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Pressure — time dependency of vacuum degassing as a rapid method for viability assessment using tetrazolium chloride: a comparative study of 17 *Pinus* species

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Summary

Vacuum degassing of seeds in tetrazolium chloride (TZ) solution has been proposed as an alternative and rapid method for assessing pine seed viability. In this study, we determined the effects of both suction level and the subsequent period of stain development at 30°C, on the viability estimates for *Pinus caribaea* and *P. pinea.* Our results demonstrated that three, 10 min cycles of degassing increased viability estimates, with the highest suction level we applied (96 kPa) being the most effective. In addition, at 96 kPa of suction the subsequent period of stain development could be reduced to 3 h whilst still retaining an equivalent estimate of viability to both a standard ISTA TZ test (18 h at 30°C) and germination level. Subsequently, for a further 17 *Pinus* seed lots, the estimate of viability from three cycles of degassing at 96 kPa followed by 3 h of stain development, was compared with both an 18 h ISTA test and germination. These results indicated that compared to germination levels. degassing provided an equivalent or improved estimate of viability to the ISTA method. Consequently, the application of degassing at 96 kPa, followed by 3 h of stain development at 30°C should allow rapid and reliable viability assessment for a wide range of *Pinus* species.

Introduction

Tetrazolium (TZ) salts have been used for over 60 years as a rapid seed viability assessment (Kuhn and Jerchel, 1941). A topographical method of assessment was developed first for cereals (Lakon, 1942) and later for coniferous species (Lakon, 1949). The TZ test has been used as a diagnostic of seed quality problems in a range of species, including numerous crops (Moore, 1962), grasses and legumes (Porter *et al.*, 1947), cotton (Lambou, 1953), conifers (Parker, 1953) and orchids (Van Waes and Deberg, 1986). Primarily it is used to rapidly estimate seed viability and is an important method for distinguishing between dead and dormant seeds (Gosling 2003). The test can also be used to examine the effects of environmental conditions on seed viability, such as responses to desiccation and storage (Dickie *et al.*, 1991; Kovach and Bradford, 1992).

The test has been widely adopted, e.g. by the Association of Official Seed Analysts (AOSA) and the International Seed Testing Association (ISTA) who have published international recommendations for testing seed viability in 247 taxa using TZ (ISTA, 2003). In the TZ test, a colourless solution of tetrazolium chloride, is reduced by dehydrogenase enzymes within viable tissues to form a stable, non-diffusible and distinctive red dye

(2,3,5-triphenyl formazan) (Seidler, 1991). The distribution and extent of red stained areas in treated seeds are examined when assessing seed viability; in the absence of intact enzyme systems, i.e. in dead tissues, there is no stain development.

The current ISTA guidelines for *Pinus* species, involve cutting a third from the distal end of the seeds to open the embryo cavity (for hard coated species, the coat is removed first) followed by incubation in TZ solution at 30°C for 18 h (ISTA, 2003, 2005). However, vacuum degassing seeds in TZ solution has been suggested as a possible method for both improving the uniformity of seed staining and shortening the period required for determining seed viability for both *Abies* and *Pinus* (Knierim and Leist, 1988; Savonen, 1999). For *Pinus sylvestris,* vacuum degassing of dry seeds in TZ solution at a suction of 24 kPa, followed by 4 h of incubation in TZ solution, resulted in equivalent levels of staining to treatments where seeds were incubated in TZ solution for 24 or 48 h without the application of degassing (Savonen, 1999). Whilst, these results suggest that degassing may increase the rate of stain permeation into seed tissues (and hence the rate of stain development) the necessary controls i.e. a direct comparison of de-gassed and control seeds subjected to the same stain development times were not included. Also, the effect of additional suction levels on stain development, the applicability of degassing to other *Pinus* species and how well it performs in comparison to the current ISTA guidelines for *Pinus* species are all unknown.

Consequently, in this paper we characterise the pressure-time dependency of vacuum degassing on two further *Pinus* species and then validate optimal test conditions for another 15 pine species.

Materials and methods

Seed lot details

Seed lots of 17 *Pinus* species (see table 1), with a range of viability levels, were obtained from commercial sources and stored at 15% RH and 15°C until use.

