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Original article

The response of dehydrated Douglas fir (*Pseudotsuga menziesii*) pollen to three *in vitro* viability assays and their relationship to actual fertility

JE Webber [1], M Bonnet-Masimbert [2]

[1] *British Columbia Ministry of Forests, Research Branch Laboratory, 1320 Glyn Road, Victoria, BC V8W 3E7, Canada;*
[2] *INRA, Station d'Amélioration des Arbres Forestiers, Centre de Recherche d'Orléans, Ardon, 45160 Olivet, France*

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Summary — *In vitro* viability response of Douglas fir pollen stored for various periods (1 to several years) was related to actual seed set. Three assay types that provided useful relationships to seed set were respiration (RESP), percent leachate conductivity (%COND) and percent germination (CLASS 1 + 2). Before developing the relationship to seed set, media effects on germination, leaching time for conductivity and pollen hydration effects for all assays were studied. Both simple linear and non-linear regression analyses were compared to percent filled seed per cone (%FSPC) as determined from controlled crossing pollinations. Media type had a significant effect on germination response which, in the time of the test (48 h), appeared to be related to osmotic rather than metabolic effects. Hydrating stored dehydrated pollen for 16 h at 100% RH and 25 °C prior to the analysis had a significant effect on improving the response for conductivity and germination, but had no significant effect on respiration. Hydration effects were also apparent on the correlation coefficient *(r)* using simple linear regression. For unhydrated and hydrated pollen, the *r* values for assay response and %FSPC were 0.70 and 0.85 for RESP, –0.36 and –0.86 for %COND, and 0.07 and 0.83 for CLASS 1 + 2 germination, respectively. Using non-linear regression models, the coefficient of determination (r^2) values for assay response of unhydrated and hydrated pollen against %FSPC were 0.76 and 0.83 for RESP, and 0.24 and 0.82 for %COND, and 0.61 and 0.84 for CLASS 1 + 2 germination, respectively. The regression equations developed for respiration, percent conductivity and germination can be applied to Douglas fir pollen lots when used for controlled crossing pollinations but may not result in expected seed set values when the pollen lot is expected to also compete with outcross pollen.

***Pseudotsuga menziesii* / Douglas fir / pollen / respiration / germination / viability / fertility / seed-set**

Résumé — Réponse du pollen de sapin de Douglas *(Pseudotsuga menzesii)* à 3 tests de viabilité *in vitro* et relation avec la fertilité réelle de ce pollen. *Les valeurs du coefficient de détermi-*

nation (r^2) pour le pollen sec et le pollen réhydraté pour la réponse aux différents tests et %FSPC sont respectivement (tableau V) de 0,76 et 0,83 pour RESP (respiration), à 0,24 et 0,82 pour %COND (pourcentage de conductivité) et 0,61 et 0,84 pour la germination (CLASS 1 + 2). Par ailleurs, à travers une expérience de dilution de pollen, il apparaît que la relation entre le pourcentage de pollen vivant et le %FSCP n'est pas linéaire (fig 5). Au-delà d'un seuil voisin de 40–50% de pollen vivant, il n'y a plus d'amélioration du %FSCP. D'un point de vue pratique, les équations de régression développées pour la respiration (fig 6), le pourcentage de conductivité (fig 7) et la germination (fig 8) peuvent être utilisées pour estimer la qualité de lots de pollen de sapin de Douglas utilisés pour des croisements contrôlés. Toutefois, ces courbes peuvent ne pas se traduire par le résultat attendu en terme de rendement en graines si un lot donné de pollen se trouve en situation de compétition avec un autre lot, ce qui n'était pas le cas de cette série d'expérimentations.

Pseudotsuga menziiesii ***/ sapin de Douglas / pollen / respiration / germination / variabilité / fertilité / lot de graines***

INTRODUCTION

As advanced generation Douglas fir seed orchards become established, the need to protect potential genetic gain becomes more important. In the Pacific Northwest, the threat of inferior gametic infiltration into orchard populations is a constant concern and estimated levels of contamination range from 6–56% (Smith and Adams, 1983; El-Kassaby and Ritland, 1986a; Wheeler and Jech, 1986a). Asynchronous flowering (El-Kassaby and Ritland, 1986b), disproportionated fecundity among clones (El-Kassaby *et al,* 1989), and inbreeding (Woods and Heaman, 1989) can also reduce the genetic efficiency (see Adams, 1983; El-Kassaby *et al,* 1984) of orchard seed. One approach to reducing the effects of contaminating pollen and improving genetic efficiency is supplemental mass pollination (SMP).

SMP has been successfully used to improve the balance of paternal contribution (El-Kassaby and Ritland, 1986b), improve seed yields (Webber, 1987) and reduce the negative impact of selfing and contamination (El-Kassaby and Ritland, 1986b; Wheeler and Jech, 1986b). However, success of SMP is dependent on many factors (see Bridgwater *et al,* 1991) not least of which is ensuring that the pollen applied has, at least, comparable fertility potential (ability to set seed) to that of competing pollen.

Pollen management procedures for handling Douglas fir pollen have been tested and, in particular, successful storage technique are now used routinely (Webber, 1987; Webber and Painter, in preparation). However, methods for assessing pollen viability *in vitro* and relating the results to seed set remain rudimentary. The objectives of this study are to optimize the response of 3 viability assays (respiration, leachate conductivity and germination) using stored Douglas fir pollen and to relate these responses to actual seed set. The study also considers the effect of pollen hydration on *in vitro* assay response and its relationship to actual seed set.

MATERIALS AND METHODS

Selection of pollen lots

Douglas fir (*Pseudotsuga menziesii* (Mirb) Franco) pollen was collected over many years from both the tree breeding and seed orchard programs. All pollen lots (referred to as a family of pollen grains arising from a single clone or seedling) were stored at a pollen moisture content of < 8% and at –20 °C in evacuated containers

(see Webber, 1987; Webber and Painter, in preparation). Storage period for each pollen lot varied and ranged from 1–5 yr.

In vitro *viability assays*

Germination

Media type

The procedure for germinating Douglas fir pollen initially followed the technique described by Ho and Sziklai (1972). Their medium was adapted from Brewbaker and Kwack (1963) and included H_3BO_3 (0.1 mg/ml), $Ca(NO_3)_2.4\ H_2O$ (0.3 mg/ml), $MgSO_4.7\ H_2O$ (0.2 mg/ml) and KNO_3 (0.1 mg/ml). The germination medium was a 10% dilution (10B) of the Brewbaker and Kwack (1963) solution which also contained sucrose (5 or 10%) and/or indole acetic acid (10 ppm). This 10B medium was satisfactory to germinate Douglas fir pollen, but the presence of sucrose also facilitated the growth of contaminants associated with the pollen. Antibiotics (nystatin and chloramphenicol) have been used to reduce the growth of contaminants (Charpentier and Bonnet-Masimbert, 1983) but for the short incubation periods used (see below), they were not required in the germination medium.

The first media experiments compared the germination of 8 dehydrated stored Douglas fir pollen parents in 4 aqueous solutions: deionized water (H_2O), 10% sucrose (10S), 10% Brewbaker and Kwack (1963) solution (10B), and 10% polyethylene glycol (PEG molecular weight 4000...10P) using the procedures described below. Agar (1.0% agar in 10% Brewbaker's solution) was also considered as a solid medium, with and without added constituents, but it was not used in subsequent trials because germination was slower and scoring response was more difficult. In a second experiment, the germination response of the same dehydrated lots were compared in 4 concentrations of PEG-4000 (10, 20, 30, and 40%) with or without the inclusion of the 10B.

Germination procedure

For comparing germination media types, 3 ml of medium were added to 35-mm Petri dishes and 10 mg of pollen sprinkled over the surface. The Petri dish lid was replaced and the dish was then placed in a larger Petri dish (90 mm) containing absorbent paper saturated with water. The lid of the larger Petri dish was secured and germination allowed to proceed at 25 °C for 48 h. No particular precautions were taken to either exclude light or use specified photoperiods.

After 48 h, germination was scored based on the percent of grains in each of 4 categories: Class 1, pollen grains elongated greater than twice the original hydrated diameter of the grain; Class 2, pollen grains showed signs of elongation but were still less than twice their hydrated diameter; Class 3, pollen grains showed no sign of elongation; and finally, any pollen grains from either of the 3 classes showing any amount of plasmolysis or other damage were scored as Class 4 (see fig 1). The actual number of germinating pollen grains counted followed the procedures suggested by Stanley and Linskens (1974) for determining significant response differences at the 95% confidence level. For pollen lots germinating in the 50% range, ≈ 300 grains were observed and for lots germinating either > 90% or < 10%, ≈ 100 grains were counted. All results were expressed as percent germination of either Class 1 or Class 1 + 2 grains.

Conductivity

Leaching of pollen lots followed the procedures of Ching and Ching (1976) in which 100 mg of pollen was soaked in 30 ml deionized water (specific conductance < 2 µS/cm) at 25 °C for 60 min with constant shaking. Initially, the leachate was filtered or centrifuged to remove the residual pollen debris. However, it was determined that removing the residue had little if any effect on conductivity measurements and simply letting the pollen suspension settle for 5 min prior to measurement was sufficient. The conductance of the filtrate was determined using a standard conductivity meter (Orion Model 101) with an immersion cell (platinum electrodes). All measurements were made at 25 °C.

