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Automated monitoring of pollen quality in Douglas fir using digital images: applications for handling and storage

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Abstract

Background: Conservation and breeding programs for forest species require efficient pollen quality monitoring strategies to maximize collection, storage and pollination efforts.
Question: Can the ImageJ image analysis software be implemented to efficiently monitor pollen viability and germinability for quality testing?
Studied species: *Pseudotsuga menziesii* (Mirb.) Franco
Methods: Tetrazolium staining and germination on 10 % Brewback media was used to test Douglas fir pollen quality. Two macros for automated counting were developed with ImageJ free software using digitized microscopic images, and were tested to evaluate provenances, germination in different media, dehydration/rehydration and freeze-storage treatments, and finally to compare pollen quality between mature trees and GA-induced strobili from juvenile trees.
Results: Automated monitoring of pollen quality was feasible because the software suitably distinguishes viable from non-viable pollen grains due to differential tetrazolium staining that translates into pixel density, whereas germinability was assayed based on pollen size and circularity. This tool was used to guide the selection of appropriate pollen handling conditions and compare pollen production in adult and GA-induced juvenile trees. Pollen flash-freezing and storage at -80 °C showed recovery between 74 and 98 % of its initial germinability after rehydration; however, viability detected by tetrazolium is reduced, suggesting that handling and storage affect the reliability of the test.
Conclusions: The flexibility of the Fiji software allowed the development of an efficient and accurate macro for monitoring *P. menziesii* pollen quality, reducing invested time and effort involved, which supports future research on its conservation physiology and its successful application for germplasm production.
Key words: imaging, macros, pollen conservation, pollen germinability, *Pseudotsuga menziesii*, tetrazolium, viability.

Resumen

Antecedentes: Los programas de conservación y producción de semilla en especies forestales requieren estrategias eficientes para el monitoreo de polen que maximicen los esfuerzos de recolección, almacenamiento y polinización.
Pregunta: ¿Puede implementarse el software de análisis de imágenes Image J para monitorear eficientemente la viabilidad y germinabilidad del polen?
Especie de estudio: *Pseudotsuga menziesii* (Mirb.) Franco
Métodos: Se utilizó tinción por tetrazolio y el medio Brewback 10 % para evaluar la calidad de polen. Se desarrollaron macros para la automatización del conteo mediante el software libre Image J usando imágenes digitalizadas. Se comparó el polen de distintas procedencias, madurez de árboles, medios de germinación, deshidratación/rehidratación y almacenamiento por congelación.
Resultados: El software distingue adecuadamente polen viable del no viable mediante la tinción diferencial que se traduce en densidad de pixeles, mientras que para germinación se consideró el tamaño y circularidad. La herramienta permitió guiar la selección de condiciones apropiadas para el manejo de polen, así como comparar la producción de árboles adultos y juveniles inducidos con GA. El congelamiento de polen permitió recuperar entre 74 y 98 % de la germinación después de la rehidratación; aunque la detección de viabilidad por tetrazolio disminuyó, mostrando que el manejo y almacenamiento afectan la confiabilidad de la prueba.
Conclusiones: La flexibilidad del software Fiji permitió el desarrollo de macros eficientes y precisas para monitorear la calidad del polen de *P. menziesii*, al reducir el tiempo y esfuerzo implicados. Esto apoya investigaciones futuras sobre la fisiología de su conservación y aplicación exitosa para la producción de germoplasma.
Palabras clave: análisis de imágenes, conservación de polen, macros, *Pseudotsuga menziesii*, tetrazolio, viabilidad.

Forest genetic improvement programs in coniferous species rely on the abundant production of quality seed for use in reforestation. Most conifers are mast-seeding, exhibiting synchronized cycles of seed-producing and non-producing years, that vary between species and populations, and have important reproductive and ecological implications under natural conditions (Redmond *et al.* 2019). However, these cycles inevitably affect the availability and number of seeds from select trees that can be supplied for plantation efforts. Enhanced seed production has been achieved in species of *Pinus* (Kong *et al.* 2018), *Picea* (El-Kassaby & Reynolds 1990), *Larix* (Lee *et al.* 2011), and especially *Pseudotsuga* (El-Kassaby & Ritland 1986, Muñoz-Gutiérrez *et al.* 2010), with varying levels of success, and mostly in seed orchards, through cultural practices that range from promoting abundant strobili in juvenile and mature trees to pollen collection, and performing controlled pollinations between selected individuals (Crain & Creg 2018). As ensuring high-quality pollen production is critical when assessing reproductive function and efficiency, and for maintaining seed orchard yield, it is an essential part of directed breeding and tree improvement programs (Li *et al.* 2021).

Monitoring pollen quantity (production), viability, and germination capacity over time provides valuable insight into strobili induction practices. It is also crucial to follow pollen collection and handling procedures -along with short or medium-term storage strategies- to sustain controlled pollination efforts, especially if there is a lack of synchrony in the development of the male and female gametes (Araújo de Oliveira *et al.* 2021). Common approaches for assessing pollen viability include various staining techniques, *in vitro* assays to detect respiration, electrical conductivity of leachates, and electric properties of pollen membranes, *in vitro* germination, *in situ* germination and seed set evaluation (Webber & Bonnet-Masimbert 1993). The suitability of these approaches depends on their efficiency, reliability, and the resources available regarding specific project or research needs and constraints (*i.e.*, equipment, pollen availability).

Viability stains and germinability tests remain the most practical and sought-after techniques for discriminating between viable and non-viable grains, even if they are not a guarantee of successful fertilization and seed set. Viability stains are used to visualize specific compounds, cellular compartments, or metabolic activity associated with aborted and non-aborted pollen grains (Stanley & Linskens 1974, Rodriguez-Riano & Dafni 2000, Tello *et al.* 2018). Tetrazolium salt-based dyes are the most frequently used and rely on the action of dehydrogenase enzymes in metabolically active cells to catalyze the colorless 2,3,5-triphenyl tetrazolium chloride to produce a red or purple color that clearly distinguishes living from non-living colorless cells (Stanley & Linskens 1974). Concomitantly, pollen germination tests are based on calculating the percentage of pollen grains that produce a pollen tube when exposed to a liquid, either water or a more complex medium, that may include salts and sugars as osmoticums, as well as growth regulators (Webber & Bonnet-Masimbert 1993, Dumont-BéBoux & von Aderkas 1997).

The evaluation of pollen quantity and quality using viability stains and germinability tests can be a laborious process with poor reproducibility, making high-throughput image-based analysis an attractive and useful alternative to shorten the time and effort involved in processing, while allowing easy storage of the images, electronic counts, and calculations. The ImageJ (Fiji is Just ImageJ) analysis platform, an open-access, user-friendly and intuitive software focused on biological image analysis, is particularly amenable to performing pollen quantification tasks quickly (Schindelin *et al.* 2012, Tello *et al.* 2018, Ayenan *et al.* 2020).

The automation of the analysis is achieved through macros -codes that contain the complete process steps- which can be easily applied to a series of images to obtain a single file containing all the results. It is a relatively straightforward process, based on setting the analysis parameters and using the differences in pixel density between the object (pollen) and the background to detect the size and circularity of the object of interest. Reproducible pollen counts consistent with manually obtained data have since been achieved in numerous species (Costa & Yang 2009, Tello *et al.* 2018).

A further step consists of implementing the software to estimate viability. The open-source code PollenCounter (also from Fiji) was initially created to automate phenotyping of grape pollen grains after Alexander's staining, also by analyzing pixel density (Tello *et al.* 2018). This code was subsequently optimized to assess pollen viability in heat-stressed tomatoes (Ayenan *et al.* 2020), and a significant correlation was found with results obtained from

manual processing, indicating that the analysis can be adjusted to different species. However, image-based analysis for estimating germinability is mostly underwhelming; neither Fiji nor PlantCV, a semi-automated software designed for this purpose (Castillo *et al.* 2022), allow full automation due to the additional complexity conferred by pollen tube growth. Notwithstanding, such software may be useful for automating evaluation in species where pollen morphology during germination follows a more uniform or predictable pattern.

Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) is one of the most ecologically and economically important conifers native to North America, which grows naturally from Canada to central Mexico, with plantations having been introduced to Europe, New Zealand, Australia, and Chile (Hermann & Lavender 1999). Unlike Canada and the United States, where Douglas fir forms large and continuous forests, populations in Mexico grow in small and isolated stands among other coniferous and hardwood species. This has resulted in a highly fragmented and discontinuous distribution (Ventura-Ríos *et al.* 2010), typical of rear-edge populations (Gugger *et al.* 2011). The ecological, morphological, phenological, and genetic differentiation of these southern-limit populations reflects local adaptations to topographic and environmental variables (Ventura-Ríos *et al*. 2010), suggesting their potential role as a reservoir of genetic diversity in changing climates, that can be valuable to genetic improvements and breeding efforts (Gugger *et al*. 2011). However, high anthropogenic pressure due to land-use change, inappropriate forest management practices, and pest attacks, has reduced the density of trees of reproductive age, thereby increasing inbreeding, which has taken its toll on reproductive success and natural regeneration (López-Upton *et al*. 2015).