Seed germination

For each species, four replicates of 25 seeds each were sown on the surface of 1% agar in water in clear plastic sandwich boxes (dimensions: 6 x 11 x 17 cm). Species were incubated at 20°C with an eight hour photoperiod except for *P. contorta* var. *murrayana,* which was incubated at 25°C (see Flynn *et al.,* 2004). For four species *(P. coulteri, P. lambertiana, P. monticola* and *P. sabiana),* which had germinated to very low levels (<10%) after six weeks, seeds were subsequently stratified at 5°C for six weeks before returning to 20°C. Germination, assessed as radicle emergence by greater than 2 mm was scored weekly for a total of 12 weeks (excluding stratification), at the end of which a cut test was performed with soft seeds being deemed inviable (Gosling, 2003).

Seed preparation prior to viability assessment

Seeds of each species were prepared for viability assessment, using tetrazolium chloride, according to the ISTA guidelines (ISTA, 2003, 2005), as appropriate, for either hard or thin shelled *Pinus* species (see table 1 for species' seed-type allocation). For the hard-

Table 1. Species of *Pinus* used for comparison of viability assessments. Also presented are weight per 1000 seeds, initial germination level and the difference between viability assessed using the ISTA methods for hard- and thin-coated pines and germination level and between viability assessed by degassing (degassing followed by 3 h of stain development at 30°C) and germination.

Pinus species	Weight per 1000 seeds (g)	Seed pre-treatment[1]	Germination ± S.E. (%)	ISTA – G[2]	ISTA (incl. patchy) – G[3]	DG – G[4]
P. attenuata	24.0	T	83 ± 3	-16	+17	+12
P. caribaea	16.9	T	68 ± 3	-5	-7	+6
P. caribaea aged[5]	16.9	T	22 ± 6	+1	+64	+4
P. contorta var *murrayana*	5.7	T	80 ± 2	-38	-38	+7
P. coulteri	309.8	H	96 ± 1	-64	+4	-13
P. jeffreyi	133.8	H	87 ± 3	-7	+13	-2
P. lambertiana	226.9	H	81 ± 4	-26	+19	-16
P. merkussi	23.1	T	0 ± 0	0	0	0
P. monticola	25.0	T	96 ± 1	-86	+4	+3
P. muricata	8.9	T	91 ±2	-45	+4	-1
P. patula	8.6	T	37 ± 2	-27	+37	-17
P. pinea	727.0	H	97 ± 2	+3	+3	+3
P. pinea aged[5]	727.0	H	50 ± 5	+5	+50	0
P. ponderosa	31.6	T	45 ± 2	-28	+55	-4
P. pseudostrobus	13.1	T	74 ± 3	-18	+15	-3
P. radiata	25.0	T	81 ± 1	-50	+11	-18
P. sabiana	710.5	H	47 ± 5	+51	+53	+47
P. sibirica	195.9	H	0 ± 0	0	0	0
P. sylvestris	9.3	T	82 ± 3	-38	+15	+12
Total number of negative / positive values:				13 –ve / 4 +ve *	–ve / 15 +ve**	8 –ve / 8 +ve[n.s.]

*$P<0.05$, **$P<0.01$., n.s. not significant. Significance levels were assed using a Sign-test (Sokal & Rohlf, 1995)

[1]H = hard-shelled species, T = thin-skinned species method from ISTA, Table 6A (2005)

[2]The difference between viability assessed using the ISTA TZ method and observed germination levels (G)

[3]The difference between viability assessed using the ISTA TZ method (assuming patchily stained seeds to be viable) and observed germination levels (G)

[4]The difference between viability assessed using the degassing method (96 kPa followed by 3 h at 30°C) and observed germination levels (G)

[5]Seeds were artificially aged at 45°C and 60% RH for 4 weeks to reduce seed viability

shelled species, the coat was removed and the 'seed' transversely cut one third from the distal end. For the thin coated species, the seed coat was left intact and seeds were only cut transversely one third from the distal end.

Tetrazolium staining

A 1% aqueous solution of 2,3,5-triphenyl tetrazolium chloride buffered to pH 7 using potassium dihydrogen orthophosphate (KH_2PO_4) and disodium hydrogen orthophosphate dehydrate ($Na_2HPO_4.H_20$) was prepared according to international guidelines (ISTA, 2003, 2005). For each species, four replicates of 25 seeds each, prepared as above, were stained for 18 h at 30°C (in the dark) in TZ solution (ISTA, 2003, 2005). Subsequently seed viability was assessed according to ISTA guidelines (2003, 2005): seeds with unstained areas, on either the embryo or female gametophyte, were scored as inviable.