A time of leaching experiment was also completed for the hydrated pollen lots only. In this test, all lots were weighed (100 mg), hydrated and then leached for 1, 2, 4, 6 and 24 h. After leaching, the conductivity of the leachate was determined and then expressed as a percentage of the total leachate (hot conductivity). After cold (25 °C) conductivity was determined, the solution was boiled for 60 min, cooled to 25 °C, deionized water added as required to make the

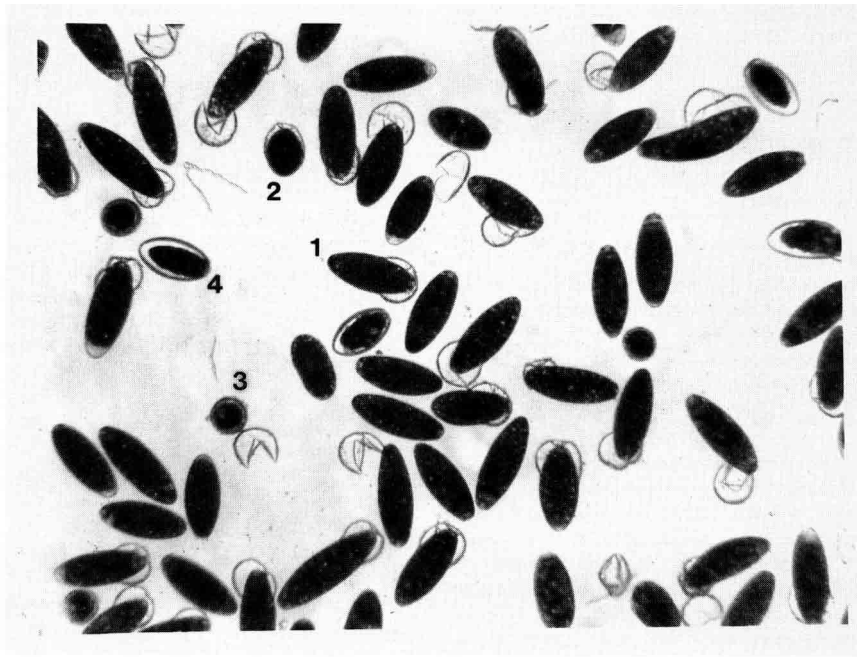

Fig 1. The 4 germination classes of Douglas fir pollen after 48 h using the medium 10B20P. Class 1 grains elongate > twice the hydrated diameter of the ungerminated pollen; Class 2 grains show elongation less than twice the unhydrated diameter; Class 3 grains show no elongation; and Class 4 grains are any grains showing damage (*ie* plasmolysis).

total vol 30 ml, and the conductivity recorded. All cold conductivity (COND) results were expressed on a dry weight basis of the pollen sample used (*ie* μS/cm/g dw). Where the results were expressed as percent conductivity (%COND), the ratio of cold to hot leachate conductivity was determined.

Respiration

Measurement of oxygen uptake by pollen in an aqueous solution followed the procedures of Binder and Ballantyne (1975). Depletion of oxygen in 3 ml vol deionized water was determined by a YSI oxygen probe (Model 5331 Clark type polarographic electrode) using a YSI standard water bath assembly (Model 5301) and oxygen monitor (Model 5300). Uptake was measured at a constant 30 °C and the output recorded on a strip chart recorder using 1 V as full range (100%).

About 100 mg of pollen (dw) was added to 3 ml deionized water contained in the cuvette of the water bath assembly and allowed to equilibrate at 30 °C for 3 min with constant stirring. After equilibration, stirring was stopped, the electrode was inserted making certain all air bubbles were excluded, and stirring resumed. Oxygen uptake was recorded for a minimum of 5 min using a chart speed of 1 cm/min. The rate of oxygen consumption was calculated using the percent change in volume of dissolved oxygen for a 5-min period and the solubility of oxygen in air-saturated water at 1 atm pressure as 5.48 μl O_2/ml at 30 °C (Lessler, 1969). Variation in oxygen solubility due to changes in atmospheric pressure during any particular test were small and, therefore, ignored. Results for oxygen consumption (RESP) were expressed as μl O_2/min/

gdw where g dw was the dry weight of pollen used.

Pollen preconditioning

All pollen lots tested were previously stored and, therefore, in a dehydrated state (< 10% moisture content). Hydrating Douglas fir pollen prior to the assay has been shown to increase both the germination (Charpentier and Bonnet-Masimbert, 1983; Jett and Frampton, 1990) and conductivity (Webber and Bonnet-Masimbert, 1989) response. The effect of preconditioning pollen by hydration or *in vitro* assay response and the correlation between assay response and filled seed per cone was considered in the 2 regression experiments described below.

Where pollen hydration technique was used, the procedures of Charpentier and Bonnet-Masimbert (1983) were followed. Pollen lots to be hydrated were first weighed and then exposed to a saturated atmosphere for 16 h at 25 °C. Hydrated pollen was assayed immediately after treatment and the response compared to a sample of the unhydrated pollen. All assay responses were based on the dry weight of pollen used. The dry weight of pollen was then calculated from the known percent moisture content of the pollen prior to hydration. Mellerowicz and Bonnet-Masimbert (1986) demonstrated that hydration of pollen prior to pollination had no effect on filled seed per cone. Consequently, hydration as a factor in field fertility trials was not considered.

Pollen moisture content effects on simple linear regression

Ten samples of Douglas fir pollen lots were randomly selected from previously stored lots. Pollen lots were hydrated for 16 h and then tested using the 3 *in vitro* assays described. These tests were completed ≈ 2 wk prior to field pollinations. Field fertility trials (see also section on *In vivo fertility)* used the following design: 10 pollen lots applied in replicate (2 bags per lot) to each of 8 seed-cone trees. Seed-cone trees (clones) were randomly selected among those trees with a sufficient crop to provide a minimum of 20 pollination bags each containing 3–6 seed-cone buds.

Non-linear regression analysis

Effect of diluting douglas fir pollen on filled seed per cone

Fertility response is seldom linear to viability response. To determine the effect of a range of pollen viabilities on seed set, a single Douglas fir pollen lot with a high fertility potential (collected from Cowichan Lake Research Station) was diluted with neat-killed pollen (4 h at 85 °C). Pollen dilutions ranged from 100% live to 100% dead (13 separate dilutions). Each dilution was tested on each of 2 trees using 2 replicates (bags) per tree. Pollination technique was slightly different than described in In vivo *fertility*. In this test, syringe pollinators were used. The syringe plunger was replaced with a small glass tube attached to a rubber bulb. When squeezed, the rubber bulb provided a slight pressure within the syringe barrel and propelled pollen out of the syringe needle towards the receptive flower. All other aspects of bagging, cone collections and seed extraction were as described. Average seed yield values were expressed as filled seed per cone (FSPC).

The effect of viability on filled seed per cone

Ninety Douglas fir pollen lots were selected from both tree breeding and seed orchard collections. Pollen samples from each lot were removed from storage and placed in glass vials with tight-fitting lids. Moisture contents were determined and oxygen uptake measured according to the technique described. Lots were not hydrated before testing. All 90 lots were ranked by oxygen uptake ($\mu l\ O_2/min^{-1}\ g^{-1}$ dw) and then arbitrarily classed into 4 viability categories: poor (0–4), low (5–12), moderate (13–21), and good (> 22). Within each category, 10 pollen lots were randomly selected.

The selected 40 pollen lots were tested using respiration, conductivity and germination assays as previously described. Each lot was tested in both its hydrated and unhydrated state. Non-linear regression procedures (see *Statistical analyses* for details) were used to estimate coefficients of determination between RESP, COND, %COND, CLASS 1, CLASS 1 + 2 and percent filled seed per cone (%FSPC).

Each of the 10 pollen lots from each viability class (40 lots) was field tested for fertility using 4 full-sibling seedlings from the Canadian Pacific Forests Products low elevation seed orchard in Saanichton, BC. A total of 80 isolation bags containing either 2 or 3 seed cones per bag were placed on each of the 4 trees. Each of the 40 pollen lots were randomly assigned to 2 replicates on each tree. For regression analyses, mean values bulked by replicate and clone were used (*ie*, $N = 4$).

Pollinations were completed using the procedures described in *In vivo* fertility. Cones were harvested by replicates but cones were kept separate and hand extracted individually. All potential seed per cone were extracted from each cone and the filled seed per cone determined by X-ray analyses as described below.

In vivo *fertility*

All pollen lots tested for *in vitro* viability were also tested for *in vivo* fertility using controlled crossing pollinations. Specific details for each test are given for each experiment. Common to all tests was the bagging and pollination technique.