Douglas fir is subject to special protection by the Mexican government (SEMARNAT 2010), indicating the urgency for better management programs in natural populations and to promote commercial plantations and germplasm-producing units to support conservation and harvesting strategies (López-Upton *et al.* 2015). Silvicultural practices that promote the induction of male and female strobili in young trees have been explored, mainly through the application of gibberellic acid 4/7 (GA4/7) (Muñoz-Gutiérrez *et al.* 2010). Nevertheless, as is common in conifers, pollen production in young trees is a limiting factor due to its scarcity (Muñoz-Gutiérrez *et al.* 2010); therefore, operational seed production necessitates collecting pollen from mature trees. This represents the additional challenge of obtaining, transporting and storing until use, making monitoring pollen quality throughout the handling period essential to ensure its fertility (Webber & Bonnet-Masimbert 1993).

Upon shedding, Douglas fir pollen is mostly spheroidal, lacking bladders, pores, or furrows, and between 90-100 μm in diameter (Owens & Morris 1990). The exine is thin and smooth, and generally splits open and is "thrown off" when moistened, allowing the pollen grain to swell and elongate considerably (up to five times its size) (Dumont-BéBoux & Von Aderkas 1997). This elongation represents a pre-germinative stage that leads the pollen grain that settles in the micropyle towards the micropylar canal. Once in proximity to the nucellus, germination *per se* occurs, with the formation and growth of the pollen tube to complete fertilization (Owens & Morris 1990).

Pollen tube formation *in vitro* is rare and requires the addition of flavonoids or other complex compounds to mimic chemical signals believed to be produced by the nucellus to help direct tube growth (Dumont-BéBoux & Von Aderkas 1997). Notwithstanding, the pre-germinative stage is readily replicated with the addition of a drop of water or medium, allowing the differentiation of four morphotypes, according to pollen grain shape and length: type 1 is elongated, with a minimum of twice the length of a hydrated grain; type 2 is oval, with up to a 50 % increase of the size of a hydrated grain; type 3 is a round grain with no elongation; and type 4 are grains with evidence of damage (plasmolysis). Of these, type 1 and 2 pollen are considered to reflect overall germination capacity and are related to higher seed sets in controlled pollination assays (Webber & Bonnet-Masimbert 1993) and may represent a useful approximation to assay pollen quality. Furthermore, the simplicity with regard to shapes and elongation patterns provides an opportunity for automated digital evaluation.

To generate an automated method that could be useful for future management and conservation programs aimed at seed production and genetic improvement in *P. menziesii*, we sought to standardize a procedure to count viable and non-viable pollen grains, as well as the four morphotypes of the pre-germinative stage, using the free ImageJ (Fiji is Just ImageJ) software.

Materials and methods

Pollen collection. Douglas fir pollen samples were obtained from two sources in Central Mexico: 1) Adults, corresponding to three samples (a1-a3), were obtained from a mix of six trees of approximately 80 years of age, from San Pedro Nexapa, Amecameca, Estado de México (19° 07' 66" N and -98° 70' 65" W; 2,800 m asl). After performing an initial test to check viability and germinability, the three samples were mixed to form a pool to increase the amount of pollen available for experimentation, and its moisture content was determined (MC: 29 %) (Webber & Painter 1996). An additional sample (a4) was provided at a later time and was kept independently. 2) Induced samples were collected at four different dates from trees between 7 and 8 years of age treated with gibberellic acid (GA4/7) to promote strobili initiation, at the Parque Ecoturístico Bosque Esmeralda, Ejido Emiliano Zapata, Amecameca, Estado de México (19° 07' 14" Lat. N and -98° 44' 06" Long. W; 2,640 m asl): i1 (March 1st), i2 (March 17th), i3 (March 22nd), and i4 (April 3rd). All samples were obtained from strobili that were still closed when collected, then kept in paper bags and allowed to dry for 24 hr at room temperature before extraction. Pollen samples were stored at 4 °C in airtight tubes until use.

Standardization. To guide the creation of a macro to automate pollen counts, the effectiveness of the viability staining method and germination media were evaluated, while assessing the correct way to capture the images and select working parameters for the software. Counts were performed in randomly selected fields at 10X magnification under an Axiostar Plus microscope (Carl Zeiss, Oberkochen, Germany). Images were digitized with an Axiocam MRC camera and AxioVision software (Axio Vs40 v. 4.8.2.0).

Pollen viability staining with tetrazolium salts.- The pollen viability staining was based on Vieitez-Cortizo (1952). Approximately 3 mg of pollen was placed in an Eppendorf-type tube with 200 μL of 2,3,5-triphenyltetrazolium chloride solution (TTC; 0.2-1.0 % solution in 1/15 mol phosphate buffer, pH of 7.2), and incubated for 90 min at 55 °C in an FD 56 drying oven (Binder™, Tuttlingen, Germany). Red/purple pollen grains were considered viable, whereas clear/colorless cells were non-viable. Ten images were obtained per sample. The percentage of viability was calculated using the formula:

$$\frac{Viable\ pollen}{Non-viable\ pollen} \times 100$$

Pollen germination.- Three media were tested: 1) double distilled water (ddH$_2$O); 2) Brewbaker and Kwack (BK) medium 100 % (Webber & Bonnet-Masimbert 1993) (10 % sucrose, 100 mg/L H$_3$BO$_3$, 300 mg/L Ca(NO$_3$)$_2$, 200 mg/L MgSO$_4$, 100 mg/L KNO$_3$, and H$_2$O; pH 5.3); and 3) BK 10 % medium (pH 5.3). Approximately 3 mg of pollen was placed in an Eppendorf tube and 200 μL of the appropriate medium was added. Incubation was performed at 26 °C for 48 hr in a 100A Stabil-Therm Dry Type Bacteriological Gravity Convection Incubator (Blue-M, New Columbia, Pennsylvania, USA). For each medium, 30 images were obtained. Germination percentage was calculated with the formula:

$$\frac{Type\ 1-2\ pollen}{Total\ pollen} \times 100$$

Automated viability and germination analysis.- To adapt the program ImageJ (Fiji is Just ImageJ) (v. 1.53) (Schindelin *et al.* 2012), all images were saved in a predefined folder to execute the batch mode analysis (Tello *et al.* 2018). The size and circularity ranges of the pollen grains were obtained using the function *Analyze>Analyze particles*, where a value of 1 indicates a perfect circle while values close to 0 express the elongation of the object.

Viability test.- The command path selected was *Image>Color>Split channels*, resulting in three channels: red, green, and blue. The red channel was chosen to identify total pollen, while for the green channel for the detection of viable pollen (Tello *et al.* 2018). Depending on the lighting and pixel density of the image, the I*mage>Color>Threshold* was adjusted. The image was converted to black and white with *Process>Binary>Convert to mask*. The *Process>Binary>Fill holes* option was used to fill the blank spaces in the objects. The *Watershed* function was used to separate closely spaced grains. The *Analyze>Analyze particles* tool was used to count pollen grains. In the red

channel, pollen was counted with a size of 3,000 to 30,000 pixels and a circularity of 0.30 to 1.00, while for the green channel, the same size but a circularity of 0.66 to 1.00 were established.

Germination test.- The *Image>Type>8 bit* command path was selected to convert the image to grayscale, and the same steps were followed as in the viability test. The threshold was adjusted, and the *Exclude on Edges* option was selected in order to avoid miscounting pre-germinated pollen as non-germinated, forming two groups: germinated pollen grains (type 1 and 2) and non-germinated pollen grains (type 3 and 4). The size range for germinated pollen grains was from 16,000 to 40,000 pixels with a circularity of 0.20 to 0.97, while for non-germinated pollen grains it was 3,000 to 16,000 pixels, with a circularity of 0.20 to 1.00.

A macro was made to automatically analyze all images: 150 for viability and 90 for germination (Supplementary Material). The analysis comprised 3,391 pollen grains for the viability test, with an average of 22.6 grains per image, whereas germinability was evaluated from a total of 1,611 pollen grains with an average of 17.9 grains per image. The results of the pollen counts were obtained in a Microsoft Excel file (2007).

Validation. The macro was used to expand the analysis to a larger set of images to verify correspondence between automated and manual counting, as well as to finetune experimental conditions: 150 images were obtained from viability assays that tested pollen from different sources (a1-4 and i1-4) and dehydration and rehydration treatments (pool); 160 images were used to evaluate pollen germinability from the different sources (a1-4 and i1-4) or different growth media (pool).

