlings. This finding will allow for the determination of the actual seed transmission rate of such a seed lot.

In this peer validation study, four seed health laboratories from France, USA, The Netherlands and Israel participated.

Materials and Methods

Characterization of seed lots

Two melon (*Cucumis melo*) seed lots, with variable levels of SqMV natural contamination and one non-contaminated seed lot were selected by the laboratory of HM Clause in France. Prior to the peer validation study, the seed lots were characterized based on the results of a DAS-ELISA prescreening and a grow-out test. The DAS-ELISA prescreening test was performed on 20 subsamples of 100 seeds following the method described in the ISTA Rule 7-026. The grow-out test was performed on a 1,000 seeds sample size following the proposed method that determined the virus seed transmission rate (STR) and gave the final characterization in terms of contamination level in each seed lot. The details of the characterization of these 3 seedlots are given in Table 1.

The percentage of actual seed transmission rate (%STR) for the initial characterization of seed lots as well as in the grow-out test was calculated using the following formula which combines results of the grow-out and DAS-ELISA tests:

$$\%\mathrm{STR} = \frac{\mathrm{No.\ ELISA\ positive\ symptomatics} + \mathrm{No.\ ELISA\ positive\ symptomless}}{\mathrm{Total\ No.\ emerged\ plants}} * 100$$

where “symptomatics” are the plants with typical and the plants with atypical SqMV symptoms and “symptomless” are the plants that did not express any SqMV symptoms on their leaves.

The number of ELISA-positive symptomless plants in the ELISA-positive pools was calculated from the contamination rate that was obtained from the “Quality Impurity Estimation” sheet of SeedCalc8 (http://www.seedtest.org/en/statistical-tools-for-seed-testing-_content---1--1143--279.html). In order to obtain this rate, the number of pools that were tested by ELISA, their size (number of pooled plants) and the number of the pools that were found positive were entered in this sheet. If needed, a second calculation of the contamination rate was performed with a different size of pools to approach the actual total number of symptomless plants that were tested positive. The contamination rate corresponding to the actual number of tested symptomless plants was then revealed through intrapolation in a (x-y) graph showing (x = contamination rate (%), y = No. of symptomless tested plants). The number of ELISA-positive symptomless plants out of the total tested in pools was finally calculated in the “rule of three”. The overall characterization results of the three seed lots are

given in Table 1. The “healthy” and “medium” contaminated seed lots were untreated whereas the “low” contaminated seed lot was treated with Thiram fungicide.

Seed samples and subsamples

Participating laboratories received 1,000 seeds in total from each of the low and medium lots and 500 seeds from the healthy lot. Each of these samples was broken down to subsamples of 20 seeds resulting in 125 subsamples in total for each laboratory. This practice aimed to ensure the clear identity of the pools of the symptomless plants.

The subsamples were prepared in the SNES sampling department with the use of the rotary divider machine based on the thousand seed weight of the corresponding lot. Subsamples were coded and their correspondence to seed lots was known only to the test coordinator. However, the codes to subsamples within the same seed lot were given in continuous numbers and not randomly. This aimed to reduce the chances of cross contamination between melon plants of different lots as they can grow tall enough to bend and touch each other.

In addition, each laboratory received 2 extra subsamples of approximately 50 seeds from the healthy seed lot. The emerged seedlings of one subsample were mechanically inoculated and served as the positive control plants and the emerged seedlings of the second subsample remained untreated and served as negative control plants. The identity of these two subsamples was known to laboratories as they required special manipulation. The peer validation study was performed during the June to August period to ensure the recommended environmental conditions in the greenhouse although the participants were located in very different geographical locations.

Grow-out method description

i. Seed sowing and greenhouse incubation

Participating laboratories filled 125 plastic trays with well-watered potting soil. Each tray was labeled with the number of one of the received seed subsamples. Seeds of each subsample were then sown in approximately 2 cm depth into the corresponding tray and were covered with a thin layer of vermiculite. The seeds of the 50-seed subsamples that came from the healthy lot and were designated to serve as negative and positive control subsamples were

sown first followed by the rest of the subsamples. Laboratories changed gloves between each subsample during sowing.

Trays were placed in an insect-proof greenhouse to avoid transmission of virus by beetles. Adequate space was kept between the trays to reduce the chances of cross contamination between subsample plants. The greenhouse temperature was maintained at 24-30°C during the day and 16-22°C during the night until seedlings emergence. After this time and until the final reading the temperature was maintained at 24-35°C. After emergence the plants were inspected every 3-5 days without handling.

ii. Mechanical inoculation of positive control plants

When the 1st true leaf of each melon plant began to emerge, (approximately 10 days after sowing) laboratories prepared the virus inoculum as follows: the dehydrated SqMV-infected melon leaves (~ 1 g fresh weight) provided by the test organizer, were ground into 4 ml of virus extraction buffer (0.53 g Na_2HPO_4 $2H_2O$ and 0.2 g $(C_2H_5)_2NCSSNa$ $3H_2O$ in 100 ml distilled water) in a mortar with a pestle. After that 0.075g of carborundum powder were added and all ingredients were mixed well. The virus inoculum was placed on ice and the inoculation procedure was performed in a short time.

A drop of the virus inoculum was placed on the cotyledons surface of all plants of the positive control-labeled tray and was smeared with fingers. For this purpose, plastic gloves and/or finger tip gloves were used. Light pressure was applied while smearing to avoid damaging the leaf tissue. Cotyledons were rinsed with tap water 5 min after the inoculation and plants continued their greenhouse incubation with the rest.

iii. Collection of plant-tissue samples and DAS-ELISA confirmation

When the majority of plants reached the stage of 3-4 true leaves (approximately 18-24 days after sowing) plants were evaluated individually. Typical SqMV symptoms are the systemic mosaic or vein banding in leaves and sometimes the leaf deformation (ICTVdB Management, 2006). However, it is possible to observe atypical SqMV symptoms such as discoloration of leaves and development of spots on them (Lecoq *et al*., 1998).

Collection of plant-tissue samples started from the Negative Control plants, continued with the rest of subsample plants and ended with the plants of the Positive Control. Aseptic materials (*e.g*. scalpel, forceps, cork borer) and disposable gloves that were changed after a collection of each plant-tissue sample were used to avoid cross contamination between the collected plant-tissue samples.