Seed-cone trees (either grafts or full-sibling seedlings ranging in age from 10–20 yr old) were selected on the basis of crop intensity and vigour from various orchards or clone banks on Vancouver Island. In particular, the clone banks at Cowichan Lake Experimental Station, Cowichan Lake, BC and the seed orchard of Canadian Pacific Forest Products, Sannichton, BC were used. On each selected seed-cone tree, pollen-cone buds on each sample branch were removed and seed-cone buds were isolated in pollination bags prior to bud burst. In all cases, large, white pollination bags (obtained from DRG Packaging Ltd, Toronto, Ontario) with plastic windows were used for initial isolation. Smaller brown "corn-tector" bags (product No 402, obtained from Lawson Pollination Bags, Northfield, IL) were used to isolate fewer seed-cone buds (2–3) on sample branches. Placed within each bag was a 1-cm cube of no-pest strip (supplied by various manufacturers but all having the active ingredient of 18% Dichlorvos) to prevent insect damage.

Optimal time to pollinate Douglas fir seed-cone buds for maximum seed yields is within 2–4 d beyond bud burst (Owens *et al*, 1981; Owens and Simpson, 1982; Webber, 1987). For consistency, all seed-cone buds were pollinated at 2 d beyong burst using ≈ 0.2 ml pollen. Pollen was applied using a compressed nitrogen driven pollination device (contact senior author for details). In the fall, mature seed cones were collected when the bracts began to flex and the cones started to turn brown. Seed cones were dried and hand extracted. All seed with a developed seed coat were separated from the non-developed ovules and counted. This represented the total potential seed per cone (PSPC). The number of filled seed per cone (FSPC) was determined by X-ray analyses using Kodax Industrex 620 paper and a Hewlett–Packard (Faxitron series Model 43855A) operating at 15 kVp for 2 min. The percent filled seed per cone was calculated from the ratio of FSPC to PSPC and expressed as %FSPC.

Statistical analyses

All statistical analyses were completed using SAS PC (SAS Institute Inc, 1988). To determine significant differences (a level of 0.05) between media types by germination class, χ^2 statistics were used. For the 4 media types, individual pairs were compared using the output of Proc Probit. The critical P value was calculated using the Bonferroni correction (0.05/6 = 0.0083). Means with the same letter (see figs 2, 3A,B) were not significantly different at the critical P value of 0.0083. For comparing the effect of hydration on assay response, a paired t-test was used.

For *in vitro* assays, the experimental unit was a pollen lot (defined as a family of pollen grains arising from a single clone including 1 or several ramets). For field fertility trials, controlled crossing pollination technique was used and individual clones were the experimental unit and cones were the sampling unit. Linear and non-linear regression, analyses were completed on the average filled seed per cone per replicate (where applicable) then averaged per clone (tree level) or bulked by clones (orchard level).

For simple linear regressions, the variables RESP, COND, % COND, CLASS 1 and CLASS 1 + 2 by hydration level were compared against %FSPC. For non-linear regressions, the variables RESP, CLASS 1 and CLASS 1 + 2 were

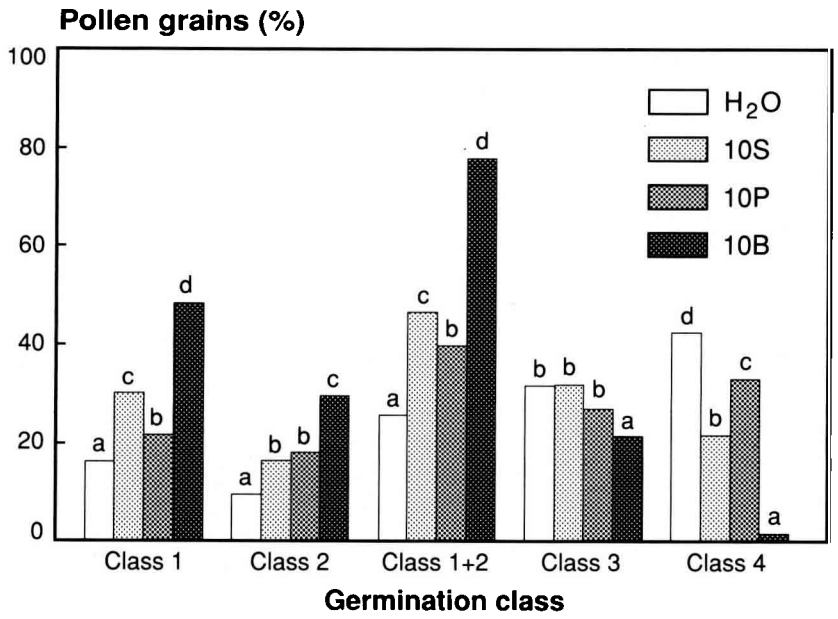

Fig 2. Germination response of unhydrated Douglas fir pollen to 4 media types by germination class: de-ionized H_2O; 10% sucrose (10S); 10% Brewbaker and Kwak (1963) solution (10B); and, 10% PEG-4000 (10P). Media types with the same letter are not significantly different at the critical P value of 0.0083 ($N = 8$).

compared with %FSPC using a logistic function in the form of:

$$y = \frac{a}{1 + e^{c+bx}}$$

For conductivity data, a hyperbola function was used in the form of: $y = ae^{bx}$

For each equation, the parameters a, b and c were approximated by iterating the best fit using Proc Nlin (non-linear) procedures. The coefficient of determination (r^2) was calculated from the corrected (CSS) and residual sum of squares (RSS), *ie*:

$$r^2 = \frac{CSS - RSS}{CSS}$$

The value S_{y-x} was also determined from the square root of the residual mean square error term and represents an average estimate of error about any point on the curve of predicted values (see figs 6–8).

RESULTS

Germination medium

Figure 1 gives examples of the 4 classes of germinating Douglas fir pollen. Figure 2 shows the average germination response (by class) of 8 Douglas fir pollen lots in each of 4 media types: deionized water

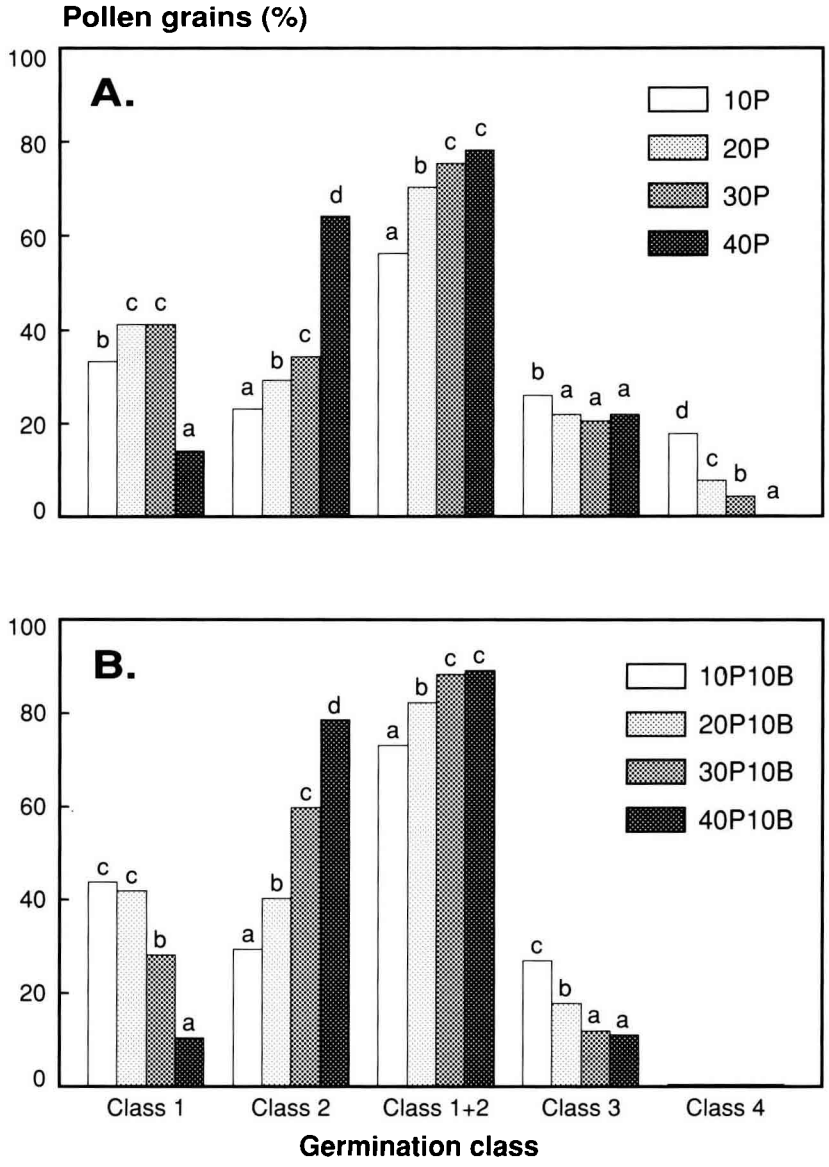

Fig 3. Germination response of unhydrated Douglas fir pollen to 4 concentrations of PEG-4000 solutions (10–40% PEG depicted as 10P-40P) alone (**A**) and with (**B**) Brewbeker and Kwack (1963) solution (10B) by germination class. Media types with the same letter are not significantly different at the critical *P* value of 0.0083 ($N = 4$).