Pollen dehydration.- Either saturated $MgCl_2$ (15 g dissolved in 5 mL of distilled water) (Connor & Towill 1993) or silica gel (Dinato *et al*. 2018) was poured into Petri dishes (90 × 15 mm) in which a smaller petri dish (60 × 15 mm) was introduced as a support for ~10 mg of pollen (pool sample) placed on aluminum trays. The external Petri dish was covered, sealed with Parafilm® and incubated at 24 °C for 4 hr. Weight was recorded every hour until 10 % MC was obtained. After this time, ~5 mg of the pollen was vacuum sealed in a commercial vaccum sealer (85 kPa Automatic Food Sealer, KOIOS) and stored at 4 °C for 24 hr. The remainder was rehydrated in a humid chamber for 3 hr at 26 °C. Viability was assessed in ten images for each sample. A total of 2,638 pollen grains were analyzed, with an average of 17.5 grains per image.

Germination media.- Seven different media were tested, consisting of either BK 100 % and BK 10 % supplemented with sucrose 10 %, or mannitol 5 %, or indole-3-acetic acid 10 ppm (IAA) (Table 1). The percentage of germination was calculated from 10 images of each medium. A total of 3,300 pollen grains were analyzed, with an average of 20.6 grains per image.

Evaluation. The full protocol was implemented to compare pollen quality under various conditions. All pollen underwent rehydration before testing.

Pollen quality in adult and induced samples.- Pollen viability and germination were compared between samples obtained from mature trees (using a1-4 as replicates) and trees induced with GA_4 (using i1-4 as replicates). A total of 120 images were analyzed.

Refrigerated pollen viability through time.- Viability of the "Pool" sample was tested before and after storage at 4 °C for a period of 4 months (n = 40).

Pollen quality of the adult and induced samples stored at -80 °C for 50 days: Undehydrated pollen (~5 mg) was placed inside 7 × 13 cm Mylar bags and vacuum sealed. Samples were frozen with liquid nitrogen for 2 min and stored at -80 °C for 50 days. Before testing, the samples were thawed at ~70 °C for 3 min and rehydrated for 3 hr in a humid chamber. Only samples with the highest percentages of germination and viability before freezing were used. Ninety images were analyzed for viability and germination.

Table 1. Tested media for pollen germination. AIA: Indole-3-acetic acid. BK: Brewbaker and Kwack medium.

Media	BK 100 % medium	BK 10 % medium
+ 5 % mannitol	✓	✓
+ 10 % mannitol	✓	
+ 5 % sucrose	✓	
+ 10 % sucrose		✓
+ 10 % sucrose + AIA		✓
+ 5 % mannitol + AIA		✓

Statistical analysis. Normality and homogeneity of variances of the data for all variables (viability and germination) in the different experimental stages were verified with the Shapiro-Wilk and Fligner-Killeen tests, respectively (α: 0.05). For standardization data, *t*-test was used to determine significant differences (α: 0.05) between automated and manual counts, and the Pearson correlation coefficient assessed the two datasets. A Bland-Altman plot was calculated to estimate the accuracy between the two approaches.

The Kruskal-Wallis H test and *post hoc* Wilcoxon signed-rank test were used to determine significant differences in the percentage of pollen germination between the different media. For macro validation, the nonparametric Mann-Whitney U test was used to determine significant differences (α: 0.05) between the results of manual and automated counting. The Spearman correlation coefficient was used to calculate the association between the two counts. For evaluation, the t-test was used to analyze the differences in pollen viability and germination between adult and induced trees. The t-test along with the nonparametric Mann-Whitney U test were used to determine significant differences in pollen viability and germination before and after storage in each sample.

RStudio v. 1.4.1106 was used for all statistical analyses (RStudio Team 2020).

Results

Standardization. Automated viability and germination testing required standardization of staining conditions and the appropriate medium for pollen grains to germinate. Tetrazolium staining allowed the status of pollen grains to be clearly distinguished, where viable grains showed stronger pigmentation than non-viable grains, on a very light background that did not interfere with subsequent image analysis (Figure 1A).

Selection of the germination medium was based on the germination percentages obtained, which were manually calculated (data did not show a normal distribution, $P = 0.0023$). Although double-distilled water quickly induced germination (78 %), an increasing loss of viability was observed after 24 hr, as well as contamination; whereas germination was favored in both BK 100 and 10 % medium (92 and 94 %, respectively) when compared to bidistilled water ($P = 0.0005$). Consequently, BK 10 % medium was used in all subsequent tests (no significant difference between BK 10 and 100 %, $P = 0.1852$).

Viability analysis using Fiji produced two images per field showing total and viable pollen-grain counts (Figure 1B, C). With the setting for the green channel image, the pixel density in non-viable grains was decreased, enabling viable grains to be distinguished (Figure 1C). For the germination test (Figure 1D), two images were obtained per field, where germinated pollen grains (Figure 1E) and non-germinated grains (Figure 1F) could be observed, differentiated by their size and circularity.

In the macro standardization (240 images), both viability and germination data showed a normal distribution (viability manual counting, $P = 0.0943$; automatic counting, $P = 0.1612$; germination manual counting, $P = 0.3301$; automatic counting, $P = 0.0592$). *t*-test showed no significant differences between manual and automatic calculations of viability ($P = 0.8831$) and pollen germination ($P = 0.9776$) (Figure 2).
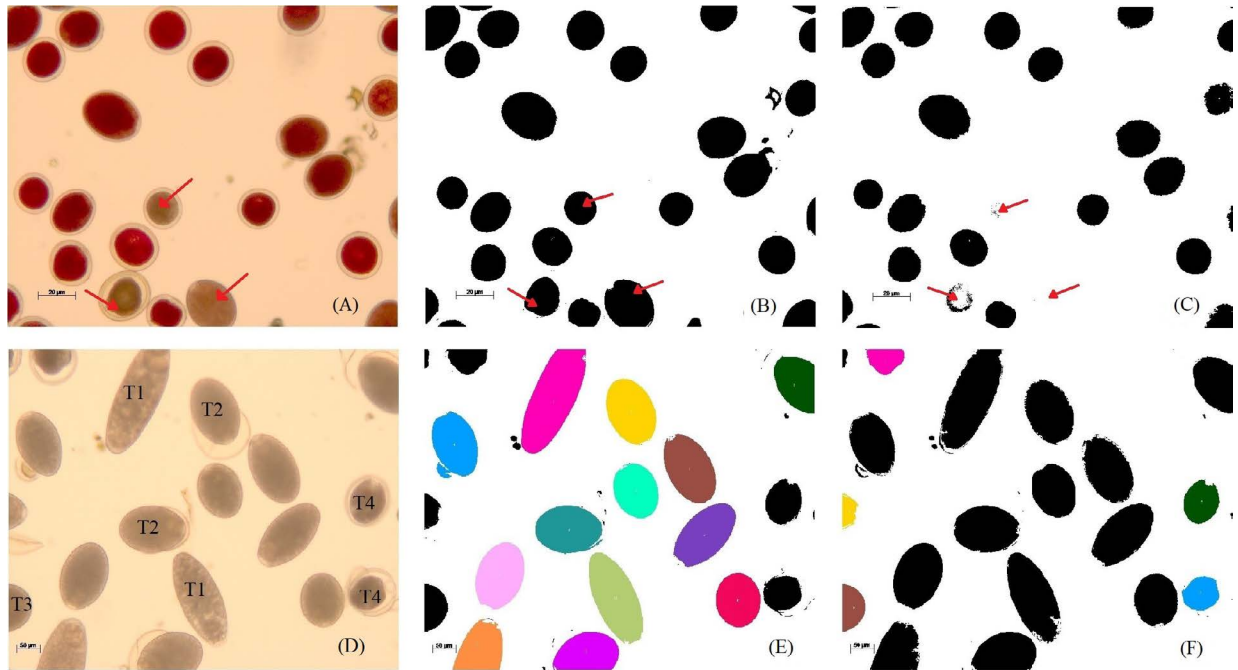
Figure 1. Viability and germination test of *Pseudotsuga menziesii* pollen grains. (A) Staining with tetrazolium chloride. Automated counting: (B) the red channel shows total pollen; and (C) the green channel shows only viable pollen. Arrows indicate non-viable pollen. (D) Germination test in 10 % BK medium, showing the different types of pollen morphologies: type 1 (T1), type 2 (T1), type 3 (T3) and type 4 (T4). Automated counting: (E) colors show germinated pollen types 1 and 2; and (F) colors show ungerminated pollen types 3 and 4.
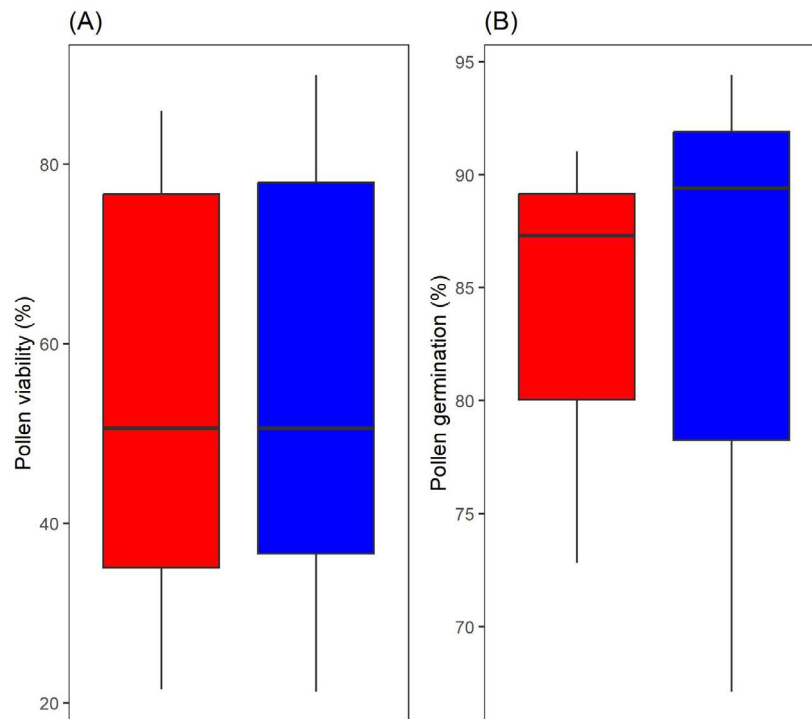
Figure 2. Box plot comparing the percentages obtained manually (blue) and automated (red) counting. (A) viability: the maximum line from the manual counting has a small deviation, the interquartile range and the median have a similar distribution. (B) germination test: there is a little difference in the distribution of the interquartile range and the median, the minimum line has the largest deviation.