For the plants of the Negative Control, a 1 cm^2 piece was cut from one of the youngest leaves of each plant. The pieces were placed in suitable containers (*e.g.* plastic extraction bags from BioReba) with a maximum of 10 pieces.

Within each subsample/tray, the collection started with the symptomless plants (plants that did not show any symptoms). As previous, a 1 cm^2 piece was cut from one of the youngest leaves of each plant and all pieces were pooled together in one suitable container (maximum of 20 symptomless plants). The collection continued with the atypical SqMV symptomatic plants, if present. These were collected individually, therefore each 1 cm^2 piece that was cut from the leaf of each plant was placed separately in a suitable container. Finally, typical SqMV symptomatic plants were collected individually, if present.

Plant-tissue samples from the Positive Control plants were collected following the same procedure for the Negative Control plants *viz.* a maximum of 10 plant-tissues was pooled in 1 container.

Plant-tissue samples were then ground in DAS-ELISA extraction buffer described in ISTA Rule 7-026 at a ratio of 1 g of plant-tissues in a 10 ml extraction buffer with the use of a suitable grinding device. Samples were stored at $4^{o}C$ and confirmed in DAS-ELISA described in ISTA Rule 7-026 the next day. Alternatively, laboratories stored the unground plant tissue samples at $4^{o}C$ and continued with the grinding, buffer addition and the DAS-ELISA confirmation step on the next day.

Due to the large number of plants for evaluation, the collection of plant-tissue samples and DAS-ELISA confirmation was done in three consecutive time periods. On day 24, plant-tissue from the 1-42 coded samples was collected, on day 26 plant-tissue from the 43-48 samples and on day 28 plant tissue from the 85-125 samples. Each collection day, a fresh plant-tissue sample of approximately 10 plants from each of the Negative and Positive Controls was collected.

Data analysis

For each subsample/tray, laboratories recorded the results of the grow-out and DAS-ELISA confirmation tests. The grow-out test results were the number of observed plants with typical SqMV symptoms and the number of observed plants with atypical SqMV symptoms out of the total emerged. The DAS-ELISA confirmation test results were: i) the number of SqMV-positive plants with typical and the number of SqMV-positive plants with atypical SqMV observed symptoms out of the total number tested and ii) the DAS-ELISA result on the pool

of symptomless plants. The subsample was considered SqMV positive when there was at least one positive DAS-ELISA result either from the symptomatic plants or the pools of symptomless..

The final result of the 1,000-seed tested sample per level was determined as follows: the sample was considered SqMV-positive if there was at least one positive result in the DAS-ELISA test (either from symptomatic or symptomless plant-tissue samples). If there was no positive result in DAS-ELISA test the seed sample was considered negative.

The norm NF EN ISO 16140 (AFNOR, 2003) was followed to evaluate the performance criteria - sensitivity, specificity and accuracy - for each contamination level using the final result of the 1,000-seed tested samples. This evaluation was performed by comparing the expected results of all laboratories with those obtained. The results were in the form of positive and negative agreements and deviations (Appendix II-B).

For each contamination level, concordance (reproducibility of qualitative data) was evaluated using the final result of the 1,000-seed tested samples in the method developed by Langton *et al*. (2002). For this evaluation, the definitions developed by Josefsen *et al*. (2004) were followed *viz*. "*the percentages of finding the same result positive or negative from two similar samples analysed in the same or different laboratories respectively and under standard repeatability conditions*".

Results and Discussion

All participating laboratories submitted their generated results in the provided data record sheet. The results of each laboratory are presented in Table 2 per contamination level.

In the samples of the healthy seed lot, laboratories 1 and 4 recorded zero plants with typical observed SqMV symptoms in the grow-out test, while laboratories 2 and 3 recorded 7 and 1 plants from this category, respectively. However, all plants from the healthy lot that were observed having SqMV typical symptoms were confirmed being SqMV-negative in the DAS-ELISA test. This probably reflects variation between laboratories in the interpretation of what is considered a SqMV typical symptom. Regarding the plants with atypical SqMV observed symptoms in the same lot, a variable number was recorded in all laboratories. All of these plants except one in laboratory 3, were confirmed being SqMV-negative in the DAS-ELISA test. Yet, the ELISA positive result in the healthy seed lot could be also attributed either to a cross contamination while processing the ELISA sample, or in the grow-out, or a

very low SqMV infection level that was not detected in the characterization test. Finally, the pools of symptomless plants in the healthy seed lot in all laboratories were confirmed being SqMV-negative in the DAS-ELISA test.

Laboratory 1, recorded one DAS-ELISA positive result on a Negative Control sample (raw data not shown) which was attributed by the laboratory to a cross-contamination while processing the ELISA sample as its well was near to the Positive Control's.

Regarding the low and medium contamination level lots, all laboratories recorded a variable number of plants with typical and atypical SqMV symptoms with laboratory 3 having the highest number. This laboratory didn't have previous experience with the grow-out method. Moreover, nutrient deficiency of plants was reported which made the observation of SqMV symptoms difficult. Laboratory 4 also reported nutrient deficiency of plants and plants with etiolating symptoms which implies that there was insufficient light in the greenhouse.

The highest number of SqMV-positive pools of symptomless plants was recorded by Laboratory 1 in both low and medium contaminated levels compared to the rest of laboratories. All of the participating laboratories were well versed in ELISA testing. Therefore, the variations seen are a result of ELISA methods between the laboratories. The most obvious and logical source of their between variation is the different antisera used in each laboratory. Antisera against viruses are available from various suppliers and it is known that their quality may differ. Differences are not so evident in the basic detection of the virus in medium and high levels of the virus but more in the levels of background. The differences in background could change the cut off levels and the ability to detect low levels of virus. Laboratory 1 used antisera provided by PRI, laboratory 2 by Envirologix and laboratories 3 and 4 by Prime Diagnostics. In addition, the quality of microtiter plates, incubation conditions and handling are factors that can have an impact on ELISA results. The interpretation of the DAS-ELISA response as positive or negative was done by each laboratory based on their equipment, software and threshold values instructed by the antisera supplier. Finally, as each laboratory used its own available grinding tools to grind the plant-tissue samples variation in tissue maceration might have been a factor.

