DON) found that orchard stock grew faster in two out of three trials in comparison with control, and the removal of 20% of the genetically inferior clones resulted in an additional gain in volume of 4%. Progeny tests of selected clones of Radiata pine grown in Australia (ELDRIDGE, 1974) showed an average superiority over the commercial check of 4% for height and 5% for DBH, corresponding to 11% superiority in volume. Open pollinated progeny tests of Scots pine (*Pinus sylvestris* L.) established in different parts of Hungary (MATYAS, 1974) from seed of selected clones, at the age of ten years were 10% superior in volume over the commercial check. All results indicate that a 10% improvement in volume for the first stage of selection (initial selection of Plus trees) plus an additional 10% from roguing the seed orchard after progeny testing. Limited selection by culling the poorest families has been also recommenced, for ponderosa and western white pines, to begin at the age of 10 years (STEINHOFF, 1974). The evaluation of the progeny tests have been made in relative young age (10 years), and the estimated gain may be different than realized gain at the end of the rotation and further evaluations have to be made as the materials become older.

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The Impact of Extraction and Storage Conditions on the Viability of Radiata Pine Pollen

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Abstract

The impact of extraction temperature, moisture content and storage temperature on *in vitro* pollen germination and tube growth, was determined in a sequence of experiments.

Pollen extracted at 25 °C, dried to 10% moisture content and stored in a refrigerator at 4 °C for almost a year retained a germination level of 84%, compared with its level of 93% immediately after extraction.

Pollen which was extracted or stored under conditions different from the above lost a greater amount of viability. There were significant interactions between extraction temperature, moisture content and type of storage: while pollen in less than optimal condition lost viability very rapidly under poor storage modalities, pollen extracted and dried as above was able to tolerate poor storage relatively well.

Key words: Pinus radiata, seed orchard, pollen viability and germination, thawing, rewarming and freezing.

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Introduction

Developments in New Zealand seed orchard management over the last decade have led to the introduction of controlled-pollinated (CP) orchards; in which initially pollen mixes, but subsequently single-parent pollens were applied to isolated female strobili (CARSON *et al.,* 1992).

The scale of pollen usage is high. At Amberley orchard in 1993 for example, 110 litres of pollen were collected for controlled pollinations. Much of the pollen that is collected is stored for usage the following year.

Associated with the developments of CP orchards have been incidences of both increased conelet abortions, and reduced seed yields per cone. Poor pollen quality has been implicated as a possible cause of both of these problems, leading to the recognition of the need to evaluate impact on pollen viability of the methods being used for its extraction and storage.

Such research was carried out as one component of a Master of Forestry Science thesis at the University of Canterbury (SIREGAR, 1994). The aim of this study was to evaluate the sequential impact on germination and pollen tube growth of a range of extraction and storage conditions. The results are reported here.

Literature Review

There exists quite a substantial international literature on conifer pollen, going back more than 40 years. Recent substantial contribution regarding the management of pollens include USDA (1993) and WEBBER and PAINTER (1996). A number of studies in those references report developments in collection, storage and testing of coniferous pollens.

Mandatory for any study of pollen management is a reliable and repeatable pollen viability assay. The major methods used globally are chemical staining (for enzymes associated with metabolic activities), electrical conductivity (a measure of pollen leachate into an aqueous solution), respiration (a measure of oxygen uptake under controlled conditions), and *in vitro* germination (a measure of pollen grain growth under controlled conditions). In loblolly pine MOODY and JETT (1990) have shown a good correlation between germination on agar and respiration rate and pollen germination *in vivo.*

In USA researchers (JETT et al., 1993) have distinguished between pollen viability *in vitro* or *in vivo,* and pollen efficiency which is the capacity of the pollen to fertilise the egg and produce a viable embryo. While it is clearly the latter trait which is important to tree breeders, the time involved in measuring pollen efficiency in *Pinus* is several years.

Concerning radiata pine in New Zealand, there was up to now no comparative testing of methods; and *in vitro* germination on agar has been the standard technique used. When this study began, this technique had a low level of repeatability and thus was not adequate for the study proposed.

The timing collection of pollen catkins is important as they must to be mature enough to open, but not to have already shed pollen. Considerable research on the effect of collection time has been reported for *Pinus taeda* by JETT et al. (1993). The result was evidence that this factor more deeply affected extractability than viability. With Douglas-fir, „forcing“ of pollen catkins in an early development stage led to poorly developed and infertile pollen (WEBBER and PAINTER, 1996).