Furthermore, a high positive correlation between manual and automatic counting which was obtained for the viability ($P = 9.89e-12$, $r = 0.98$) and germination ($P = 0.0169$, $r = 0.99$) tests, which was further confirmed by the Bland Altman analysis. Differences between the measurements made with both approaches were within 95 % confidence intervals and the points were distributed without any pattern (Figure 3A, C).

Validation. The viability tests performed on different samples did not show a normal distribution (manual counting $P = 0.0002$, automatic counting $P = 0.0003$); nonparametric Mann-Whitney U test revealed no significant differences between manual and automatic counting ($P = 0.9314$) and a high positive correlation between both methods ($P = 1.42e-10$, $r_s = 0.97$). Viability varied between provenances and tree maturity from 42 to 83 %, with lowest values observed for induced trees (Figure 4A); whereas germinability ranged between 37 and 83 %, reflecting the results obtained for viability (Figure 4B).
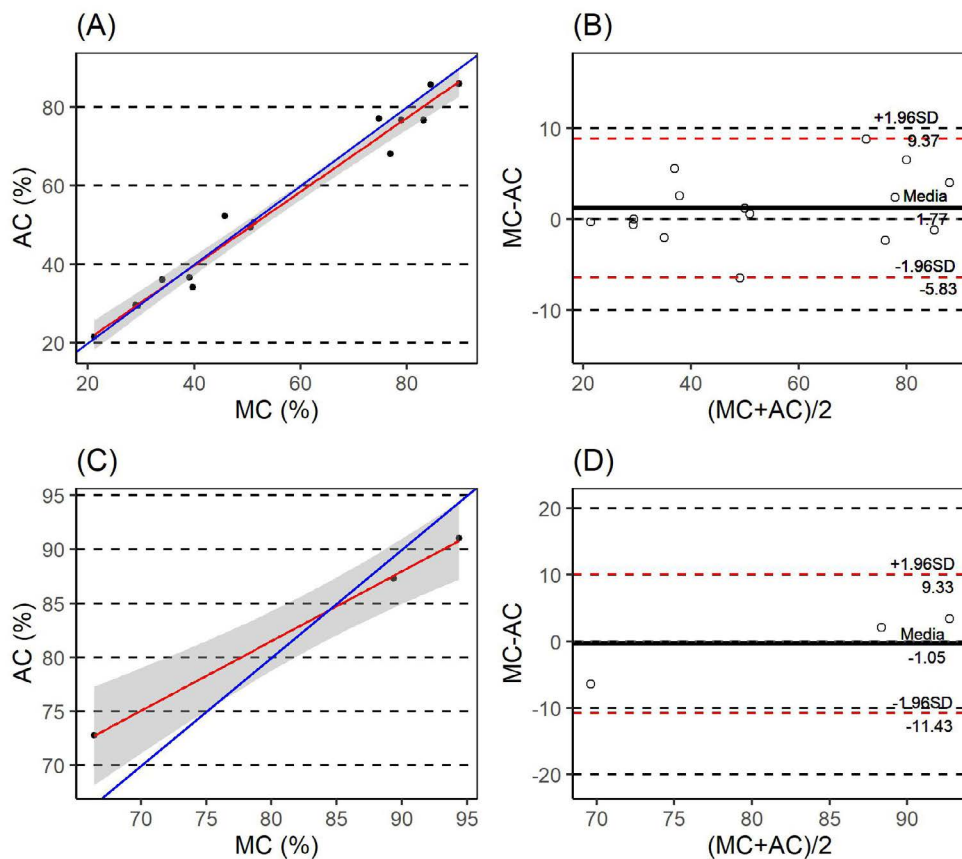

Figure 3. Dispersion diagram showing the correlation between manual (MC) and automated (AC) counting for (A) viability test; (C) germination test. Manual counting is indicated with the red line, automated counting with the blue line. Bland Altman plot showing the differences between the two counting methods versus the mean of the two methods in the viability test (B) and germination test (D). Confidence interval limits are presented as red dotted lines.

Dehydration had a negative impact on pollen viability, with the use of silica gel being the least aggressive -showing 25 % decrease- while over 50 % of viability was lost under $MgCl_2$ and vacuum. Prior to staining, rehydration for 3 hr allowed a partial recovery, suggesting that it is a necessary step for adequate monitoring of viability (Figure 5A). Non-germinability testing on different media of the adult, induced (90 images) and Pool (70 images) samples showed non-normally distributed data (manual counting $P = 0.0001$, automatic counting $P =$

0.0003); a high correlation between both counting methods ($P = 4.57e-15$, $r_s = 0.98$) was observed with no significant difference between them ($P = 0.3422$). Incorporating sugars and AIA had a positive impact when compared to double-distilled water ($P < 0.05$) but was similar to BK 100 and 10 % (Figure 5B, $P > 0.05$). Furthermore, a significantly higher germination percentage was found using BK 10 % compared to the medium with 10 % sucrose + AIA ($P = 0.0038$), with with 5 % mannitol ($P = 0.0057$), or mannitol 5 % + AIA ($P = 0.0175$), so continued supplementation of the basic medium was not considered, particularly due to its tendency to become contaminated in the presence of sugars.

Evaluation. The standardized tools for quality evaluation were applied to compare pollen from induced and mature trees. Additionally, we tested the usefulness of flash-freezing and storage at -80 °C as a medium-term conservation strategy.

Pollen quality in induced trees.- Viability and germinability of pollen from adult and induced trees showed a normal distribution (viability: adult trees $P = 0.6994$, induced trees $P = 0.8638$; germination: adult trees $P = 0.6412$, induced trees $P = 0.7274$); the subsequent t-test showed that these had significantly higher values in adult trees ($P = 0.0351$ and $P = 0.0291$, respectively). In addition, induced trees showed a greater variation in both parameters (Figure 6), reflecting the decrease in quality throughout the season, being at its highest in February and tapering off towards May (Figure 4A, B).
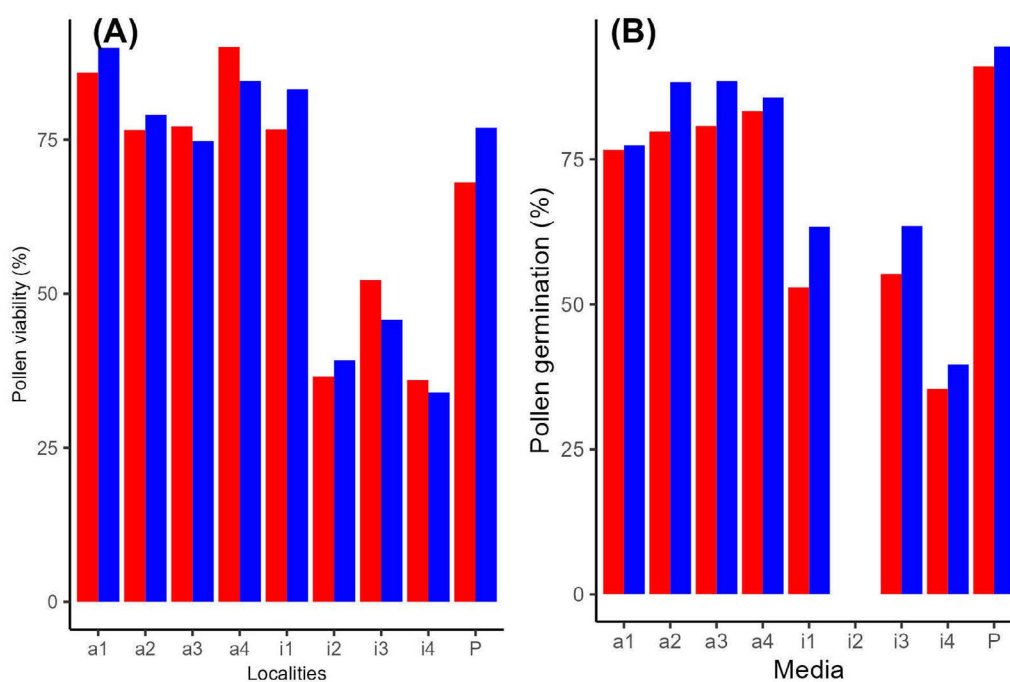