For vacuum degassing, seeds (4 replicates of 25 seeds each) of a thin- *(P. caribaea)* and thick-shelled species *(P. pinea),* prepared as above, were submerged in TZ solution and degassed for three periods of 10 min each at 24 kPa of suction (Savonen, 1999) in a PD3 Plate degasser attached to a XDS 5 dry pump (BOC Edwards, Crawley, UK.) in an air-conditioned room at c. 20°C. Between each 10 min period of degassing the suction was released for a 1 min interval (Savonen, 1999). Subsequently, seeds were moved to 30°C, in the dark, for 2, 3, 4, 7 and 17.5 h to allow the stain to develop. For control treatments, seeds submerged in TZ solution were kept in the dark on an adjoining laboratory bench during the degassing treatment and then moved to 30°C at the same time as the treated seeds.

In a second experiment, the effects of differing suction levels on stain development were tested on seeds of *P. caribaea* and *P. pinea.* Seeds (4 replicates of 25 seeds each) were prepared as above and then degassed in TZ solution at 0 (control), 24, 48 or 96 kPa of suction for three periods of 10 min, separated by 1 min, before being transferred to 30°C for 3 h.

Since 96 kPa of suction was found to be the most effective treatment, a comparison of the 18 h ISTA method, three 10 min cycles of vacuum degassing at 96 kPa followed by three hours of stain development at 30°C and germination was made for 17 additional *Pinus* seed lots, of varying viability, as follows: firstly, two inviable seed lots to act as controls *(P. merkussi* and *P. sibirica),* secondly viable seed lots of 13 further species and thirdly as additional controls, reduced viability (artificially aged) *P. caribaea* and *P. pinea* seeds. Seeds were artificially aged at 45°C and 60% RH (achieved using lithium chloride solution) for 4 weeks in sealed electrical boxes.

Statistical analyses

Two-way ANOVA on arc-sine transformed viability levels was used to assess the effects of time and degassing on viability assessment for each of *P. pinea* and *P. caribaea.* For both *P. caribaea* and *P. pinea,* one-way ANOVA on arc-sine transformed data followed by Tukey's pair-wise comparisons was conducted to test for significant differences between germination level, viability level using the ISTA method and degassing using 0, 24, 48 and 96 kPa followed by three hours of staining at 30°C. Across species, a sign test (Sokal and Rohlf, 1995) was used to assess whether the two TZ test methodologies provided

an accurate assessment of viability. The test was applied by calculating the number of species for which viability level was (1) greater (2) lower or (3) the same as the observed level of germination. Subsequently, the significance of the number of species for which the estimate of viability differed from the estimate of germinability was tested using the binomial distribution (Sokal and Rohlf, 1995).

Results and discussion

Stain development time, suction level and viability in P. caribaea and P. pinea

Vacuum degassing at 24 kPa for three periods of 10 min followed by stain development at 30°C revealed that, for both *P. caribaea* and *P. pinea,* the level of staining was significantly affected by both the duration of stain development (Two-way ANOVA, $P<0.05$) and by the degassing treatment (Two-way ANOVA, $P<0.05$). Thus, for both species degassing yielded a higher estimate of viability than in the control (standard non-degassing method) (figures 1 A and B; Two-way ANOVA $P<0.05$), with the estimate of viability increasing with stain development time (figures 1 A and B). Thus, by 4 h *post* degassing the number of fully stained seeds was close to that of the 17.5 h non-degassed treatment (figure 1), i.e. a saving of 13.5 h, enabling the test to be completed within one day.

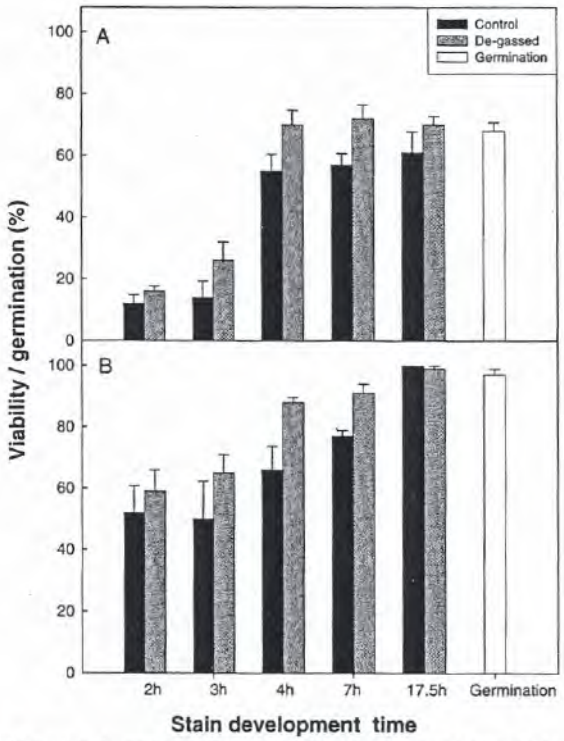

Figure 1. The effect of vacuum degassing at 24 kPa of suction in TZ solution, followed by varying periods of subsequent stain development at 30°C, on viability assessment of (A) *Pinus caribaea* and (B) *P. pinea* seeds. For comparison, germination percentage is also presented. Bars are +1 S.E. of the mean.