(H_2O); 10% sucrose (10S); 10% Brewbaker and Kwack (1963) solution (10B); and, 10% polyethylene glycol-4000 (10P). Media type had a strong effect on the proportion of damaged pollen grains (Class 4). The percent of Class 4 grains for the 4 media types were all significantly different from one another with 10B showing the lowest proportion (0.08%) followed by 10S (21.6%), 10P (33.1) and H_2O (42.4%). The proportion of pollen grains not germinating (Class 3) was also lowest for the medium 10B. There was no significant difference between the percentage of Class 3 grains for the other media types. The proportion of germinating Class 1, 2 and 1 + 2 grains was significantly highest in 10B compared to the other 3 media types.

Figure 3 contrasts the germination response (by class) of 4 levels of PEG-4000 concentrations alone (fig 3A) and with the 10B medium (fig 3B). With PEG alone (fig 3A), there was a steady decrease in Class 4 damaged grains with increasing concentration of PEG (all significantly different from one another). The lowest concentration of PEG (10P) yielded the highest proportion of Class 3 (non-germinating) grains which was significantly different from the other three. As the concentration of PEG increased, the proportion of Class 1 grains showed a significant increase from 10P to 20P, no significant difference between 20P to 30P, then a significant decrease with the 40P media. For the proportion of Class 2 grains, there was a significant increase over the range of 10–40% PEG. Comparing Class 1 + 2 grains with media type, there was a significant increase over the range of 10–30% PEG but no significant difference between 30–40% PEG.

The addition of 10% Brewbaker's solution to the 4 PEG concentrations completely eliminated the Class 4 grains (fig 3B). Also, the addition of 10B to the 4 concentrations of PEG further lowered the proportion of Class 3 grains but only at the 3 higher levels of PEG. For the 10P10B and 20P10B media, there was no significant difference between the proportion of Class 1 grains but there was a significant decrease over the 30P10B and 40P10B media. Correspondingly, the proportion of Class 2 grains increased significantly over the 4 media types. Likewise, Class 1 + 2 grains increased significantly over the 10P10B to 30P10B media but showed no further significant increase for the 40P10B media.

Based on these data, the media 20P10B was selected for testing the germination of Douglas fir pollen *in vitro*. Although the 30P10B and 40P10B media yielded the highest proportion of Class 1 + 2 grains (88.1 and 89.0%, respectively), they also yielded significantly lower proportions of Class 1 grains (28.3 and 10.5%, respectively). There was no significant difference between the proportion of Class 1 grains for the 10P10B and 20P10B media but the proportion of Class 1 + 2 grains was significantly higher for 20P10B.

Conductivity analyses: leaching time

Figure 4 shows the response of percent conductivity (%COND) by viability class for 40 hydrated Douglas fir pollen lots over 5 leaching times. The 4 viability classes were distinguished from each other by percent conductivity after 1 h. The poor viability class pollen lots had much higher %COND values while the moderate and good viability class pollen lots produced the lowest %COND values and showed the least differences. Over a 6-h period, %COND values rose gradually for all viability classes and after 24 h, the values approached 80% of the total leachable material. The coefficient of determination (r^2) values for both COND and %COND against %FSPC were calculated for each of the 5 leaching times using the corrected

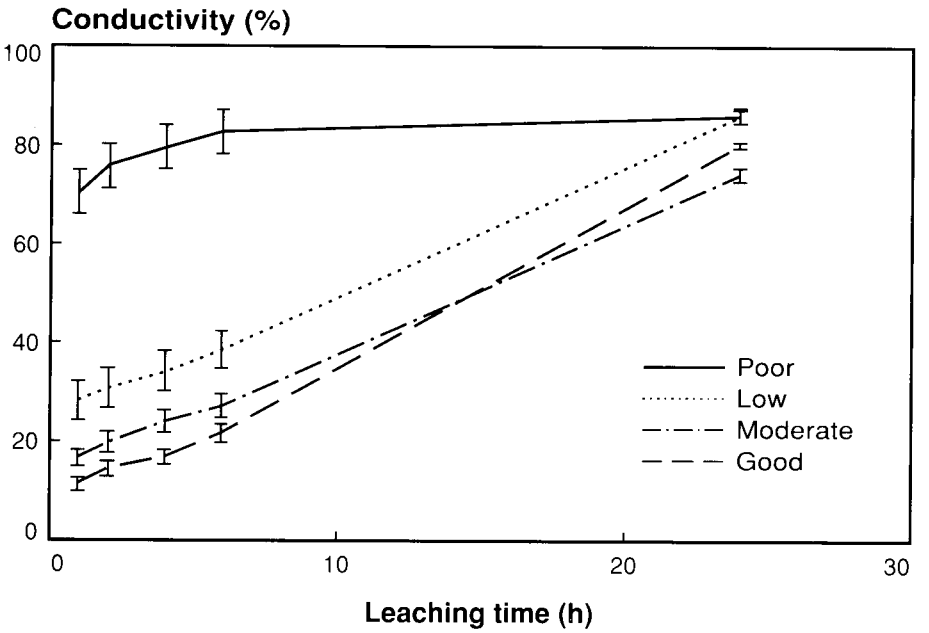

Fig 4. Mean percent conductivity values of 40 hydrated Douglas fir pollen lots segregated into 4 viability classes (10 lots per class) and leached for 5 extraction periods ($N = 10$).

and residual sum of squares from the output of SAS non-linear regression procedures (see *Statistical analyses).* Table I shows a slight decline of r^2 values for both COND and %COND up to 6 h leaching with a large drop in r^2 at 24 h. Based on these data, a 1-h leaching time yielded %COND values which when used in the hyperbolic function described under *Statistical analysis,* will explain nearly 82% of the variability in %FSPC.

Simple linear regression: the effect of pollen moisture content

Table II compares the mean (± SE) assay response for respiration, conductivity, percent conductivity and germination (Class 1 and 1 + 2) for 10 Douglas fir pollen lots

Table I. Coefficients of determination (r^2) for 4 hydrated Douglas-fir pollen lots segregated into 4 viability classes comparing conductivity (COND) and percent conductivity (%COND) values leached for 5 different periods against mean seed set (%FSPC) from each of 4 clones ($N = 4$).

Leaching time (h)	*Coefficient of determination (r^2)* [1]	
	COND	*%COND*
1	0.753	0.817
2	0.728	0.799
4	0.704	0.780
6	0.654	0.749
24	0.083	0.184

[1] r^2 values were calculated from the output of Proc Nlin (SAS version 6.03) using the corrected sum of squares (CSS) and the residual sum of squares (RSS): r^2 = (CSS–RSS)/(CSS).

that were either hydrated or unhydrated. Average moisture content of the 10 dehydrated lots was 3.5%. After 16-h exposure at 100% RH and 25 °C, the average moisture content was 25.9%. Only respiration showed no significant improvement in response due to hydration. Both conductivity and germination responses were significantly improved by hydration. Total leachate (511.9 *vs* 315.0 μS/cm/g dw) and percent conductivity (57.4 *vs* 35.0%) were lower and germination response for Class 1 (10.0 *vs* 48.5%) and Class 1 + 2 (17.7 *vs* 67.4%) were higher when exposed to 100% RH for 16 h at 25 °C prior to the assay.

Table III shows the correlation coefficient *(r)* derived from simple linear regression analyses for mean assay response (both hydrated and unhydrated pollen) against seed set (FSPC and %FSPC). In all cases, hydrating pollen lots prior to the assay improved *r* values. For respiration, *r* values were less affected by hydration state than those for conductivity or germination. As expected, the *r* values for mean assay response against seed set were considerably better if the seed-cone parent trees were bulked (N = 10) than if the seed-cone trees were considered as a separate factor (N = 80, data not shown).

Non-linear regression analysis

The effect of diluting Douglas fir pollen

Figure 5 shows the relationship between FSPC and the percent live pollen for each of 13 dilutions. Each value point represents the average of 2 replicates on each of 2 seed-cone clones. As the proportion of live pollen rose from 0 to 50%, there was a steady almost linear increase in FSPC. However, beyond ≈ 40–50% live pollen, no corresponding increase in FSPC was observed.

In terms of a threshold level, this corresponded to ≈ 35–40 FSPC. For Douglas fir, this represents ≈ 55% PSPC based on an average potential of 64–70 seeds per cone (Ho, 1980) arising from 32–35 ovuliferous scales per cone (Owens *et al,* 1991). Assuming all other factors equal, higher vi-

Table II. Mean assay response (± SE) for 10 Douglas fir pollen lots comparing hydration state for respiration (RESP), conductivity (COND), percent conductivity (%COND) and germination (Class 1, Class 1 + 2). (N = 10).

	Assay response		p *value* [1]
	Unhydrated	*Hydrated*	
RESP [2]	16.7 (2.5)	17.9 (2.3)	0.134
COND [3]	511.9 (39.0)	315.0 (61.6)	< 0.001
%COND	57.4 (2.5)	35.0 (2.3)	< 0.001
Clas 1	10.0 (3.6)	48.5 (7.0)	< 0.001
Class 1 + 2	17.7 (5.3)	67.4 (8.9)	< 0.001

[1] *p* value derived from an ANOVA using pairs as blocks; [2] units for respiration are μl O_2/min/g dw; [3] units for conductivity are μS/cm/g dw.