Figure 4. Percentage viability (A) and germination (B) of locations determined manually (blue) and with Fiji software (red). a1-4: Adults, i1-4: Induced, P: Pool.

Medium-term storage.- The viability of the refrigerated Pool sample was determined over time, and it was found to decrease by ~ 50 % within four months of storage (Figure 7). Although all material was rehydrated, freezing reduced the estimation of viability detected by tetrazolium staining between 13 and 29 % (Figure 8A); in contrast, frozen pollen retained between 74 and 98 % of its germinability (Figure 8B).
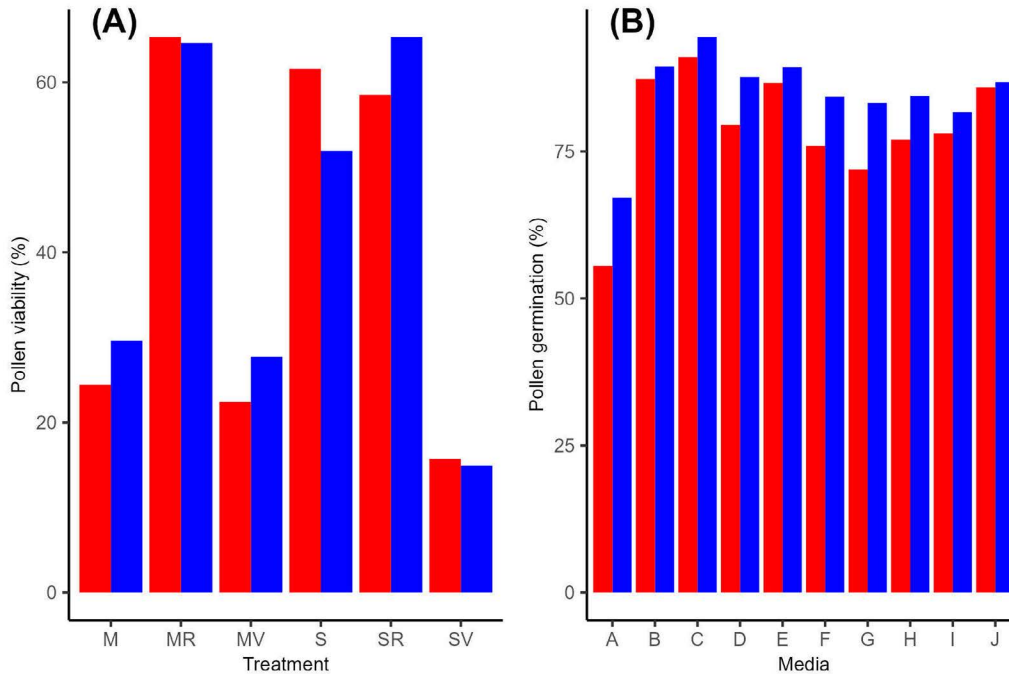
Figure 5. Germination percentage of provenances (A) and on different media (B) calculated manually (blue) and with Fiji software (red). M: dehydrated with magnesium chloride, S: dehydrated with silica gel, R: rehydrated, V: vacuum stored. A: bidistilled water, B: BK 100 % medium; C: BK 10 % medium. D to F: BK 100 % medium as a base. D: 5 % mannitol; E: 10 % mannitol; F: 5 % sucrose. G to J: BK 10 % medium as a base. G: 10 % sucrose; H: 10 % sucrose + AIA; I: 5 % mannitol; and J: mannitol 5 % + AIA.

Discussion

Standardization and validation. Assessing pollen quality through the automated analysis of digital images represents an efficient approach to monitor many samples; in the present study, manual counting of 30 images took about 45 min, while the software performed it almost immediately (~60 seconds, which also includes the generation of Excel databases), reducing time and manual labor. Although many methods exist for quantifying the number of pollen grains, few have been adapted to discern viability, and even fewer are able to quantify germinability in an easy, inexpensive, and reproducible manner (Costa & Yang 2009). Here, we applied Fiji software (Schindelin *et al.* 2012), which, by using the *Analyze particles* tool, was effectively adapted to viability staining and to the detection of morphometric changes used for assessing germinability in *P. menziesii* pollen. Furthermore, the procedure was reliable and reproducible as the programming language allowed the recording of commands to create a macro (Schindelin *et al.* 2012), which was later applied to a collection of images obtained from pollen tested under various conditions.

Staining with tetrazolium proved to be a good alternative for determining the viability of fresh *Pseudotsuga* pollen because of its rapidity, reproducibility, and accuracy. Its adaptation to the Fiji software was straightforward since viable and non-viable pollen are easily discernible in the green channel, whereas total pollen number is counted by using the red channel. The use of TTC presented the additional advantage of staining only the pollen, leaving a clear background, which improved and facilitated the handling of the images. This contrasts with what was observed with either Alexander or Peterson staining, where the background takes on a pink color that generates noise that must be removed during image processing (Tello *et al.* 2018). Adapting the macro to other similar stains, such as MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide), is feasible but would require testing and optimization, since viable pollen grains may show irregular black lines on its surface (Rodriguez-Riano & Dafni 2000), which can pose difficulties for automation.
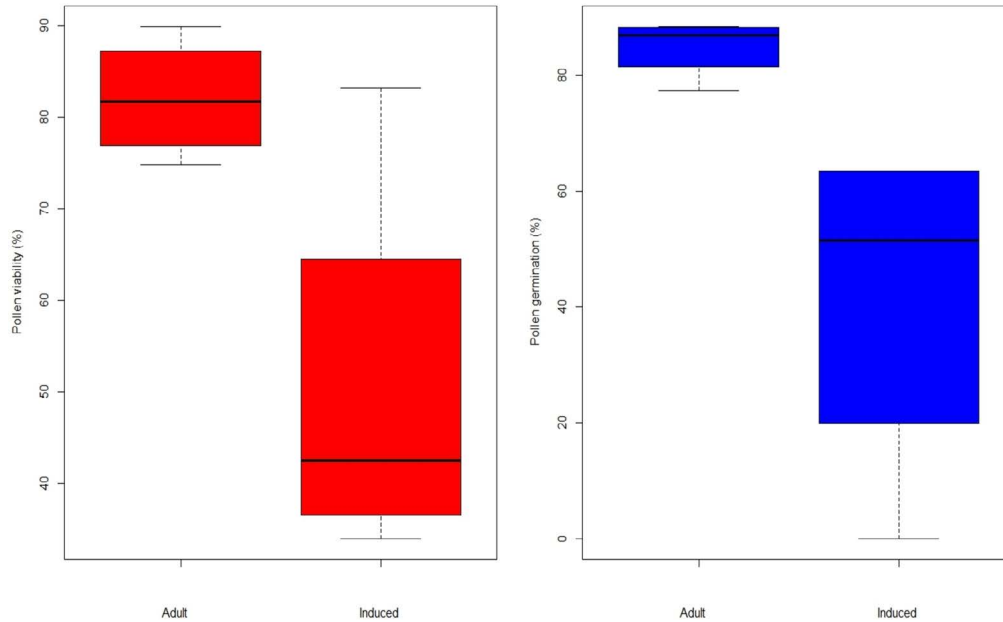
Figure 6. Comparison of viability (red) and germination (blue) percentages between adults and induced tree samples.