Specificity and accuracy values of the Healthy level were less than 100% demonstrating that there was a false positive result obtained (Table 3). Nevertheless, for the Low and Medium levels sensitivity and accuracy values were 100% demonstrating that there was no false negative result (Table 3). Regarding the concordance value this was 100% for the two

positive levels but only 50% for the Healthy lot due to the false positive result obtained (Table 3).

Conclusions and Recommendations

All laboratories found the SqMV that was present in the positive seed lots showing that the proposed method is able to detect and confirm infectious SqMV in the seed lot.

In this study, it was shown that SqMV infection can be symptomless as pools of symptomless plants were SqMV-positive in the DAS-ELISA test.

Confirmation in a DAS-ELISA test of the visually observed symptoms on the plants is a necessary step as SqMV can express atypical or no symptoms. It is also necessary because it is possible to have plants infected with other seed-transmitted viruses whose symptoms may be confused with SqMV symptoms or plants with symptoms of nutrient deficiency.

Performing the grow-out in the recommended environmental conditions will ensure the SqMV multiplication and thereafter its detection in the plant tissue samples.

Use of appropriate precautions throughout the grow-out and DAS-ELISA tests will minimize or prevent the potential for cross contamination. Moreover, the use of negative control in the grow-out and ELISA tests is important to identify possible cross-contaminations.

In order to harmonise with the sample size of the prescreening method described in the ISTA Rule -7-026, a sample of 2,000 seeds of a lot should be tested in the grow-out.

In this study melon seeds were used. However, all cucurbits could be evaluated in a greenhouse grow-out and symptoms evaluated with no problem. Yet, the ELISA confirmation test on plant tissue samples should be evaluated and verified for the various cucurbits being evaluated in the grow-out test before accepting results.

The grow-out and DAS-ELISA confirmation of SqMV in a seed lot previously found SqMV-positive in the prescreening ISTA Rule 7-026 allows seed health laboratories to assess whether the seed lot will result in diseased plants.

Acknowledgements

The input of participating laboratories from France, The Netherlands, U.S.A. and Israel is greatly acknowledged. Special thanks to S. Gregoire for designing the experiment, to HM-Clause S.A. in France and USA for providing testing material and running initial tests and to GEVES-SNES for preparing the seed samples.

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Table 1. Characterization of the three melon seed lots used in the peer validation study.

Seed lots	Contamination level	DAS-ELISA on seeds (ISTA Rule 7-026)	DAS-ELISA on grow-out collected plants	Indicative Seed Transmission Rate (STR) %
1	Healthy	0+/20[1]	0+/0 symptomatics[2] and 0+/50 pools[3]	0%
2	Low	20+/20	2+/8 symptomatics and 2+/50 pools	0.43%
3	Medium	20+/20	5+/5 symptomatics and 3+/50 pools	0.91%

[1] X+/Y tested seed subsamples in DAS-ELISA.
[2] X+/Y tested plants with typical and plants with atypical SqMV symptoms in DAS-ELISA.
[3] X+/Y tested pools of symptomless plants in DAS-ELISA.

Table 2. DAS-ELISA results per contamination level in each laboratory.

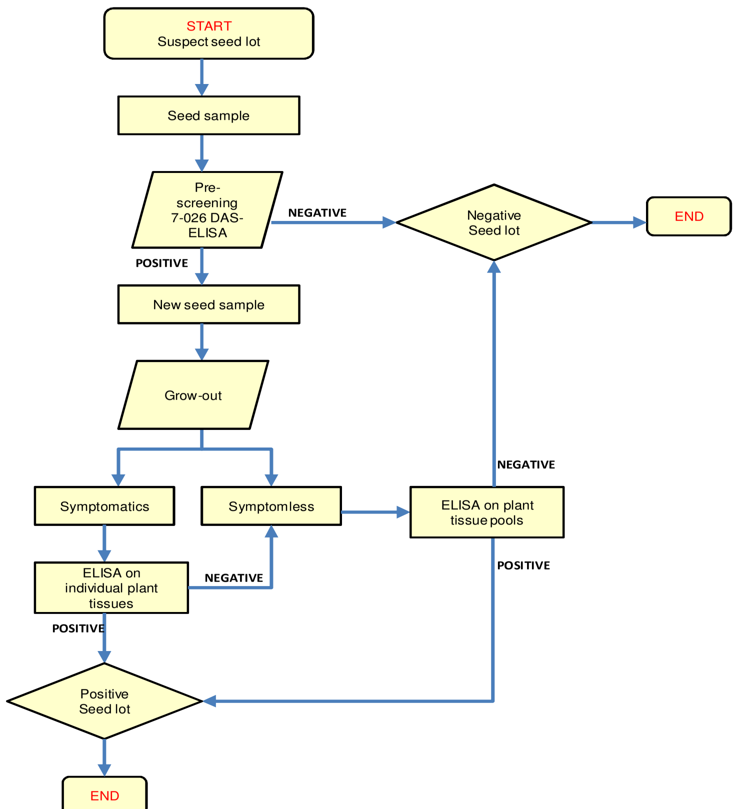
	Laboratories			
	1	2	3	4
Seed lots	DAS-ELISA on grow-out collected plants	DAS-ELISA on grow-out collected plants	DAS-ELISA on grow-out collected plants	DAS-ELISA on grow-out collected plants
Healthy	0+/0 typical [1]	0+/7 typical	0+/1 typical	0+/0 typical
	0+/1 atypical [2]	0+/44 atypical	1+/112 atypical	0+/9 atypical
	0+/25 pools [3]	0+/25 pools	0+/25 pools	0+/25 pools
Low	7+/7 typical	0+/2 typical	4+/6 typical	3+/3 typical
	1+/6 atypical	0+/14 atypical	1+/225 atypical	0+/41 atypical
	10+/50 pools	1+/50 pools	1+/50 pools	1+/50 pools
Medium	6+/6 typical	13+/14 typical	5+/6 typical	5+/6 typical
	1+/1 atypical	0+/16 atypical	2+/293 atypical	5+/61 atypical
	14+/50 pools	4+/50 pools	3+/50 pools	1+/50 pools

[1] X+/Y tested plants with SqMV typical symptoms in DAS-ELISA.
[2] X+/Y tested plants with SqMV atypical symptoms in DAS-ELISA.
[3] X+/Y tested pools of symptomless plants in DAS-ELISA.