The impact of extraction temperature and relative humidity (RH) on the viability of pine pollens has been examined by a number of workers since the researches of DUFFIELD (1954). The used extraction temperatures appear to range from a lower level of 22 °C (*P. radiata;* VERGARA et al., 1995) to a top value of 38 °C (*P. taeda;* JETT et al., 1993). The literature suggests that, when assessed directly after extraction, there is little, if any, effect of extraction temperature on viability.

Frequently, RH is not controlled during pollen extraction and is variable through the extraction processes. 30% to 50% RH appears a commonly used range for conifers, e.g. JETT et al. (1993); WEBBER and PAINTER (1996). There are limited data published on the impact of extraction RH on pollen viability, although DUFFIELD (1954) found that high extraction RH's reduced viability.

Much pollen used in tree improvement programmes or in the production of genetically improved seeds is stored prior to use; there have been a lot of investigations on a number of species for the impact of pollen moisture content (MC) and storage methodology on subsequent viability. Both in Sweden and in S.E. USA, facilities have been developed where dry pollen was used as an appropriate storage MC in a part of the extraction process (JETT et al., 1993).

In the case of loblolly pine (JETT et al., 1993) pollen is dried to less than 10% MC for storage. For Douglas-fir (WEBBER and PAINTER, 1996) 4% to 8% MC was shown to be optimal. Operational temperatures for pollen storage, with different species and companies, range from 2 °C in a refrigerator to –196 °C in liquid nitrogen; through –20 °C to –25 °C in a freezer. The general result is that the longer is the storage period, the lower is the appropriate temperature (JETT et al., 1993). When using other method than liquid nitrogen, it is necessary to store pollen in a desiccator to maintain its low MC.

Conifer pollen which has been stored at low MC needs to be rehydrated in order to restore its metabolic activity before use and before viability testing (WEBBER, 1991). This rehydration process has proved to be the key point to obtain repeatable *in vitro* germination values.

Materials and Methods

A critical pre-requisite to this research was the development of a reliable and highly repeatable method for assessing pollen viability. The method developed is reported in detail in SIREGAR (1994). It involved the direct culture of rehydrated pollen. The rehydration process involved placing the pollen in an open petri dish in an airtight container at 90% RH at 25 °C for four hours. Such rehydrated pollen was then spread thinly on a 1% agar medium containing 2% sucrose, and incubated at 25 °C for 48 hours. After that time, the germination status of 200 randomly selected grains in each of two replicates was assessed, and (in some experiments) the length of 100 pollen tubes was measured. A pollen grain was classified as germinated when its tube had elongated to a length which was greater than the diameter of the pollen grain. If the two replicates differed in germination percent by more than 15%, the test was repeated.

A sequence of experiments was designed to investigate: (i) extraction temperature; (ii) the impact of MC on short-term storage; (iii) the impact of MC and temperature on long term storage; and (iv) the handling of pollen after storage at –20 °C. The pollens used in the experiments were collected from seven year old ramets of five clones growing at the Amberley seed orchard in Canterbury. Approximately six litres of catkins were collected from each clone, at the development stage when catkins are traditionally collected. At this stage, pollen is just beginning to shed and the catkins release a little yellow liquid when crushed. Pollen extraction from catkins was carried out in controlled environment rooms set a constant 70% RH. These rooms were used because of their accurate temperature-holding capacity. However, because these rooms were not designed for drying biological material, it was not possible to reduce RH below this level. The extraction was terminated on the basis of visual acceptance that all catkins were fully open. Pollen was dried under vacuum using silica gel as desiccant. Moisture contents were measured gravimetrically following WEBBER (1991). Long-term storage was performed in two ml sealed containers, grouped inside a 50 ml jar containing silica gel.

The complete trial involved 700 treatments, each replicated twice. Thus 1400 separate pollen germinations were carried out over a period of almost a year. Detailed modalities were as follows:

Exp. 1. (test of extraction temperature): five clones x three extraction temperatures (20 °C, 25 °C, 30 °C).

Exp. 2. (test of extraction temperature and supplemental drying): five clones x three extraction temperatures (20 °C, 25 °C, 30°C) x five moisture contents (30%, 15%, 10%, 7%, 3%).