Since by 3 h of stain development time, the level of viability had not reached that observed with the standard 18 h ISTA method, 3 h was chosen to determine whether altering the suction level could further improve staining for *P. caribaea* and *P. pinea*. For both species, the estimate of viability increased significantly with increasing suction (up to 96 kPa) (One way ANOVA, $P<0.05$), with 96 kPa resulting in levels of staining that were not significantly different to either the 18 h ISTA method or the germination test ($P>0.05$, table 1; figures 2A and B). Consequently for these species, a 3.5 h test (0.5 h degassing at 96 kPa followed by 3 h at 30°C) provides both a reliable estimate of viability and yields a substantial saving of time in testing.

For dry *Pinus* seeds, there is a small air gap between the seed coat and the surface of the female gametophyte and consequently for 'thin' coated species, this may potentially restrict the uptake of TZ solution and hence the effectiveness of staining (Savonen, 1999). In addition, for all *Pinus* species, there may also be an air space in the embryo cavity which could also restrict uptake of TZ solution. Presumably the improvement observed

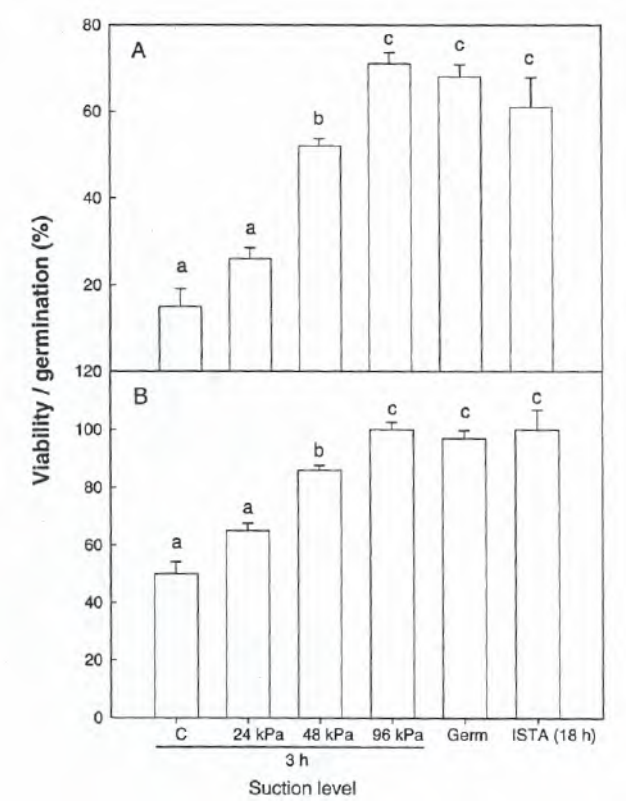

Figure 2. A comparison of seed germination, viability assessment using the standard ISTA TZ method for *Pinus* species and viability assessed using vacuum degassing of TZ solution at a range of suctions followed by 3 h of stain development at 30°C for (A) *Pinus caribaea* and (B) *P. pinea* seeds. Differing letters above bars indicate a significant difference between treatment means as assessed by One-way ANOVA followed by Tukey's pair wise comparisons. Bars are +1 S.E. of the mean.

following degassing, particularly at the highest level of suction applied, results from the removal of this intra-seed air, which when the suction is released is rapidly replaced by TZ solution resulting in a larger surface area for the uptake of solution into seed tissues and hence more rapid tissue stain development (Savonen, 1999). In addition, it has previously been noted (Wood *et al.*, 2005) that some seeds of species in families with oily seeds stained poorly. However, we show here that oily pine seeds (Flynn *et al.*, 2004) respond well to rapid viability assessment with TZ when degassing is incorporated into the method.