Table III. Correlation coefficients from a simple linear regression model comparing hydrated and unhydrated pollen for respiration (RESP), conductivity (COND), percent conductivity (%COND) and germination (Class 1, Class 1 + 2) against mean seed set response (FSPC and %FSPC) from 8 seed-cone clones ($N = 8$).

	Correlation coefficient (r)			
	Unhydrated		*Hydrated*	
	FSPC	*%FSPC*	*FSPC*	*%FSPC*
RESP	0.66	0.70	0.84	0.85
COND	−0.23	−0.37	−0.75	−0.86
%COND	−0.20	−0.36	−0.73	−0.86
Class 1	0.04	0.10	0.67	0.78
Class 1 + 2	0.07	0.07	0.71	0.83

ability of pollen is associated with higher FSPC. However, there is a limit beyond which increasing viability is not associated with increasing FSPC. For Douglas fir pollen used in controlled crossing pollinations, it appears that pollen can be diluted to ≈ 50% before any reduction in FSPC is observed.

With regards to pollen viability, it is not certain whether a pollen lot yielding fewer seeds per cone compared to a more fertile pollen lot has fewer live pollen grains (assuming a pollen grain is either fertile or not) or if all the grains are just less fertile (assuming that the fertility of a pollen grain can vary). Our *in vitro* viability assays cannot distinguish between the 2 possibilities. However, this pollen dilution study does indicate the FSPC response of a pollen lot in which the pollen grain is either alive or dead. Under these conditions, a pollen lot with < 50% functional grains is associated with decreasing fertility. Presumably under these conditions any viability assay (*ie* germination) would be a direct indication of the proportion of fully functional pollen grains (see fig 8).

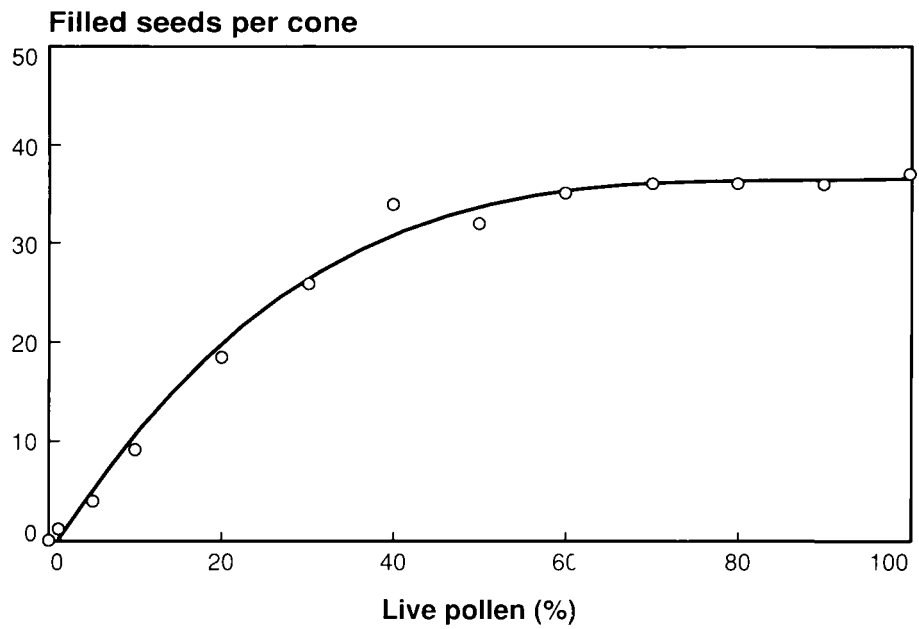

Fig 5. The effect of diluting live Douglas fir pollen with heat-killed pollen on seed set (FSPC) ($N = 2$).

The effect of pollen viability

To determine the effect of viability and the non-linear response of FSPC, 40 pollen lots, representing 10 from each of 4 viability classes were all tested under the same conditions. Figures 6–8 show scatter plots for %FSPC against respiration, percent conductivity and percent germination (Class 1 + 2), respectively for 40 pollen lots (segregated into 4 viability classes). Also shown is the curve of predicted values derived from the non-linear regression equation (parameters shown), the coefficient of determination (r^2), and the S_{y-x} value (an average estimate of error about any point on the curve).

In general, the %FSPC from each of the 4 viability classes sort out according to their respective rating. The good lots (good and moderate classes) produced the best %FSPC and the poor lots (poor and low classes) yielded the lowest %FSPC. Comparing the 3 viability assays with the 4 viability classes, there was considerable variation in ranking between lots within a class but average values for lots by class were ranked according to their respective class. The rank order for respiration is expected since the original ranking of the 90 lots

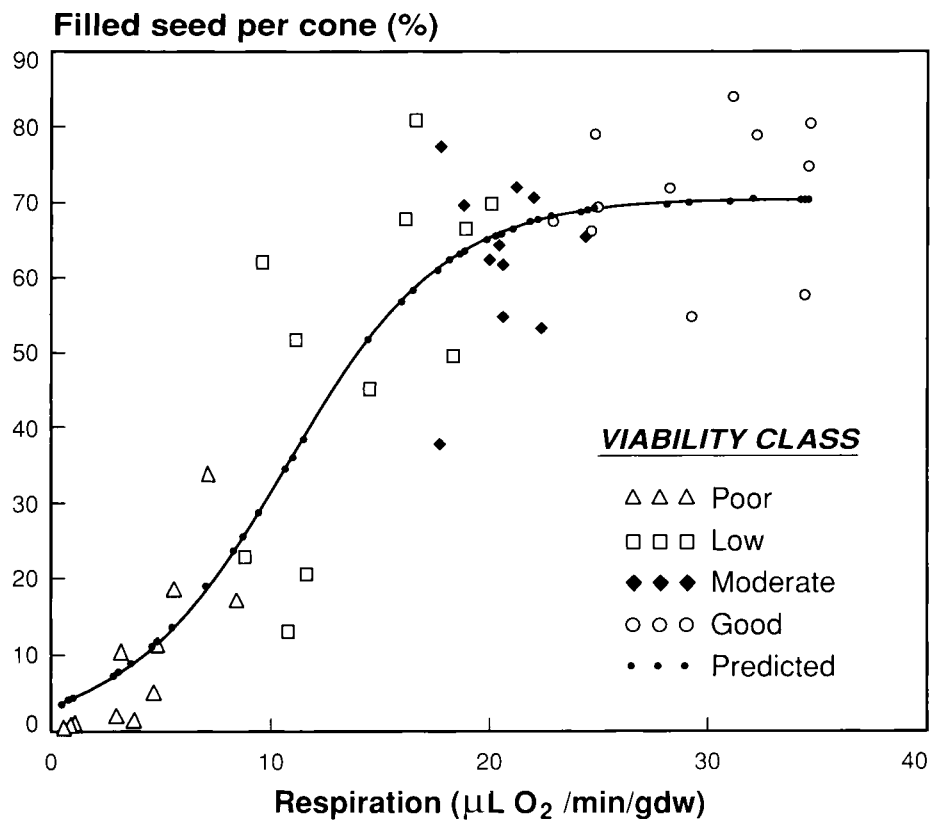

Fig 6. The relationship between %FSPC (mean values from each of 4 clones) and respiration values for 40 hydrated Douglas fir pollen lots segregated into 4 viability classes ($N = 4$). The parameters for the logistic function are $a = 70.4$, $b = -0.28$ and $c = 2.98$ with values for $S_{y-x} = \pm 11.7$ and $r^2 = 0.832$.