Exp. 3. (test of temporary storage): five clones x one extraction temperature (25 °C) x four moisture contents (44%, 30%, 20%, 10%) x two storage temperatures (ambient, 4 °C) x two times (2 weeks and 4 weeks).

Exp. 4. (test of long-term storage 0.9 year): five clones x three extraction temperatures (20 °C, 25 °C, 30 °C) x five moisture contents (30%, 15%, 10%, 7%, 3%) x six storage treatments (ambient, 4 °C, –20 °C, plus three post-storage treatments from –20 °C which examined rewarming, thawing, and refreezing).

where:

% Moisture content was defined as $(wt_w - wt_d)/wt_w$ x 100

wt_w = weight of pollen whose MC% to be determined

wt_d = weight of pollen after drying at 85°C for four hours

The designs were :

Exp. 1 randomised complete block.

Exp. 2. Split-plot (as main plot were clones and extraction temperature; as sub-plot was moisture content).

Exp. 3. split-split plot (as main plot were clones and moisture content; as sub-plot was storage temperature; as sub-sub-plot was length of storage).

Exp. 4. split-split plot (as main plot were clones and extraction temperature; as sub-plot was moisture content and as sub-sub-plot was storage conditions).

Data analysed from experiments 1 to 3 were germination percent. In experiment 4, germination percent and pollen tube length were assessed. Percentage data were transformed into arcsine and analysed by PROC ANOVA. DUNCAN's test was used to differentiate between values at the 5% level of confidence (SAS Institute, 1987).

Results

The complete results are presented in SIREGAR (1994). To provide a succinct paper which reports the major findings, several data compactions have been made in this present paper. Individual clone values are not presented. Firstly, clonal differences were statistically significant in every experiment, except number 2, and clone x treatment interactions were frequently significant. Secondly, for a better readability, only data judged to provide representative and useful coverage of the experiments as a whole are presented in figures and tables. Thus, e.g. following the results of experiment 1, a number of combinations of extraction temperature and moisture content are omitted.

Figure 1 presents the key results from Experiment 1. The higher was the extraction temperature, the lower was the MC of the newly extracted pollen. At a temperature of 25 °C or above, extraction was complete in three days, with little negative impact on germination. At 20 °C however, the time was extended to four days to obtain comparable amounts of pollen. The most suitable extraction temperature from the perspective of a seed orchard manager was close to be 25 °C as it maintained a high germination percentage combined with a reasonable extraction time.

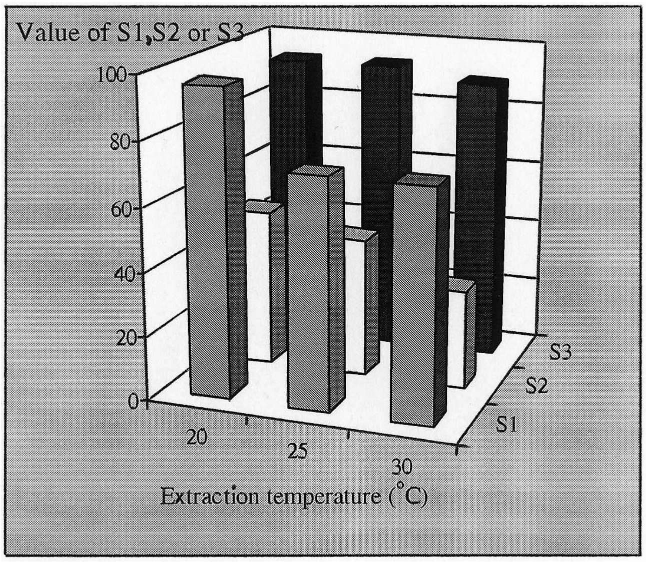

Figure 1. –Effect of extraction temperature on moisture content and germination percentage of pollen (S1 = Time needed for extraction (hour), S2 = Moisture content (%), S3 = Germination percentage).

Figure 2 indicates the mean germination percentage of pollen extracted at 25 °C, followed by drying. Even with drying down to 3% MC, the losses in immediate viability from the original 93% germination were small and non-significant.

Figure 3 indicates the effect on viability of short-term storage of pollen for 4 weeks before use. Storage for this time lenght may be necessary prior to the pollination of 2nd-cycle strobili. The results showed clearly that pollen with a 10% moisture content could be stored in better conditions with a 30% MC, irrespective of whether storage was at ambient temperature or in a refrigerator at 4 °C. At ambient temperature, the difference was very much marked.