Table 3 Statistical evaluation of final results.

Contamination Level	Sensitivity (%)	Specificity (%)	Accuracy (%)	Concordance (%)*
Healthy	N/A	75	75	50
Low	100	N/A	100	100
Medium	100	N/A	100	100

Appendix I: Flow chart showing the Grow-out and DAS-ELISA confirmation tests in practice.



Validation study re. Moisture Determination in Forest Tree Seed

Proposal to remove the requirement that seed of *Pinus* spp. with a thousand seed weight of greater than 200 g need to be cut before moisture determination and for tree species with a thousand seed weight of greater than 200 g to change the working sample amount of seed that needs to be used for moisture determination from five intact seeds to 5 g of seed.

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SUMMARY

The International Rules for Seed Testing currently require that seed of *Pinus* spp. with a thousand seed weight of greater than 200 g is cut before moisture determination; and that for tree seeds with a thousand seed weight of greater than 200 g, two replicates of five seeds are used for moisture determination. This validation study was undertaken with the aim of determining whether cutting was need for seed of *Pinus* spp. with a thousand seed weight of greater than 200 g (scope 1) and whether for tree seeds with a thousand seed weight of greater than 200 g 5 g of seed could be used for each working sample rather than five intact seeds (scope 2). The species used for scope 1 was *Pinus cembra* and for scope 2 *Quercus ilex*. For each scope two seed lots and three moisture levels were used. For scope 1 moisture was determined in whole and cut seed and for scope 2 moisture was determined was using an amount of seed equivalent to 5 intact seeds for each replicate and 5 g of seed for each working sample. Six laboratories participated in the validation study.

The results of the validation study showed that the methods for Scopes 1 and 2 are yielding comparable moisture results in terms of the moisture determined and the repeatability and reproducibility of the determination. This validation study therefore supports the proposal that for *Pinus* spp. where the TSW is greater than 200 g there is no need to cut the seed prior to moisture determination and that whole seed therefore be used for the moisture determination. And that for tree seeds where the TSW is greater than 200 g two working samples of 5 g be used rather than two replicates of an amount of seed equivalent to 5 intact seeds

AIM

The aims of this validation are to verify whether:

1. cutting is compulsory for the seeds of *Pinus* spp. when the TSW is > 200 g. (scope 1)
2. for tree seeds where TSW is > 200 g, the two working samples of 5 g (or 10 g) rather than 2 replicates of 5 seeds can be tested (scope 2).

MATERIALS AND METHODS

1. Species used

Seed of two species for which cutting is obligatory because the TSW is > 200 g) will be used for this validation: *Pinus cembra* and *Quercus ilex*.

Scope 1:

Pinus cembra will be used as the representative species for the *Pinus* genus to verify if cutting is compulsory for the seeds of this genus when the TSW is > 200 g.

Scope 2:

Quercus ilex will be used to determine if it is possible to test two working samples of 5 g (or 10 g) of seeds instead of 2 replicates of 5 seeds.

2. Participating Laboratories

Six laboratories from six countries participated in the validation

ITML0600 – contact Sergio Pasquini
CZDL0200 – contact Marta Dohnalová
GBDL01 – contact Jane Taylor
DEDL0400 – contact Andrea Jonitz
FRDL0200 – contact Céline Herbert
BEDL0200 – contact Anja Ritserveld

In this report to maintain anonymity each participating laboratory has been randomly assigned a number between 1 and 6.

3. Preparation of samples

Sample preparation for both scope 1 (*Pinus cembra*) and scope 2 (*Quercus ilex*) was the same. For each species two seed lots were used and for each seed lot three moisture levels were assessed; low moisture (*Pinus cembra*: 5 and 4%; *Quercus ilex*: 20 and 14%), the natural moisture of the seed (*Pinus cembra*: both 7%; *Quercus ilex*: 40 and 43%) and high moisture *Pinus cembra*: 19 and 10%; *Quercus ilex*: 40 and 48%). For each seed lot a total of 900 seeds was used. The 900 seed lot was split into three 300 seed sub lots, one for each moisture level. At the end of the moisture adjustment these sub lots were further subsamples to give a total of 60 smaller lots for each species to be distributed to the participating laboratories and for verification of moisture homogeneity:

a. 2 seed lots x 6 laboratories x 3 moistures = 36 lots for distribution to the laboratories

b. 2 seed lots x 3 moistures x 4 lots = 24 lots for verification of moisture homogeneity.

The homogeneity of the seed lots was assessed by determining the moisture of four randomly selected packets for each seed lot at each moisture level. For both scopes

both the current pre-drying sample preparation method and the proposed method was used to determine the moisture content.

The low and high moisture contents were achieved as follows:

a. On receipt the moisture content of each lot was determined using the low temperature oven method (17 hours at 103°C). The weight that the seed needed to reach either after drying or hydration to be at the required moisture was then calculated.

b. The seed lot was split into three sub lots of 300. The moisture content of one sublot was reduced to the required lower moisture content by drying the seeds in an oven operating at 40°C until the weight calculated to be equivalent to the required moisture content of was reached. When the required weight was reached the seeds were mixed thoroughly, a subsample taken to verify the moisture content had reached the predetermined percentage. The sub lot was then subsampled into lots of 30 seeds which were placed in 116 x 188 mm 12/20/50 micron laminated polyester/aluminium foil/polythene packets which were then heat sealed. The moisture content was verified using the low temperature oven method.

c. The second 300 seed sublot was placed in a room germinator at 20°C and 85-95% RH until the weight calculated to be equivalent to the required moisture content was reached. When the required weight was reached the seeds were mixed and subsampled as described for the low moisture treatment.

d. The moisture content of the third sub lot of 300 seeds was not adjusted. This is the "natural" moisture seed lot.