Usefulness on other Pinus species

Comparing the results of degassing at 96 kPa followed by 3 h at 30°C with the ISTA TZ method and germination for *P. caribaea, P. pinea* and an additional 17 *Pinus* seed lots, indicated that with de-gassing there was no tendency for viability to be systematically either over- or under-estimated compared with germination levels (Sign-test, $P>0.05$, table 1, figure 3). However, with the ISTA method there was a significant tendency for viability to be under-estimated compared to germination (Sign-test, $P<0.05$, table 1, figure 3). Thus, TZ under-estimated viability for 13 seed lots and over-estimated viability for 4 seed lots. For one species *(P. sabiana)* the estimate of viability from both TZ methods was higher than observed germination levels. Since for this species all non-germinated seeds were viable, as assessed by a cut test, this disparity is likely to reflect seed dormancy

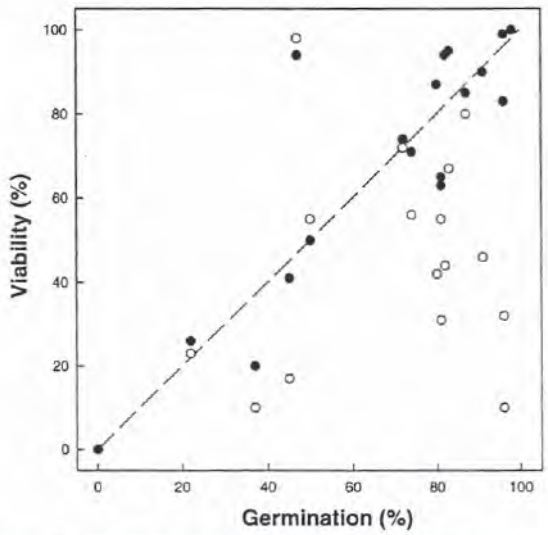

Figure 3. A comparison of viability level, as assessed using either degassing at 96 kPa of suction followed by 3 h of stain development at 30°C (closed symbols) or the ISTA method (18 h of stain development at 30°C) (open symbols) with germination level for 17 species of *Pinus* as well as aged *P. caribaea* and *P. pinea* seeds. The dashed line indicates the expected relationship if viability level matches germination. Note that the closed symbol at 0% germination and 0% viability refers to two seed lots (*P. merkussi* and *P. sibirica*) where germination and viability from both assessment methods was 0%. In addition the closed symbol at 97% germination (*P. pinea*) masks the corresponding open symbol for *P. pinea* i.e. viability from degassing was the same as for the ISTA method.

or the use of unsuitable germination conditions (Steiner *et al.*, 1999; Wood *et al.*, 2005) rather than an over-estimation of viability. However, for all other species non-germinated seeds were scored as non-viable following a cut test. The use of both artificially aged seeds (i.e. reduced vigour seed lots) and the inclusion of two inviable seed lots and the match between germination and viability for these batches (table 1) demonstrates that the improvements from degassing do not result from artificially high estimates of viability as an artefact of the degassing and instead presumably result from an improvement in TZ permeation or penetration or both into seed tissues. Nonetheless, although degassing resulted in substantial improvements in both the level and rate of seed staining across the study species, this method should be further validated on multiple seed lots (of contrasting viability levels), of one or more species, before wider adoption.

The results also demonstrate that for some species, e.g. *P. muricata, P. monticola, P. coulteri* and *P. radiata* assuming patchily stained seeds to be viable results in the estimate of viability being closer to both actual germination and viability, as assessed by degassing (table 1). This preponderance of patchily stained, but viable, seeds which stain fully following degassing suggests that stain permeation into *Pinus* seeds can be problematical and is improved by degassing, as previously shown by Savonen (1999) with *Pinus sylvestris*. However, across the species, assuming that patchily stained seeds are viable resulted in a significant and systematic over-estimation of viability (15 of 17 seed lots where viability was over-estimated; Sign test $P<0.05$, table 1). Consequently, although for some *Pinus* species subjected to the standard ISTA TZ viability test, patchiness may be indicative of incomplete stain development (permeation) it can also reflect seeds being non-viable. Thus, this finding supports the current ISTA guideline of treating patchily stained *Pinus* seeds as non-viable.

Conclusions

1. The use of de-gassing at 96 kPa followed by 3 h of stain development at 30°C can, for pines, result in an equivalent or better estimate of seed viability as the current ISTA guidelines.
2. This rapid (14.5 h faster) method can reduce the number of patchily stained seeds.
3. Further studies should investigate the wider applicability of this technique for more diverse species, particularly those with intra-seed air spaces / pockets (e.g. Rubiaceae).

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