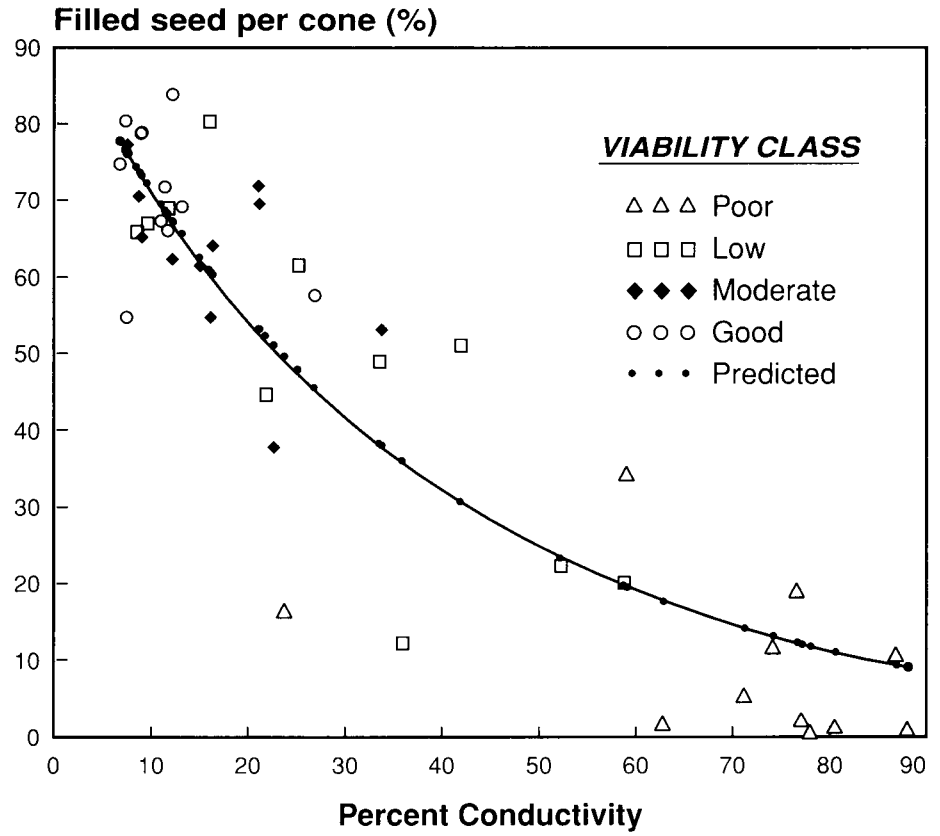

Fig 7. The relationship between %FSPC (mean values from each of 4 clones) and percent conductivity values for 40 hydrated Douglas fir pollen lots segregated into 4 viability classes ($N = 4$). The parameters for the hyperbolic function are $a = 93.0$ and $b = -0.026$ with values for $S_{y-x} = \pm 12.0$ and $r^2 = 0.817$.

was done using respiration values from unhydrated lots. Table IV shows the mean response for each of the 4 classes to each of the 3 viability assays plus the FSPC and %FSPC values by viability class. The improved response for conductivity and germination with hydration is again apparent. The 3 assays (RESP, %COND and CLASS 1 + 2) as well as FSPC and %FSPC all rank according to their respective viability classes with the single exception of germination CLASS 1.

Considering individual pollen lots by viability class, all of the good lots and 9 of the moderate class lots produced a minimum of 50% PSPC (≈ 35 FSPC). The poor and low viability class pollen lots showed a wide range of variability. Of the 10 pollen lots in the low viability class, 6 produced a minimum of 50% PSPC (range for all 10 pollen lots was 12.5–80.4% PSPC). None of the poor viability class pollen lots produced %FSPC > 40%. For all 4 viability classes, 25 of the 40 lots produced at least

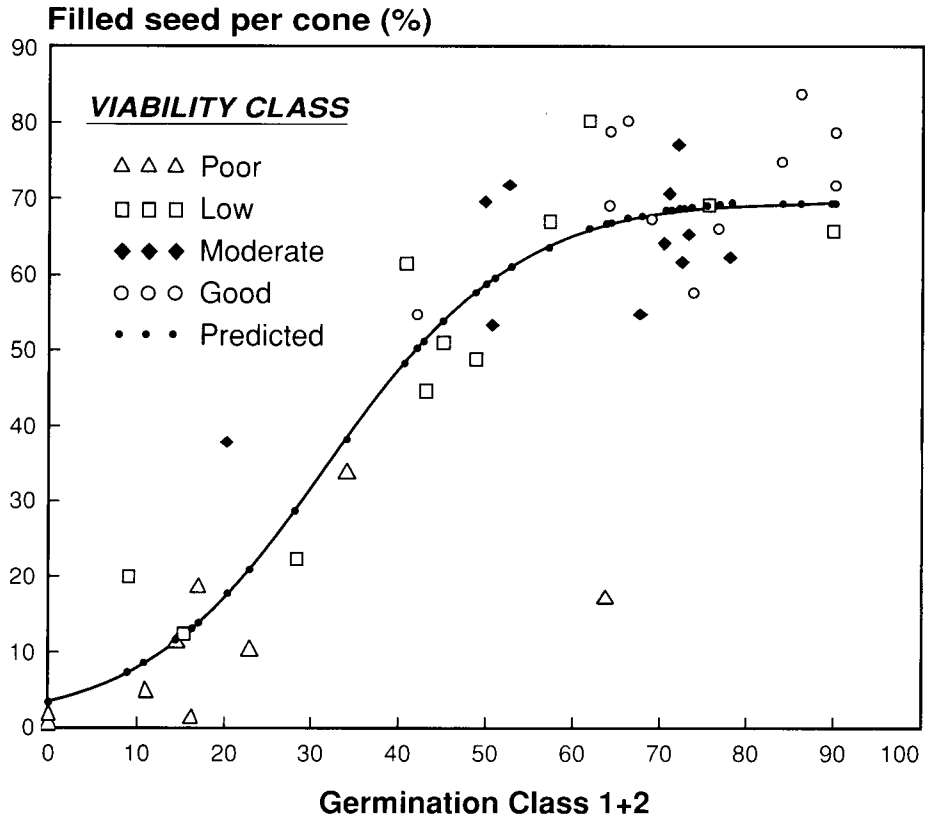

Fig 8. The relationship between %FSPC (mean values from each of 4 clones) and percent germination (Class 1 + 2) values for 40 hydrated Douglas fir pollen lots segregated into 4 viability classes ($N = 4$). The parameters for the hyperbolic function are $a = 69.7$, $b = -0.093$ and $c = 2.98$ with values for $S_{y-x} = \pm 11.5$ and $r^2 = 0.838$.

50% PSPC. This corresponded to assay ranges of 9–33 µl O_2/min/g dw for respiration, 10–45% conductivity, and 40–90% germination (Class 1 + 2).

Coefficients of determination (r^2) between each of the 3 *in vitro* assays (hydrated and unhydrated) and their corresponding %FSPC are shown table V. The importance of hydration on improving the r^2 values, especially for conductivity and germination assays, is again apparent. Coefficient of determination for respiration, percent conductivity and percent germination (Class 1 + 2) are all > 0.8, suggesting that > 80% of the observed variation in %FSPC can be explained by assay response.

For all 3 assays, classifying pollen lots into good, moderate, low and poor categories showed responses indicative of their class (figs 6–8). Both the good and moderate classes produced yields that would be considered operationally acceptable (50% PSPC or better). The poor and low viability class pollen lots produced %FSPC that, in general, were unacceptable. Furthermore,

Table IV. Mean assay response for respiration (RESP), percent conductivity (%COND), and germination (CLASS 1 and CLASS 1 + 2) for unhydrated and hydrated pollen lots segregated into four viability classes (N = 10) and mean seed set values for each viability class (N = 4).

Viability assay	*Viability class*			
	Poor	*Low*	*Moderate*	*Good*
RESP 1				
Unhydrated	2.9	8.3	17.3	26.6
Hydrated	3.8	13.8	20.5	29.6
%COND				
Unhydrated	88.8	81.5	61.6	59.2
Hydrated	70.2	28.1	16.3	11.3
CLASS 1				
Unhydrated	3.0	6.5	16.1	21.4
Hydrated	9.1	23.5	19.8	10.5
CLASS 1 + 2				
Unhydrated	10.2	43.2	56.4	64.4
Hydrated	16.3	46.5	62.8	74.3
Seed set				
FSPC	5.4	34.0	42.1	49.1
%FSPC	8.0	49.5	61.6	71.4

[1] Units for respiration are μl O_2/min/g dw.

Table V. Coefficients of determination (r^2) for 40 hydrated Douglas fir pollen lots segregated into 4 viability classes comparing hydration state for respiration (RESP), conductivity (COND), percent conductivity (%COND) and germination (Class 1, Class 1 + 2) against mean seed set (%FSPC) from 4 seed-cone trees (N = 4).

	Coefficient of determination (r^2) [1]	
	Unhydrated	*Hydrated*
RESP	0.762	0.832
COND	0.282	0.753
%COND	0.239	0.817
Class 1	0.279	0.366
Classe 1 + 2	0.608	0.838

[1] r^2 values were calculated from the output of Proc Nlin (SAS version 6.03) using the corrected sum of squares (CSS) and the residual sum of squares (RSS): r^2 (CSS–RSS)/(CSS).

the low viability class pollen lots showed considerable variation suggesting that the predictive values of these pollen lots from the regression equations will be highly variable.

DISCUSSION

In vitro *assays*

Potential fertility of Douglas fir pollen can be adequately assessed using respiration, conductivity and germination as *in vitro* tests. For germination, media type had a large effect on response. In water alone, unhydrated pollen showed extensive damaged (Class 4) grains (see fig 2). The osmotic potential of deionized water was high relative to the pollen grain. If the osmotic potential of the media is decreased (by adding sucrose, PEG or Brewbakers solution), the proportion of Class 4 grains decreased and the proportion of Class 1 and 2 grains increased (figs 2, 3). The proportion of Class 3 grains was relatively unaffected by changing osmotic potential (fig 2).