Regarding germinability assessment, only the four pre-germinative morphological types that recur during *in vitro* culture of *Pseudotsuga* pollen (Dumont-BéBoux & von Aderkas 1997) were considered for macro development. Each type is differentiated by changes in size and circularity, allowing the software to easily distinguish and count between the elongated shapes of the viable pollen (type 1 and 2) from the rounded shapes of non-viable pollen (type 3 and 4) (Webber & Bonnet-Masimbert 1993). This represents an advantage over what is observed in most species, where pollen tube elongation implies a challenge for the creation of a reproducible macro, since under *in vitro* conditions individual tubes grow at different speeds and can take irregular shapes (Webber & Painter 1996); these irregularities may necessitate extensive manual corrections during analysis (Castillo *et al.* 2022). This limitation may be the main reason for the lack of general macros to quantify germination in other species; however, the results presented here may be applicable to larch (*Larix*) pollen, which also follows a pre-germinative pattern similar to *Pseudotsuga*, as well as for other members of the genus (Ho & Rouse 1970, Takaso & Owens 1994, von Aderkas *et al.* 2012).

The reproducibility of the analyses was further tested using images from the assays that were conducted to guide decision making towards an efficient pollen management protocol (validation). These trials showed that the BK 10 % medium was sufficient to sustain efficient germination (94.4 %). Commonly, this medium is used in combination with sugars, osmoticants and hormones, known to promote the formation of the pre-germinative states as a result of changes in the physical properties of the cell membrane (Webber & Bonnet-Masimbert 1993, Dumont-BéBoux & von Aderkas 1997), yet we did not find this to be necessary. Additionally, because conifer pollen is generally considered to be tolerant to desiccation (Webber & Bonnet-Masimbert 1993), the impact of dehydration and rehydration on pollen quality was assessed. However, when analyzing the quality of dehydrated *Pseudotsuga* pollen, a decrease in viability between 25 and 50 % was evidenced. Using a rehydration step prior to staining allowed a significant recovery of the viability percentage. Similar reports in *Carya illinoinensis* (Wangenh.) K.Koch, showed rehydration could increase germination to 60 % (Conner 2011). Rehydration allows the cell to restore its metabolic activity and helps stabilize and activate membranes before undergoing further osmotic shock, thus improving pollen tube development and survival (Webber & Bonnet-Masimbert 1993).

Evaluation. Mature and induced trees.- In conifer breeding programs -particularly in sexual and asexual seed orchards- one of the conditions to be met is to achieve panmixia (random crossing among all individuals), which can be difficult when trees in the orchard are not phenologically synchronized, thus requiring management practices to

ensure stable production of high-quality and genetically diverse seed (Araújo de Oliveira *et al.* 2021). The application of gibberellic acid has been effective in inducing strobilus in Pinaceae because of its involvement in multiple physiological responses (Muñoz-Guitiérrez *et al.* 2010, Vargas-Hernández & Vargas-Abonce 2016). It is an actively pursued practice within genetic improvement programs, and for enhancing seed production in plantations. Hence, monitoring the quantity and quality of pollen is essential to ensure good fertilization and seed production (Ho 1991, Kears & Inouye 1993, Chhun *et al.* 2007, Kumar & Dwivedi 2014).

In this study, a comparison between adult and GA4/7 induced trees showed that overall viability and germination were lower in the latter, and that these decreased as the season progressed. This effect may reflect the carbon cost of promoting early pollen production and the formation of reproductive structures in induced trees (Wheeler *et al.* 1980). In natural circumstances, resource accumulation and allocation are critical prior to the formation of the strobili (Roland *et al.* 2014), where a trade-off in resource allocation occurs, negatively impacting other aspects of tree growth and development (Crain & Cregg 2018). Exogenously applied gibberellins may cause a redistribution of nutrients within shoots (Muñoz-Gutiérrez *et al.* 2012); for example, in *Pinus radiata* D. Don, treatment with GA4/7 resulted in a significant reallocation of ^{14}C and dry matter to and within the terminal bud (Ross 1983). Proper management, such as irrigation and fertilization before and after induction, as well as during untreated years, can help minimize the impact of the treatment and promote proper strobilus formation and pollen production. Furthermore, the effect of gibberellin injections on pollen production in Douglas fir has been shown to depend on the age and sexual maturity of the tree (Ross 1983), as well as the genotype (Webber *et al.* 1985), which may relate to the ability to metabolize exogenous GA4/7 (Kong *et al.* 2018). Likewise, because of hormone type and dosage, exogenous application affects pollen production in *P. sylvestris* L. by altering development of the strobili (Eriksson *et al.* 1998) and results in low pollen viability in *Picea mariana* (Mill.) B.S.P. (Ho 1991), as well as in angiosperms such as *Oryza sativa* (Chhun *et al.* 2007) and *Brassica campestris* L. (Kumar & Dwivedi 2014).

Medium-term storage.- The 50 % decrease in viability of the Pool sample after four months of storage at 4 °C demonstrates the importance of carrying out pollen conservation practices appropriate to the species. Although pollen storage under these conditions is reported to preserve its viability and germination rate (Kadri *et al.* 2017), these
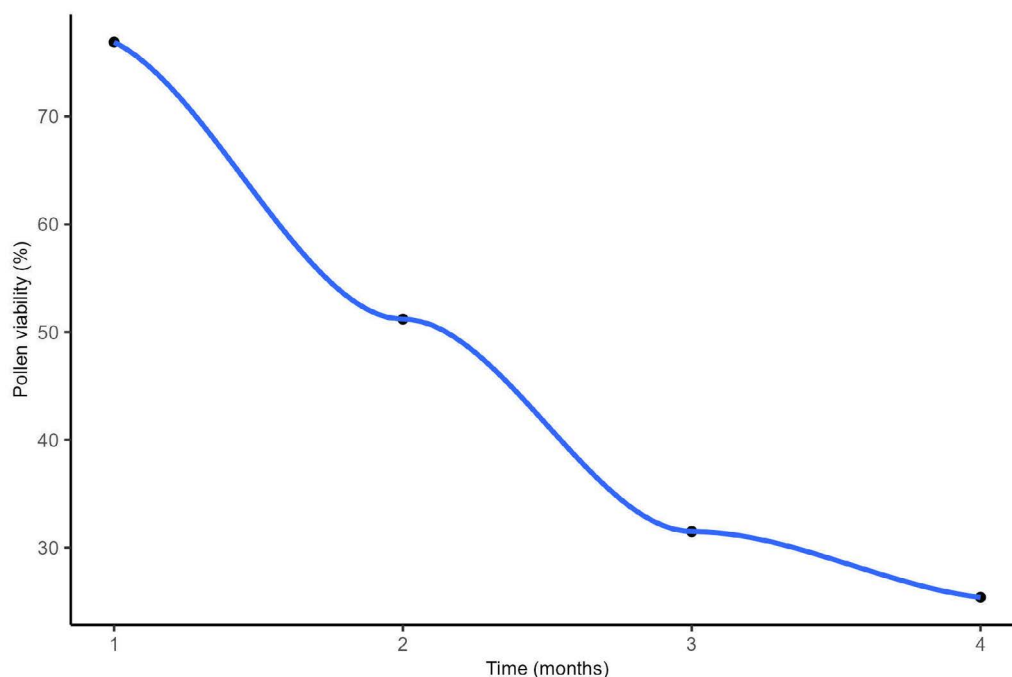

Figure 7. Percentage of "Pool" viability over 4 months.

decline over time. A decreased moisture content of 4-8 %, vacuum-sealed storage atmosphere, replacing air with nitrogen, or storage at temperature below -25 °C help promote longevity (Webber & Painter 1996), and should be tested further.

Despite the decrease in viability and germinability, our observations show that *P. menziesii* pollen was able to recover from rapid freezing with liquid nitrogen and subsequent storage at -80 °C, thus representing a feasible strategy for medium-term conservation that should be evaluated for longer periods. This agrees with reports for various species with desiccation-tolerant pollen, including conifers (Xu *et al.* 2014). Nevertheless, it is important to point out some relevant aspects of monitoring dried and frozen pollen. Firstly, assessment by tetrazolium was the most strongly affected by manipulation, usually resulting in the underestimation of viability. This contrasts with prior reports (Binder & Ballantyne 1975), although it may reflect the criteria, we used to define viability, where in addition to coloration, the circularity of the cytoplasm of the pollen grain was considered. During our tests, rehydration time may not have been sufficient to allow pollen grains to recover their shape and functionality, which may have affected the penetration of the reagent. Dehydration causes changes in the permeability of the exine and the plasma membrane, or in cytoplasmic viscosity, which can alter the abundance and function of enzyme systems (dehydrogenase) (Van Bilsen *et al.* 1994, Song *et al.* 2018), as well as limit the presence of respiratory substrates, making staining less efficient (Stanley & Linskens 1974). Under these circumstances, it is likely that evaluating germination, rather than staining, relates better to pollen fertility potential (Cook & Stanley 1960). Further exploration of incubation times and nutrient medium composition could favor adequate hydration and provide additional benefits (Webber & Painter 1996).