At the end of the sample preparation there were 60 packets of seed in total, comprising the following samples:

a. Ten packets of each seed lot to give a total of 20 packets at low moisture content.

b. Ten packets of each seed lot to give a total of 20 packets at natural moisture content.

c. Ten packets of each seed lot to give a total of 20 packets at high moisture content.

4. Scope 1 Testing Protocol followed by the Participating Laboratories:

The species used was *Pinus cembra.* Each laboratory determined the moisture content on the seed for each moisture level using two methods:

a. Applying the low temperature oven method, as described in Chapter 9 of the ISTA Rules (2012) with whole seeds used for the moisture determination and

b. Applying the low temperature oven method, as described in Chapter 9 of the ISTA Rules (2012) with cut seeds (cut in 4 pieces with scissors) used for the moisture determination.

Participating laboratories completed a results sheet sent by the organising laboratory. The detailed SOP for the validation study was included in the results sheet. Each laboratory was also asked to provide the SOP they followed for the validation and to enter their moisture data into the results sheets. The specific protocol followed by the laboratories is in Appendix One.

5. Scope 2 Testing Protocol followed by the Participating Laboratories

The species used was *Quercus ilex*. Each laboratory determined the moisture content on the seed for each moisture level using two methods:

a. Applying the low temperature oven method, as described in Chapter 9 of the ISTA Rules (2012) using the current method of taking ten intact seeds, cutting them, then mixing and subsampling two working samples for moisture determination approximately equal in weight to 5 intact seeds and

b. Applying the low temperature oven method, as described in Chapter 9 of the ISTA

Rules (2012) but cutting more than 10 g of seed and then taking two working

samples of 4.5g (± 0.5g) for the moisture determination. The specific protocol followed by the laboratories is in Appendix Two.

6. Statistical Analysis

This section of the validation report was prepared with Kirk Remund, vice-chair of the ISTA Statistical Committee

The moisture data submitted by the participating laboratories was analysed to assess the reproducibility/repeatability of the methods used in Scopes 1 and 2 and to assess if the moisture method means are statistically different at a 0.05 significance level for Scope 1 and 2.

RESULTS

1. Missing Laboratory

Laboratory 1 was removed from the statistical analysis for Scope 1 due to differences from the other laboratories.

2. Moisture determined

Scope 1: *Pinus cembra*

a. Moisture determined for confirmation of homogeneity

The seed lots were considered to be sufficiently homogeneous for the validation to proceed (Table 1 and 2).

Table 1: Moisture per cent determined for four lots (packets) of seed lot 1 for each moisture using both whole and cut seed. Individual data averaged over the duplicate working samples and the individual data averaged over the four samples lots is presented. Standard errors of the mean are given in brackets.

		Pinus cembra Seed lot 1				
Moisture (%)	Pre-drying seed treatment	Sample 1	Sample 2	Sample 3	Sample 4	Average
Low	Whole seed	5.3	5.5	5.3	5.6	5.4 (± 0.08)
	Cut seed	5.4	5.4	5.2	5.2	5.5 (± 0.05)
High	Whole seed	19.2	20.3	19.7	19.3	19.6 (± 0.26)
	Cut seed	18.9	19.0	18.0	19.3	18.8 (± 0.28)
Natural	Whole seed	7.0	7.1	7.1	7.0	7.0 (± 0.01)
	Cut seed	6.8	7.0	6.9	7.1	7.0 (± 0.08)

Table 2: Moisture per cent determined for four lots (packets) of seed lot 2 for each moisture using both whole and cut seed. Individual data averaged over the duplicate working samples and the individual data averaged over the four samples lots is presented. Standard errors of the mean are given in brackets.

		Pinus cembra Seed lot 2				
Moisture (%)	Pre-drying seed treatment	Sample 1	Sample 2	Sample 3	Sample 4	Average
Low	Whole seed	4.0	3.8	3.8	3.9	3.9 (± 0.04)
	Cut seed	4.0	4.1	4.0	3.9	4.0 (± 0.05)
High	Whole seed	10.0	9.9	10.1	10.1	10.0 (± 0.06)
	Cut seed	9.7	9.6	9.5	9.7	9.7 (± 0.05)
Natural	Whole seed	7.0	7.3	7.0	7.3	7.1 (± 0.08)
	Cut seed	7.0	7.2	6.7	7.6	7.1 (± 0.18)

b. Moisture determined by the participating laboratories.

There was no significant difference (t-test) in the moisture determined using cut seed versus whole seed for any moisture for either seed lot (Tables 3 and 4), except, the low moisture treatment for seed lot 2 (Table 4) where the moisture determined in cut seed was higher than that determined in whole seed.

Table 3: Moisture determined by the six laboratories for *Pinus cembra* seed lot 1 for each moisture using whole and cut seed. Data presented is the average of the moisture determined for the duplicate working samples. The average moisture determined by the six laboratories and with laboratory 1 removed is given.

		Pinus cembra Seed lot 1							
Moisture (%)	Pre-drying seed treatment	Laboratory 1	Laboratory 2	Laboratory 3	Laboratory 4	Laboratory 5	Laboratory 6	Average (Six Laboratories)	Average (Five Laboratories)
Low	Whole seed	7.5	5.6	5.6	5.6	5.8	5.7	6.0 (± 0.31)	5.7 (± 0.03)
	Cut seed	7.6	5.5	5.9	5.7	4.9	5.6	5.9 (± 0.37)	5.5 (± 0.17)
High	Whole seed	18.2	19.3	23,2	18.2	19.6	19.5	19.7 (± 0.75)	20.0 (± 0.85)
	Cut seed	17.2	17.8	21,3	19.5	19.2	19.0	19.0 (± 0.58)	19.4 (± 0.57)
Natural	Whole seed	8.0	7.2	7,1	6.9	7.0	7.3	7.2 (± 0.16)	7.1 (± 0.08)
	Cut seed	7.9	6.8	7,1	6.9	7.9	7.1	7.3 (± 0. 19)	7.2 (± 0.19)

Table 4: Moisture determined by the six laboratories for *Pinus cembra* seed lot 2 for each moisture using whole and cut seed. Data presented is the average of the moisture determined for the duplicate working samples. The average moisture determined by the six laboratories and with laboratory 1 removed is given.