For most conifers, the constituents of the germination media are relatively simple. Sugar (generally sucrose) solutions are most often used which appears to act as both an osmoticum and a substrate for respiration (see Stanley and Linskens, 1974; pp 67–76). Sucrose has also been reported to be an essential component of

the *in vitro* germination of *Pinus roxburghii* pollen (Dhawan and Malik, 1981) but in short-term (< 48 h) tests it may not be essential as a carbon source but rather acts as an osmoticum (Nygaard, 1977). This appears to be the case for Douglas fir pollen. For short-term germination tests, sucrose is not an essential but an osmoticum is. Among the many types of non-metabolizable substrates, polyethylene glycol can provide a wide range of water potentials (Stenter *et al,* 1981) and has been used successfully to germinate pollen of other species (Zhang and Croes, 1982; Subbaiah, 1984). Since PEG is an inert osmoticum, it is preferred.

Calcium and boron have also been implicated as important germination media constituents in some angiosperms (Johri and Vasil, 1961; Brewbaker and Kwack, 1963) and some pines (Nygaard, 1970; Dhawan and Malik, 1981). Apparently calcium is essential to maintain the structural integrity of pollen membranes (Nygaard, 1970) while the role of boron in tube growth is not known.

PEG-4000 has been used previously for pollen germination and water relationship studies, primarily because it was considered to be too large a molecule to be taken up by the cell. However, recent reports suggest that PEG-4000 may be taken up by the cell (Jacomini *et al,* 1988), in which case the osmotic potential of the medium and pollen grain would change. How PEG might affect germination of Douglas fir pollen, other than osmotic effects, is not known. The importance of water relations in the germination of pollen suggests PEG effects warrant further study.

The results of these media experiments suggest that in the early stages (< 48 h) of Douglas fir pollen germination, response may be more related to the physical properties of cell membrane hydration and elasticity than to metabolic activation. Although PEG has less effect on the overall germination response (*ie* Class 1 + 2), the proportions of Class 1 and Class 2 grains can be significantly affected by various concentrations of PEG (figs 2, 3). It is also interesting to note that the r^2 value for germination Class 1 grains is very poor (table V) even with hydration. This may be attributed to a media effect in which case, germination response must include both Class 1 + 2 grains. In addition to the stabilizing effects of PEG, the inorganic constituents of Brewbaker and Kwack (1963) medium are also important. By comparing sucrose (10S) and PEG (10P) against Brewbaker and Kwack's media (10B), the inorganic constituents of 10B produced the lowest proportion of damaged (Class 4) pollen grains (figs 2, 3). Whether the 10B medium acts principally as an osmoticum, membrane stabilizer or some combination of both is not known. Regardless, the combination of the osmotic stablizing effects of PEG and the inorganic constituents of Brewbaker and Kwack's solution (20P10B) yielded the best germination response which when used with the logistic regression equation accounted for over 80% of the variation in FSPC.

Pollen moisture content

For most angiosperm pollen, dehydration has a deterimental effect on its fertility potential (Shivanna and Heslop-Harrison, 1981). While this effect can often be reversed by rehydration, some species are more sensitive to dehydration than others. In corn pollen, for example, dehydration below 20% moisture content leads to irreversible loss of viability (Kerhoas *et al,* 1987). In conifers, however, dehydration of pollen does not have such a severe effect on fertility potential.

The importance of pollen moisture content (hydration state) for *in vitro* assay response has been clearly demonstrated by

Charpentier and Bonnet-Masimbert (1983) for Douglas fir pollen, Jett and Frampton (1990) for Loblolly pine pollen, and Foushee (1990) for western White pine pollen. For Douglas fir and Loblolly pine pollen, the benefit of a 16-h hydration period (at 100% RH) on germination response was apparent but the magnitude of the response was dependent on the pollen moisture content prior to hydration. For Douglas fir, the assay response to hydration was greater if the pollen moisture content was < 7% (Charpentier and Bonnet-Masimbert, 1983) whereas for Loblolly pine, the threshold level was ≈ 15% (Jett and Frampton, 1990).

For the pollen lots studied in these experiments, moisture content average ≈ 6–8%. The effect of rehydrating pollen prior to *in vitro* assay increased pollen moisture content of ≈ 26% and produced the greatest increase in assay response for conductivity and germination. Respiration response showed no significant increase with hydration. These results confirm earlier observations for both conductivity and germination response of Douglas fir pollen to hydration (Webber and Bonnet-Masimbert, 1989). It is now a matter of protocol to rehydrate all Douglas fir pollen lots for 16 h at 100% RH and 25 °C prior to testing.

Hydration effects were also apparent from simple linear regression analyses (see table II) with higher *r* values associated with hydrated assay response and seed set compared to its unhydrated pair (see table II). Apparently, hydration is required to both stabilize (lower conductivity values) and activate (increased germination values) pollen membranes. Respiration, however, appears to be less sensitive to membrane hydration state. Moody and Jett (1990) reported no significant effect on respiration rates due to rehydration in Loblolly pine.

Although respiration rates in Douglas fir pollen appear to be less sensitive to hydration, both the average respiration response (table IV) for pollen lots within the 4 viability classes and the correlation coefficient *(r)* and coefficient of determination (r^2) measured against %FSPC (tables III and V) all improved. However, the effect of hydration on conductivity and germination response and their relationship to %FSPC was more apparent.

Predicting potential seed yields

Previous reports (Ching and Ching, 1976) have developed the relationship between various viability assays and germination but few have actually correlated the assay with field fertility. Binder and Ballantyne (1975) reported a positive relationship between respiration and fertility and suggested that pollen lots with respiration rates of 20 nmol O_2/min/100 mg at 30 °C (equivalent to ≈ 5 µl O_2/min/g) were probably capable of producing seed. Data collected in our experiments suggest that pollen lots with respiration values of 5 µl O_2/min/g dw will produce seed (≈ 20% PSPC or 12 FSPC) but the yields would be too low for operational use. Such a low viability pollen lot could yield seed for breeding purposes using controlled crossing technique. However, low viability pollen could not be expected to compete well in open pollination where higher viability pollen also occur (Webber and Yeh, 1987; Apsit *et al,* 1989).

More recently, Moody and Jett (1990) reported r^2 values between germination and respiration rates for Loblolly pine pollen lots and total seed to be 0.88 and 0.81, respectively. Furthermore, Moody and Jett (1990) were able to generate exponential response curves for both germination and respiration rate against percent filled seed as a function of pollen age.

In Douglas fir, PSPC is limited by the number of developed ovules available. Because there is a limit beyond which any increase in pollen viability is not equally matched by an increase in FSPC, correlation analyses based on simple linear regression models do not adequately describe this non-linear response. Another problem in developing relationships between assay response and FSPC is ensuring that a wide range of pollen viabilities are included in the test to generate a good relationship.

The results shown in tables II–V confirm the beneficial effects of hydration for improving assay response and reducing the non-explainable regression variation between assay response and fertility. The logistic regression model used for respiration and germination responses and the hyperbola model used for conductivity response against %FSPC seems to fit the data well. Although it may be possible to improve the relationship by using other models, the equations shown in figures 6–8 allow us to explain over 80% (see table V) of the variation within the data. The remaining 20% of the variability is likely related to field pollination technique, pollination mechanism, and male–female interactions (Apsit *et al,* 1989).

Using the appropriate equations for predicting %FSPC from the response of respiration, percent conductivity and percent germination (see figs 6–8), it is possible to predict the seed set response from controlled crossing experiments. However, for operational pollination programs, these models may not be applicable where competition between lots of differing viabilities can occur. Under these conditions, it may be better to consider only 2 viability classes: acceptable and unacceptable.

If 50% PSPC is established as an operational seed production target, then the threshold values for accepting or rejecting a pollen lot would be 14 μl O_2/min/g dw for respiration, 25% of the total leachate for conductivity, or 45% germination for Class 1 + 2 grains. Again, it must be emphasized that these results were obtained from controlled crossing technique.

Applying these threshold values to the 40 lots, the number of pollen lots failing to meet the expected %FSPC were 5, 5 and 4, respectively using respiration, conductivity and germination assays. The number of pollen lots that met or exceeded the threshold value for respiration, conductivity and germination but did not produce the expected 50% PSPC were 3, 2 and 2, respectively. Conversely, the number of pollen lots that produced 50% PSPC but did not meet the threshold value for respiration, conductivity and germination were 2, 3 and 2, respectively.

If a pollen lot is used for controlled crossing, then it may be possible to lower the critical assay value, especially if < 50% yields are acceptable. Thus, for single lot application, lots with values > 10 μl O_2/min/g dw for respiration, < 50% leachate for conductivity, or > 30% Class 1 + 2 for germination can be expected to produce acceptable seed yields (30% or ≈ 20 FSPC). However, if lots falling within this viability range are used in polymixes or expected to compete with outcross pollen, then one cannot expected similar results. Fowler (1987) and Cheliak *et al* (1987) have studied both the biological and genetic implications of using polymixes and each recommend keeping the number of male parents within the polymix as high as possible to prevent significant distorsion of male contribution.