The two macros developed with ImageJ software (Fiji is Just ImageJ) allowed the automated evaluation of *P. menziesii* pollen viability and germination. This simplified processing of the large quantities of digital images required while standardizing conditions for effective pollen handling and storage protocols, which could streamline current and future pollination programs. Although the macros we implemented to assess *P. menziesii* pollen quality were reproducible and accurately reflected manual counts, it is important to note that they are not free from generating artifacts as a result of inconsistencies in the technical procedure between experiments, since they depend strongly on
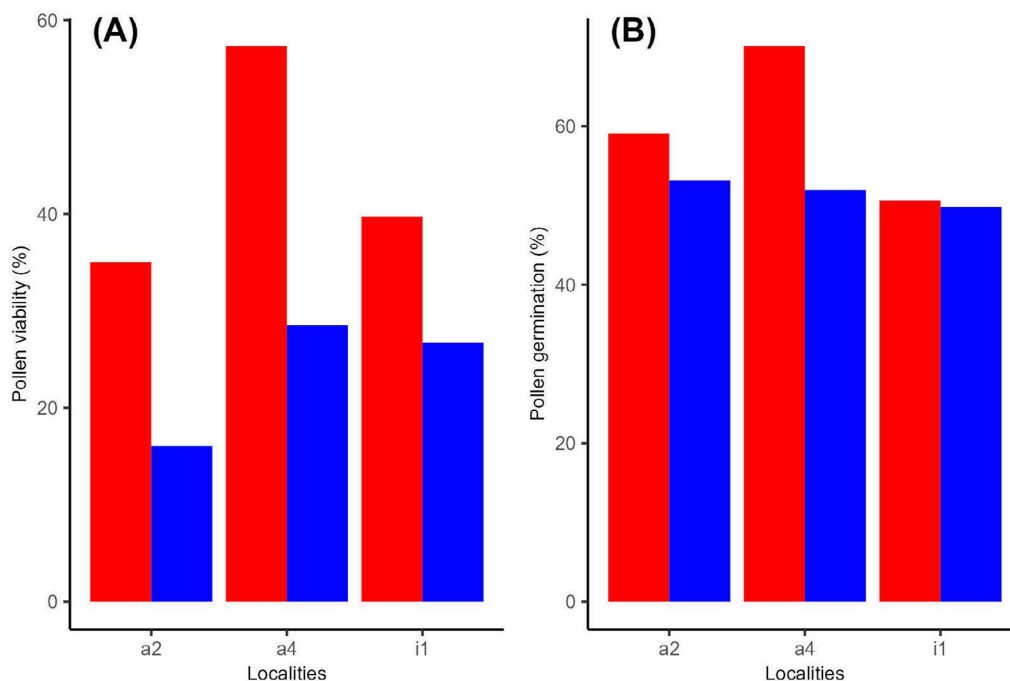

Figure 8. (A) Viability and (B) germination of samples before (red) and after (blue) freezing and storage.

high image quality. Of particular importance are the incubation times in the stain of choice (here TTC), as well as the light intensity when acquiring the images, since changes in the coloration of the pollen grains result in variations in the number of pixels. In this case, the threshold must be adjusted manually to discern between viable and non-viable pollen, affecting macro accuracy and performance when applied to the rest of the images automatically. Furthermore, to ensure high reproducibility, it is important that the pollen grains are not surrounded by particles, such as those of plant tissue, and that the grains do not overlap or are too close together. This can be achieved firstly by mixing the pollen suspension and placing a drop in the center of the slide before carefully placing the cover slide. The software can be used to correct images with overlapping grains by using the Watershed option, which separates nearby particles; however, it may produce inaccuracies when counting. In addition, the Exclude on Edges option helps to reduce the error when assessing germination since incomplete or split type 1-2 pollen grains can be taken as type 3-4, due to their proximity to the edge of an image, thereby falsely increasing the proportion of ungerminated pollen.

Supplementary material

Supplementary material for this article can be accessed here https://doi.org/10.17129/botsci.3356

Acknowledgments

Literature cited

Araújo de Oliveira AC, da Silva Lédo A, Polek M, Krueger R, Shepherd A, Volk GM. 2021. Optimization of *in vitro* germination and cryopreservation conditions for preserving date palm pollen in the USDA National Plant Germplasm System. *Plant Cell, Tissue and Organ Culture* **144:** 223-232. DOI: https://doi.org/10.1007/s11240-020-01907-1

Ayenan MAT, Danquah A, Ampomah-Dwamena C, Hanson P, Asante IK, Danquah EY. 2020. Optimizing pollen-counter for high throughput phenotyping of pollen quality in tomatoes. *MethodsX* **7**: 100977. DOI: https://doi.org/10.1016/j.mex.2020.100977

Binder W D, Ballantyne D J. 1975. The respiration and fertility of *Pseudotsuga menziesii* (Douglas fir) pollen. *Canadian Journal of Botany* **53**: 819-823. DOI: https://doi.org/10.1139/b75-099

Castillo S E, Tovar J C, Shamin A, Gutierrez J, Pearson P, Gehan M A. 2022. A protocol for *Chenopodium quinoa* pollen germination. *Plant Methods* **18**: 65. DOI: https://doi.org/10.1186/s13007-022-00900-3

Chhun T, Aya K, Asano K, Yamamoto E, Morinaka Y, Watanabe M, Kitano H, Ashikari M, Matsuoka M, Ueguchi-Tanaka M. 2007. Gibberellin regulates pollen viability and pollen tube growth in rice. *The Plant Cell* **19**: 3876-3888. DOI: https://doi.org/10.1105/tpc.107.054759

Connor KF, Towill LE. 1993. Pollen-handling protocol and hydration/dehydration characteristics of pollen for application to long-term storage. *Euphytica* **68**:77-84. DOI: https://doi.org/10.1007/BF00024157

Conner PJ. 2011. Optimization of *in vitro* pecan pollen germination. *American Society of Horticultural Science* **46**: 571-576. DOI: https://doi.org/10.21273/HORTSCI.46.4.571

Cook SA, Stanley RG. 1960. Tetrazolium chloride as an indicator of pine pollen germinability. *Silvae Genetica* **9**: 121-148.

Costa CM, Yang S. 2009. Counting pollen grains using readily available, free image processing and analysis software. *Annals of Botany* **104**: 1005-1010. DOI: https://doi.org/10.1093/aob/mcp186

Crain BA, Cregg BM. 2018. Regulation and management of cone induction in temperate conifers. *Forest Science* **64**: 82-101.

Dinato NB, Santos IRI, Leonardecz E, Burson BL, Quarín CL, de Paula AF, Fávero AP. 2018. Storage of bahiagrass pollen at different temperatures. *Crop Science* **58**: 2391-2398. DOI: https://doi.org/10.2135/cropsci2018.03.0164

Dumont-BéBoux N, Von Aderkas P. 1997. In vitro pollen tube growth in Douglas-fir. *Canadian Journal of Forest Research* **27:** 674-678. DOI: https://doi.org/10.1139/x96-219?journalCode=cjfr

El-Kassaby YA, Ritland K. 1986. The relation of outcrossing and contamination to reproductive phenology and supplemental mass pollination in a Douglas-fir seed orchard. *Silvae Genetica* **35**: 240-4.

El-Kassaby YA, Reynolds S. 1990. Reproductive phenology, parental balance, and supplemental mass pollination in a Sitka-spruce seed-orchard. *Forest Ecology and Management* **31**: 45-54. DOI: https://doi.org/10.1016/0378-1127(90)90110-W

Eriksson U, Jansson G, Almqvist C. 1998. Seed and pollen production after stem injections of gibberellin A4/7 in field-grown seed orchards of Pinus sylvestris. *Canadian Journal of Forest Research* **28**: 340-346. DOI: https://doi.org/10.1139/x97-219

Excel (12.0). 2007. Microsoft. Web site. https://www.microsoft.com/es-mx/microsoft-365/excel?rtc=1 (accessed April 5, 2022)

Gugger P F, González-Rodríguez A, Rodríguez-Correa H, Sugita S, Cavender-Bares J. 2011. Southward Pleistocene migration of Douglas-fir into Mexico: phylogeography, ecological niche modeling, and conservation of 'rear edge' populations. *New Phytologist* **189**: 1185-1199. DOI: https://doi.org/10.1111/j.1469-8137.2010.03559.x

Hermann RK, Lavender DP. 1999. Douglas-fir planted forests. *New Forests* **17**: 53-70. DOI: https://doi.org/10.1023/A:1006581028080

Ho RH. 1991. Promotion of cone production in potted black-spruce grafts, using gibberellins, heat-treatment and root-pruning. *Forest Ecology and Management* **40**: 261-269. DOI: https://doi.org/10.1016/0378-1127(91)90044-V

Ho R, Rouse GE. 1970. Pollen germination of *Larix sibirica* (Siberian larch) in vitro. *Canadian Journal of Botany* **48:** 213-215. DOI: https://doi.org/10.1139/b70-032

Kadri K, Boaaga K, Makhlouf S. 2017. Physiological characterization of some male pollinators in Tunisia and study of the effect of conditioning temperature on the viability and germination of pollen. *Journal of New Sciences* **48**: 2907-2920.