		Pinus cembra Seed lot 2							
Moisture (%)	Pre-drying seed treatment	Laboratory 1	Laboratory 2	Laboratory 3	Laboratory 4	Laboratory 5	Laboratory 6	Average (Six Laboratories)	Average (Five Laboratories)
Low	Whole seed	6.8	3.9	4,0	3,9	3.7	4.1	4.4 (± 0.49)	3.9 (± 0.05)
	Cut seed	6.7	4.1	4.2	4,2	4.3	4.3	4.6 (± 0.41)	4.2 (± 0.03)
High	Whole seed	10.2	10.0	10,2	9.9	10.3	9.9	10.1 (± 0.07)	10.1 (± 0.09)
	Cut seed	9.7	9.4	10,0	11,6	9.9	9.5	10.0 (± 0.33)	10.1 (± 0.40)
Natural	Whole seed	8.9	7.2	7.2	7.2	7.8	7.3	7.6 (± 0.28)	7.3 (± 0.12)
	Cut seed	8.6	7.2	7.4	7.3	7.2	7.0	7.4 (± 0.23)	7.2 (± 0.07)

Scope 2: *Quercus ilex*

a. Moisture determined for confirmation of homogeneity.

The seed lots were considered to be sufficiently homogeneous for the validation to proceed (Table 5 and 6).

Table 5: Moisture per cent determined for four lots (packets) of seed lot 1, at each moisture level, using both weighed and counted seed. Individual data averaged over the duplicate working samples and the individual data averaged over the four samples lots is presented. Standard errors of the mean are given in brackets.

		Quercus ilex Seed lot 1				
Moisture (%)	Amount of seed used determined by	Sample 1	Sample 2	Sample 3	Sample 4	Average
Low	seed weight	23.3	18.4	21.0	22.0	21.2 (± 1.03)
	seed number	21.0	19.8	21.5	21.8	21.0 (± 0.44)
High	seed weight	38.9	40.6	39.6	41.5	40.1 (± 0.57)
	seed number	37.6	40.2	38.2	37.8	38.4 (± 0.60)
Natural	seed weight	42.2	40.3	40.0	45.2	41.9 (± 1.18)
	seed number	40.8	41.2	41.6	41.5	41.3 (± 0.17)

Table 6: Moisture per cent determined for four lots (packets) of seed lot 2, at each moisture level, using both weighed and counted seed. Individual data averaged over the duplicate working samples and the individual data averaged over the four samples lots is presented. Standard errors of the mean are given in brackets.

		Quercus ilex Seed lot 1				
Moisture (%)	Amount of seed used determined by	Sample 1	Sample 2	Sample 3	Sample 4	Average
Low	seed weight	15.3	14.1	13.0	14.9	14.3 (± 0.50)
	seed number	15.2	13.9	13.4	14.3	14.2 (± 0.40)
High	seed weight	51.6	43.4	46.4	43.1	46.1 (± 1.97)
	seed number	56.0	43.2	46.4	43.1	47.4 (± 2.93)
Natural	seed weight	44.2	47.9	45.7	45.3	45.8 (± 0.77)
	seed number	41.7	47.9	45.7	45.3	44.2 (± 1.21)

b. Moisture determined by the participating laboratories.

There was no significant difference (t-test) in the moisture determined when the amount of seed used was determined by weight rather than by number (Tables 7 and 8).

Table 7: Moisture determined by the six laboratories for *Quercus ilex* seed lot 1 for each moisture using weighed and counted seed. Data presented is the average of the moisture determined for the duplicate working samples. The average moisture determined by the six laboratories and with laboratory 1 removed is given.

		Quercus ilex Seed lot 1							
Moisture (%)	Pre-drying seed treatment	Laboratory 1	Laboratory 2	Laboratory 3	Laboratory 4	Laboratory 5	Laboratory 6	Average (Six Laboratories)	Average (Five Laboratories)
Low	seed weight	40.8	42.5	40.5	43.3	39.6	41,3	41.3 (± 0.55)	41.4 (± 0.66)
	seed number	40.5	44.2	42.4	42.0	41.9	40,2	41.9 (± 0.59)	42.2 (± 0.64)
High	seed weight	22.8	22.2	20.4	21.1	22.2	22.8	21.9 (± 0.39)	21.7 (± 0.43)
	seed number	22.5	22.0	23.0	20.1	22.0	21,7	21.9 (± 0.40)	21.8 (± 0.47)
Natural	seed weight	38.4	39.6	41.7	39.5	39.4	39.4	39.7 (± 0.44)	39.9 (± 0.45)
	seed number	39.3	40.2	41.4	38.9	39.8	39.8	39.8 (± 0.37)	39.9 (± 0.44)

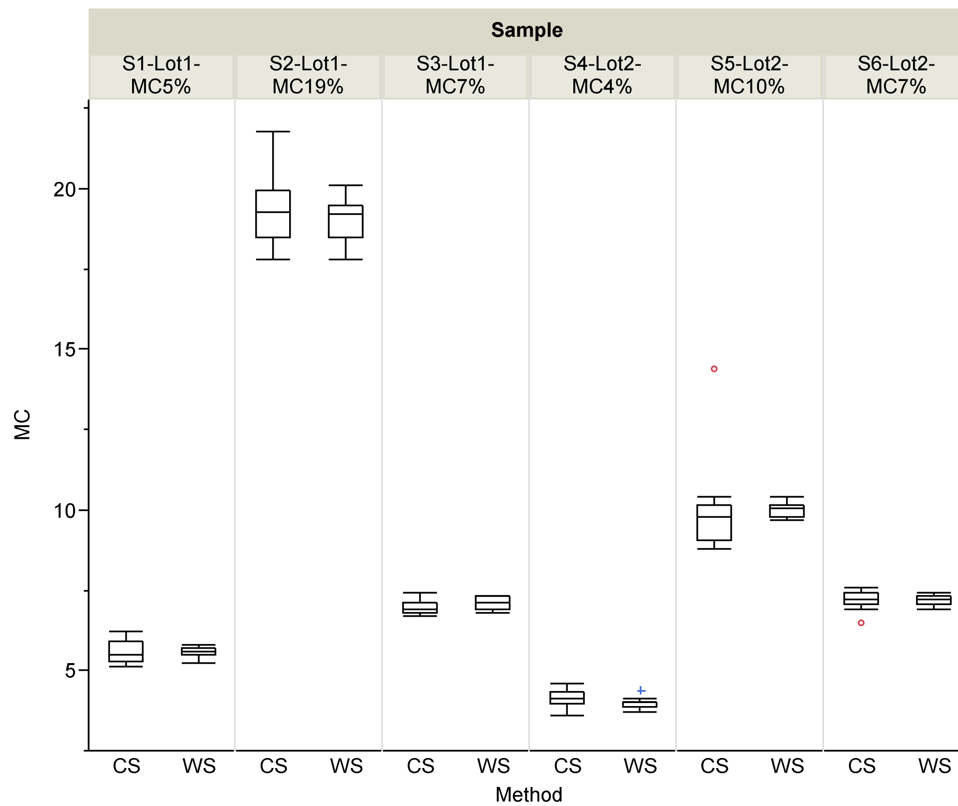
Table 8: Moisture determined by the six laboratories for *Quercus ilex* seed lot 2 for each moisture using weighed and counted seed. Data presented is the average of the moisture determined for the duplicate working samples. The average moisture determined by the six laboratories and with laboratory 1 removed is given.