It may be possible to mimic controlled crossing results under open pollination conditions but timing of pollination and application technique must be stricly controlled. In Douglas fir, the pollination mechanism is such (see Owens *et al,* 1981) that

pollen arriving first at the stigmatic tip has the avantage of completing the subsequent steps towards fertilization over pollen grains arriving later (Webber and Yeh, 1987). Pollination technique can also affect FSPC values. Pollen applicators that propel the pollen at the receptive strobili using compressed gas driven devices have consistently yielded higher FSPC values (Webber, 1991; and unpublished data) compared to more passive pollinator types (*ie*, paintbrushes and misting pollinators). It should be possible, then, to influence the proportion of applied male parents in Douglas fir using early pollination with lots of high viability and applied using compressed gas driven pollinators.

The non-linear regression models developed for respiration, conductivity and germination procedures may also be useful for estimating the relative viability of pollen lots being used in a polymix. This may have particular importance when < 10 lots are used within the mix. As the number of clones within a seed orchard is reduced to maximize genetic gain potential and the number of pollen parents are reduced to capitalize on specific traits, then the differential viability among pollen parents will become very important.

CONCLUSION

The procedures described here for the *in vitro* assay of Douglas fir pollen have also been used for other species within British Columbia's tree improvement program. Respiration and conductivity procedures are as described, but germination media varies slightly for each species.

It is now a matter of routine to store pollen from White spruce *(Picea glauca)*, Western hemlock *(Tsuga hetrophylla)*, Lodgepole pine *(Pinus contorta)* and Western larch *(Larix occidentalis)* at moisture contents < 8% (Webber, unpublished data). These species also respond similarly to hydration technique, although the hydration periods vary somewhat. Where tested, the relationships between hydrated assay response and FSPC also show some degree of improvement over the unhydrated assay response. However, considerable field testing is still required for these species to develop the predictive response for seed set that was developed for Douglas fir.

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REFERENCES

Adams WT (1983) Application of isozymes in plant breeding. *In: Isozymes in Plant Genetics and Breeding, Part A* (Tanksley SD, Orton TJ, eds) Elsevier Sci Publ, Amsterdam, 381-400

Aspit VJ, Nakamura RR, Wheeler NC (1989) Differential male reproductive success in Douglas fir. *Theor Appl Genet* 77, 681-684

Binder WD, Ballantyne DJ (1975) The respiration and fertility of *Pseudotsuga menziesii* (Douglas fir) pollen. *Can J Bot* 53, 819-823

Brewbaker JG, Kwack BH (1963) The essential role of calcium ion in pollen germination and pollen tube growth. *Am J Bot* 50, 859-865

Bridgwater FE, Blush TD, Wheeler NC (1991) Supplemental mass pollination. *In: Pollen Management Handbook, Vol II.* Proc Pollen

Manage Wrksp Macon, GA, July 17–18, 1990. Sponsored by S Res Infor Exchange Group and USDA For Serv, S For Exp Stat

Charpentier JP, Bonnet-Masimbert M (1983) Influence d'une rehydratation préalable sur la germination *in vitro* du pollen de Douglas *(Pseudotsuga menziesii)* après conservation. *Ann Sci For* 40, 309-317

Cheliak WM, Skroppa T, Pitel JA (1987) Genetics of the polycross. I. Experimental results from Norway spruce. *Theor Appl Genet* 73, 321-329

Ching TM, Ching KK (1976) Rapid viability tests and aging study of some coniferous pollen. *Can J For Res* 6, 516-522

Dhawan AK, Malik CP (1981) Effect of growth regulators and light on pollen germination and pollen tube growth in *Pinus roxburghii*. *Sarg Ann Bot* 47, 239-248

El-Kassaby YA, Fashler AMK, Sziklai O (1984) Reproductive phenology and its impact on genetically improved seed production in a Douglas fir seed orchard. *Silv Genet* 33, 12-125

El-Kassaby YA, Ritland K (1986a) Low levels of pollen contamination in a Douglas fir seed orchard as detected by allozyme markers. *Silv Genet* 35, 224-229

El-Kassaby YA, Ritland K (1986b) The relation of outcrossing and contamination to reproductive phenology and supplemental mass pollination in a Douglas fir seed orchard. *Silv Genet* 35, 240-244

El-Kassaby YA, Fashler AMK, Crown M (1989) Variation in fruitfulness in a Douglas fir seed orchard and its effect on crop-management decisions. *Silv Genet* 38, 113-121

Foushee D (1990) Effect of rehydration on *in vitro* germination of western white pine pollen. *In: Proct 14th Prog Rep Inland Empire Tree Imp Coop* (Fins L, ed) Univ Idaho, 31-35

Fowler DP (1987) In defense of the polycross. *Can J For Res* 17, 1624-1627

Ho RH (1980) Pollination mechanism and seed production potential in Douglas fir. *For Sci* 26, 522-528

Ho RH, Sziklai O (1972) Germination of Douglas fir pollen. *Silv Genet* 21, 48-51

Jacomini E, Bertani A, Mapelli S (1988) Accumulation of polyethylene glycol 6000 and its effects on water content and carbohydrate level in water-stressed tomato plants. *Can J Bot* 66, 970-973

Jett JB, Frampton Jr LJ (1990) Effect of rehydration on *in vitro* germination of Loblolly pine pollen. *South J App For* 14, 48-51

Johri BM, Vasil IK (1961) Physiology of pollen. *Bot Rev* 27, 325-381

Kerhoas C, Gay G, Dumas C (1987) A multidisciplinary approach to the study of the plasma membrane of *Zea mays* pollen during controlled dehydration. *Planta* 171, 1-10

Lessler MA (1969) Oxygen electrode measurements in biochemical analysis. *In: Methods of Biochemical Analysis* (Glick D, ed) Interscience Publ (John Wiley and Sons), NY, 1-28

Mellerowicz E, Bonnet-Masimbert M (1986) Importance de la teneur en eau du pollen pour la réalisation de croisements controlés chez le Douglas. *Ann Sci For* 43, 179-188

Moody WR, Jett JB (1990) Effects of pollen viability and vigor on seed production of Loblolly pine. *South J Appl For* 14, 33-38

Nygaard P (1970) Studies on the germination of pine pollen *(Pinus mugo) in vitro*. II. Effects of different ions. *Physiol Plant* 23, 372-384

Nygaard P (1977) Utilization of exogenous carbohydrates for tube growth and starch synthesis in pine pollen suspension cultures. *Physiol Plant* 39, 206-310

Owens JN, Simpson SJ (1982) *Further observations on the pollination mechanism and seed production of Douglas fir. Can J For Res* 12, 431-434

Owens JN, Simpson SJ, Molder M (1981) The pollination mechanism and optimal time of pollination in Douglas-fir *(Pseudotsuga menziesii). Can J For Res* 11, 36-50

Owens JN, Colangeli AM, Morris SJ (1991) Factors affecting seed set in Douglas fir *(Pseudotsuga menziesii). Can J Bot* 69, 229-238

SAS Institute Inc (1988) *SAS/Stat™ User's Guide, Release 6.03 Edn.* SAS Institute Inc, Cary, NC, pp 1028

Shivanna KR, Heslop-Harrison J (1981) Membrane state and pollen viability. *Ann Bot* 17, 759-770

Smith DB, Adams WT (1983) Measuring pollen contamination in clonal seeds orchards with the aid of genetic markers. *In: Proc 17th South For Tree Improv Conf.* Athens, GA, 69-77

Stanley RG, Linskens HF (1974) *Pollen: Biology, Biochemistry and Management.* Springer-Verlag, NY

Stenter AA, Mozafar A, Goodin JR (1981) Water potential of aqueous polyethylene glycol. *Plant Physiol* 67, 64-67

Subbaiah CC (1984) A polyethylene glycol based medium for *in vitro* germination of cashew pollen. *Can J Bot* 62, 2473-2475

Webber JE (1987) Increasing seed yield and genetic efficiency in Douglas fir seed orchards through pollen management. *For Ecol Manage* 19, 209-218

Webber JE (1991) *Interior Spruce Pollen Management Manual.* BC Ministry For Res Branch, Land Manage Rep 70, pp 25

Webber JE, Yeh FCH (1987) Test of the first-on, first-in pollination hypothesis in coastal Douglas fir. *Can J For Res* 17, 63-68

Webber JE, Bonnet-Masimbert M (1989) Influence of the moisture content of forest tree pollen on its response to different viability test. *Ann Sci For* 46, 60s-63s

Wheeler N, Jech K (1986a) Estimating supplemental mass pollination (SMP) success electrophoretically. *In: Proc 20th Canadian Tree Improvement Assoc Meeting, August 1985, Quebec, Quebec* (Caron F, Corriveau AG, Boyle TJB, eds) Can For Serv, 111-120

Wheeler N, Jech K (1986b) Pollen contamination in a mature Douglas fir seed orchard. *In: Proc IUFRO Conf Breeding Theory, Progeny Testing and Seed Orchards, Oct 13–17, 1986, Williamsburg, Virginia* (Weir RJ, ed) NC State Univ – Industry Cooperative Tree Improvement Program, 160-171

Woods JH, Heaman JC (1989) Effect of different inbreeding levels on filled seed production in Douglas fir. *Can J For Res* 19, 54-59

Zhang HQ, Croes AF (1982) A new medium for pollen germination *in vitro*. *Acta Bot Neerl* 31, 113-119