Kears CA, Inouye DW. 1993. Pollen. *In:* Inouye DW. ed, *Technique for pollination biologists.* Niwot, CO: University Press of Colorado, pp. 77-124. ISBN: 0-87081-281-5

Kong L, von Aderkas P, Zaharia LI. 2018. Effects of stem-injected gibberellins and 6-benzylaminopurine on phytohormone profiles and cone yield in two lodgepole pine genotypes. *Trees* **32**: 765-775. DOI: https://doi.org/10.1007/s00468-018-1670-7

Kumar G, Dwivedi K. 2014. Gibberellic acid-mediated male sterility during gametogenesis of *Brassica campestris* L. *Chromosome Botany* **9**: 59-63. DOI: https://doi.org/10.3199/iscb.9.59

Lee WY, Lee JS, Lee JH, Noh EW, Park EJ. 2011. Enhanced seed production and metabolic alterations in *Larix leptolepis* by girdling. *Forest Ecology and Management* **261**: 1957-1961. DOI: https://doi.org/10.1016/j.foreco.2011.02.022

Li Y, Li X, Zhao MH, Pang ZY, Wei JT, Tigabu M, Zhao XY. 2021. An overview of the practices and management methods for enhancing seed production in conifer plantations for commercial use. *Horticulturae* **7**: 252. DOI: https://doi.org/10.3390/horticulturae7080252

López-Upton J, Valdez-Lazalde JR, Ventura-Ríos A, Vargas-Hernández JJ, Guerra-de-la-Cruz V. 2015. Extinction risk of *Pseudotsuga menziesii* populations in the central region of Mexico: An AHP analysis. *Forests* **6**: 1598-1612. DOI: https://doi.org/10.3390/f6051598

Muñoz-Gutiérrez L, Vargas-Hernández JJ, López-Upton J, Gutierrez-Rangel N. 2010. Induction of reproductives structures in *Pseudotsuga menziesii*. *Agrociencia* **44**: 835-847.

Muñoz-Gutiérrez L, Vargas-Hernández JJ, Santos-Posadas HM, López-Upton J. 2012. Effect of GA4/7 and stem girdling on shoot growth in *Pseudotsuga menziesii* (Mirb.) Franco. *Revista Fitotecnia Mexicana* **35**: 239-249.

Owens JN, Morris SJ. 1990. Cytological basis for cytoplasmic inheritance in *Pseudotsuga menziesii*. I. Pollen tube and archegonial development. *American Journal of Botany* **77**: 433-445. DOI: https://doi.org/10.1002/j.1537-2197.1990.tb13574.x

Redmond MD, Davis TS, Ferrenberg S, Wion AP. 2019. Resource allocation trade-offs in a mast-seeding conifer: piñon pine prioritizes reproduction over defence. *AoB Plants* **11**: plz070. DOI: https://doi.org/10.1093/aobpla/plz070

Rodriguez-Riano T, Dafni A. 2000. A new procedure to asses pollen viability. *Sexual Plant Reproduction* **12**: 241-244. DOI: https://doi.org/10.1007/s004970050008

Roland CA, Schmidt JH, Johnstone JF. 2014. Climate sensitivity of reproduction in a mast-seeding boreal conifer across its distributional range from lowland to treeline forests. *Oecologia* **174**: 665-677. DOI: https://doi.org/10.1007/s00442-013-2821-6

Ross SD. 1983. Enhancement of shoot elongation in Douglas-fir by gibberellin A4/7 and its relation to the hormonal promotion of flowering. *Canadian Journal of Forest Research* **13**: 986-994. DOI: https://doi.org/10.1139/x83-131

RStudio Team. 2020. RStudio: Integrated Development for R. RStudio. https://www.rstudio.com/categories/rstudio-ide/ (accessed April 5, 2022)

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY. 2012. Fiji: an open-source platform for biological-image analysis. *Nature Methods* **9**: 676-682. DOI: https://doi.org/10.1038/nmeth.2019

SEMARNAT [Secretaría del Medio Ambiente y Recursos Naturales]. 2010. Norma Oficial Mexicana NOM-059-SEMARNAT-2010, Protección ambiental - Especies nativas de México de flora y fauna silvestres - Categorías de riesgo y especificaciones para su inclusión, exclusión o cambio - Lista de especies en riesgo. Diario Oficial de la Federación. 2da Sección, 30 de diciembre de 2010. https://www.dof.gob.mx/normasOficiales/4254/semarnat/semarnat.htm (accessed November 18, 2022)

Song YS, Lee SH, Jo JA, Choi SH, Seo DJ, Wi SG, Jung, W. J. 2018. Changes in the activity of peroxidase and dehydrogenase in pear pollen during germination under different storage conditions. *Horticultural Science and Technology* **36**: 777-788. DOI: https://doi.org/10.12972/kjhst.20180076

Stanley RG, Linskens HF. 1974. Viability tests. *In: Pollen: Biology Biochemistry Management.* Springer-Verlag, pp. 67-86. DOI: https://doi.org/10.1007/978-3-642-65905-8_6

Takaso T, Owens JN. 1994. Effects of ovular secretions on pollen in *Pseudotsuga menziesii* (Pinaceae). *American Journal of Botany* **81**: 504-513. DOI: https://doi.org/10.1002/j.1537-2197.1994.tb15475.x

Tello J, Montemayor MI, Forneck A, Ibáñez J. 2018. A new image-based tool for the high throughput phenotyping of pollen viability: evaluation of inter-and intra-cultivar diversity in grapevine. *Plant Methods* **14**: 1-17. DOI: https://doi.org/10.1186/s13007-017-0267-2

van Bilsen DG, Hoekstra FA, Crowe LM, Crowe JH. 1994. Altered phase behavior in membranes of aging dry pollen may cause imbibitional leakage. *Plant Physiology* **104**: 1193-1199. DOI: https://doi.org/10.1104/pp.104.4.1193

Vargas-Hernández JJ, Vargas-Abonce JI. 2016. Effect of giberellic acid (GA4/7) and girdling on induction of reproductive structures in *Pinus patula*. *Forest Systems* **25**: 7. DOI: https://doi.org/10.5424/fs/2016252-09254

Ventura-Ríos A, López Upton J, Vargas-Hernández JJ, Guerra-de la Cruz V. 2010. Characterization of populations of *Pseudotsuga menziesii* (MIRB.) Franco in central México: Implications for conservation. *Revista Fitotecnia Mexicana* **33**: 107-116.

Vieitez-Cortizo E. 1952. The use of 2,3,5-triphenyltetrazolium chloride to determine the vitality of pollen. *Anales de Edafología y Fisiología Vegetal* **11**: 1033-1044.

von Aderkas P, Nepi M, Rise M, Buffi F, Guarnieri M, Coulter A, Gill K, Lan P, Rzemieniak S, Pacini E. 2012. Post-pollination prefertilization drops affect germination rates of heterospecific pollen in larch and Douglas-fir. *Sexual Plant Reproduction* **25**: 215-225. DOI: https://doi.org/10.1007/s00497-012-0193-4

Webber JE, Bonnet-Masimbert M. 1993. The response of dehydrated Douglas fir (*Pseudotsuga menziesii*) pollen to

three in vitro viability assays and their relationship to actual fertility. *Annales des Sciences Forestières* **50**: 1-22. DOI: https://doi.org/10.1051/forest:19930101

Webber JE, Painter RA. 1996. *Douglas-fir pollen management manual*. Ministry of Forests Research Program. https://www.for.gov.bc.ca/hfd/pubs/docs/wp/wp02.htm (accessed February 3, 2022)

Webber JE, Ross SD, Pharis RP, Owens JN. 1985. Interaction between gibberellin A4/7 and root-pruning on the reproductive and vegetative process in Douglas-fir. II. Effects on shoot elongation and its relationship to flowering. *Canadian Journal of Forest Research* **15**: 348-353. DOI: https://doi.org/10.1139/x85-056

Wheeler NC, Wample RL, Pharis RP. 1980. Promotion of flowering in the Pinaceae by gibberellins: IV. Seedlings and sexually mature grafts of lodgepole pine. *Physiologia Plantarum* **50**: 340-346. DOI: https://doi.org/10.1111/j.1399-3054.1980.tb04111.x

Xu J, Li B, Liu Q, Shi Y, Peng J, Jia M, Liu Y. 2014. Wide-scale pollen banking of ornamental plants through cryopreservation. *Cryo Letters* **35**: 312-319.