		Quercus ilex Seed lot 2							
Moisture (%)	Pre-drying seed treatment	Laboratory 1	Laboratory 2	Laboratory 3	Laboratory 4	Laboratory 5	Laboratory 6	Average (Six Laboratories)	Average (Five Laboratories)
Low	seed weight	46.8	47.1	43.9	42.4	46.0	46.3	45.4 (± 0.75)	45.1 (± 0.87)
	seed number	43.3	45.1	44.4	44.8	43.1	42.8	43.9 (± 0.39)	44.0 (± 0.45)
High	seed weight	15.2	14.6	15.7	15.1	13.1	14.0	14.6 (± 0.38)	14.5 (± 0.45)
	seed number	15.2	14.5	15.5	14.8	12.6	14.3	14.5 (± 0.41)	14.3 (± 0.47)
Natural	seed weight	47.0	43.3	44.9	42.5	47.2	42.7	44.6 (± 0.87)	44.1 (± 0.88)
	seed number	38.9	41.9	41.1	43.3	44.6	38.9	41.4 (± 0.94)	42.0 (± 0.97)

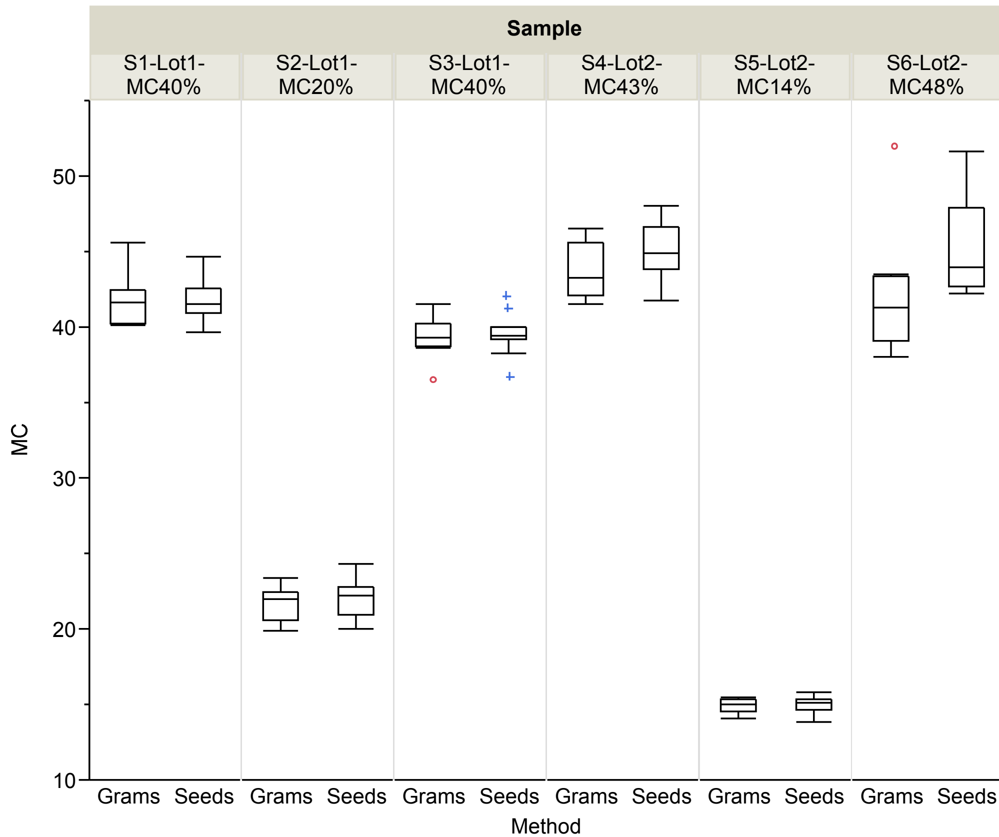
2. Box Plots

The box plots below for Scope 1 and 2 show visual agreement between the methods used.

Scope 1 Box-Plots by Sample and Method (Laboratory 1 Removed)



Scope 2 Box-Plots by Sample and Method



Note that the samples 1 through 6 for each scope are three samples selected from each of two lots at differing moisture levels.

3. Analysis of the Repeatability and Reproducibility of the Methods

Table 9 contains the estimates of repeatability and reproducibility for each method within each scope. These estimates are functions of the mixed model that was fit to the data. While a formal test of differences in repeatability and reproducibility between methods in not presently available for ISTA calculations the observed differences between methods repeatability and reproducibility in the table below are judged to be acceptable for identifying if differences exist.

Table 9: Estimates of the repeatability and reproducibility between using cut seed and whole seed for Scope 1 (*Pinus cembra*) and determining the amount of seed used for the moisture determination by weight and by number for Scope 2 (*Quercus ilex*).

Method	Scope	Random Effect	Variance Comp.	% of Total	Repeatability	Reproducibility
cut seed	1	Lab	0.07	17	0.15	0.40
		Lab*Sample	0.18	45		
		Residual	0.15	38		
whole seed	1	Lab	0.01	10	0.07	0.09
		Lab*Sample	0.02	18		
		Residual	0.07	72		
by weight	2	Lab	0.51	23	1.10	2.24
		Lab*Sample	0.64	28		
		Residual	1.10	49		
by number	2	Lab	0.00	0	0.83	3.01
		Lab*Sample	3.01	78		
		Residual	0.83	22		

Table 10 gives the repeatability estimates for each laboratory (for Scope 1 laboratory 1 is included for the calculations).

Table 10: Estimate of the repeatability for each laboratory for both scopes (1 - *Pinus cembra* and 2 - *Quercus ilex*).

Scope	Laboratory	Method	Repeatability
1	1	cut seed	0.09
	2	cut seed	0.21
	3	cut seed	0.18
	4	cut seed	0.10
	5	cut seed	0.09
	6	cut seed	0.17
	1	whole seed	0.55
	2	whole seed	0.04
	3	whole seed	0.20
	4	whole seed	0.01
	5	whole seed	0.01
	6	whole seed	0.07
2	1	by weight	0.78
	2	by weight	0.95
	3	by weight	0.47
	4	by weight	2.32
	5	by weight	1.38
	6	by weight	0.67
	1	by number	2.51
	2	by number	0.84
	3	by number	0.16
	4	by number	0.16
	5	by number	0.45
	6	by number	0.86

A model with a fixed method effect was used to assess whether the moisture determined by the two methods within each scope differ statistically. The experimental unit for these comparisons are the samples by laboratory (i.e. each sample bag). For Scope 1, there was no significant difference between method means (p-value=0.543) with an estimated 0.9% difference in between method moisture percentages. For Scope 2 there was also no significant difference between method means (p-value=0.081) with an estimated 2% difference between moisture percentages.

Based on these validation data and associated statistical analysis results, the methods for Scopes 1 and 2 are yielding comparable moisture results in terms of mean and variance (repeatability/reproducibility).

Discussion and Recommendations

The results of this validation study after statistical analysis have demonstrated that for both scopes there is no significant difference between the current method of sample preparation (i.e. cutting for seed of *Pinus* spp. with a TSW of > 200 g for scope 1 and using two replicates of five seeds for scope 2) and the proposed methods of testing whole seed of *Pinus* spp. if the TSW is > 200 g and for any species where the TSW is > 200 g using 5 g of seed rather than 5 seeds for each replicate. The statistical analysis also showed that the results obtained had comparable variance i.e. the same repeatability and reproducibility.

As a result the recommendation for Scope 1 is that for *Pinus* spp. where the TSW is > 200 g there is no need to cut the seed prior to moisture determination and that whole seed therefore be used for the moisture determination. While the *Pinus cembra* seeds are easy to cut with a scalpel on a board and the mixing and weighing was accomplished within the three minute time limit permitted removed of the cutting step will speed up the moisture determination and reduce apotential source of error (the cutting step) from the moisture determination procees.

Similarly the recommendation for scope 2 is that for tree seeds where the TSW is greater than 200 g two working samples of 5 g be used rather than two replicates of an amount of seed equivalent to 5 intact seeds. For very large seeds such as *Quercus ilex* using 5 g of seed has several advantages including that this seed weight will fit into a moisture container with a diameter of greater than 5cm and less than 8cm, whereas 5 seeds of *Quercus ilex* equates to 10-15 grams of seed and is therefore outside the amount permitted for containers with a diameter of greater than 5cm and less than 8cm, and in some cases also for the largest weight of seed permitted of 10.0 (± 1.0) g (in containers greater than 8cm in diameter). Moreover judging five seed fractions from the cut seed can be time-consuming, and lengthens the time the seed is exposed to the atmosphere before moisture determintion. The proposed change will speed up the moisture determination in the laboratory.

Acknowledgements

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Appendix One: Scope 1 detailed protocol

The detailed protocol followed by the participating laboratories to determine if cutting is needed for *Pinus* spp. where the TSW is > 200 g was:

1. Open the sealed bag.
2. Mix with accuracy the seeds in a beaker.
3. Take randomly 5 intact seeds and note the weight of the first replicate.
4. Take randomly 5 intact seeds and note the weight of the second replicate.
5 The total time requested from the opening of the bag to weighing the two replicates should be no more than 3 minutes for each sample.

SOP for cutting the seeds into pieces*

1. Open the sealed bag.
2. Cut 10 seeds into 4 pieces, first longitudinally then transversally.
3. Mix with accuracy the seeds in a beaker.
4. Take with a spoon as materials as necessary for reaching the total amount of about 5 intact seed for the first replicate.
5. Take with a spoon as materials as necessary for reaching the total amount of about 5 intact seed for the second replicate.
6. The total time requested from the opening of the bag to weighing the two replicates should be no more than 3 minutes for each sample.

Appendix Two: Scope 2 detailed protocol

The detailed protocol followed by the participating laboratories to determine if for tree seeds where the TSW is > 200 g it is possible to test two working samples of 5 g (or 10 g) of seeds instead of 2 replicates of 5 seeds was:

1. Weigh the 4 empty moisture containers.
2. Open the sealed bag.
3. Quickly take 10 seeds randomly from the bag.
4. Reseal the bag with scotch-tape.
5. Cut the seeds in 4 pieces, first longitudinally then transversally.
6. Mix with accuracy the seeds in a beaker.
7. Quickly take 2 sub-samples of pieces approximately equal to 5 intact seeds and place in 2 moisture containers.
8. Weigh the seeds in containers.
9. The total time requested from the opening of the bag to weighing the two replicates should be not more than 3 minutes for each sample.
10. Re-open the seal bag.
11. Quickly weigh out 10 g of seed.
12. Cut the seeds in 4 pieces, first longitudinally then transversally.
13. Mix with accuracy the seeds in a beaker.
14. Take at least three spoon full of cut material to reach the weight of the first replicate (4.5 grams) and place in a moisture container – repeat one more time.
15. Weigh the seeds in containers.
16. The total time requested from the opening of the bag to weighing the two replicates should be not more than 3 minutes for each sample.