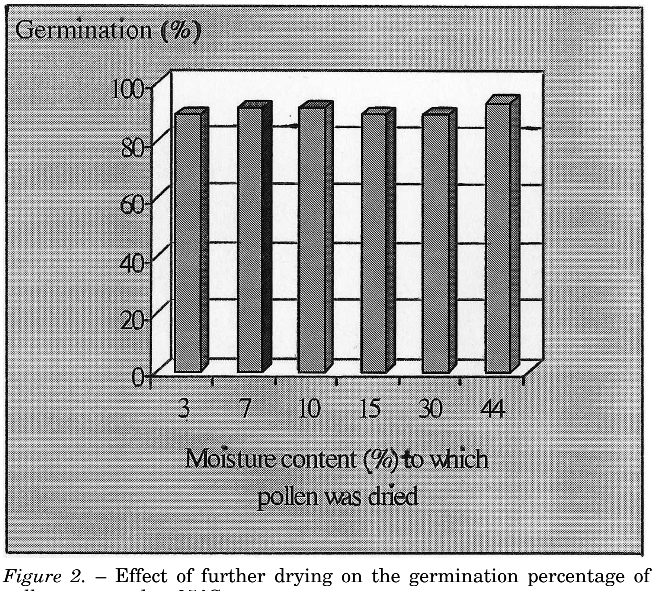

Figure 2. – Effect of further drying on the germination percentage of pollen extracted at 25 °C.

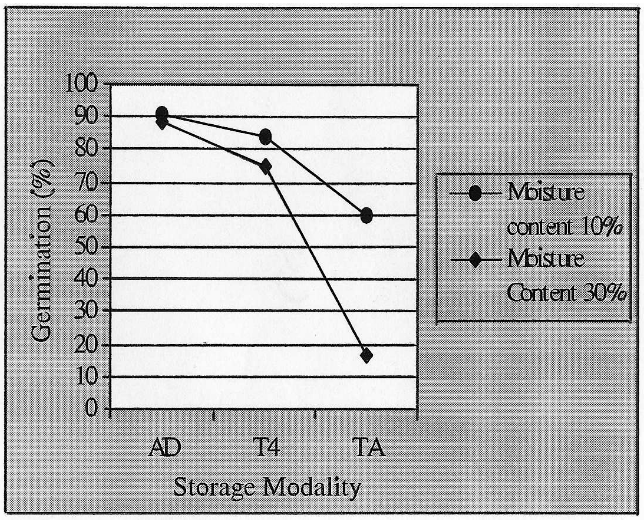

Figure 3. – Effect of temporary storage for 4 weeks on the germination percentage of pollen dried to 30% and 10% MC after extraction (AD = immediately after drying, T4 = after storage at 4 °C, TA = after ambient storage).

Table 1 presents the impact of long-term storage on germination percentage and tube growth of pollens: (i) extracted at 25 °C and dried to different MC's; and (ii) extracted at temperatures different than 25 °C and dried to 10% MC.

With regard to (i) in terms of tube length and germination percentage, pollen stored at 10% MC in either refrigerator or freezer was maintained good viability. Even after long-term storage at ambient temperature, pollen with a 10% MC had a sufficient viability to germinate *in vivo* and fertilise ovules. Drying to a level lower than 10% MC did not maintain such a high viability, under ambient conditions.

With regard to (ii), the data indicate an impact of extraction temperature on viability very significant after storage, even at the optimal MC of 10%. The highest values for germination percentage and pollen tube length, irrespective of storage method, were from pollen extracted at 25 °C.

When seed orchard managers store pollen in a freezer at –20°C, removing and replacing it daily during its use, it is submitted to a regular cycle of thawing and re-freezing. In addition, it has been suggested (MATHEWS and KRAUS, 1981) that pollen stored at –20 °C should be re-warmed in a refrigerator for 24 hours and then at room temperature for 2 hours prior to use. The data in *table 5* compare germination and pollen-tube growth from pollens (a) subject to gradual re-warming or not; and (b) following one or two episodes of thawing and freezing, followed by re-warming.

The data in *table 2* indicate that the germination of *P. radiata* pollen stored in a freezer does benefit from a gradual re-warming, but receives relatively minor damage from limited thawing and re-freezing.

Discussion and Conclusion

As measured *in vitro,* after extraction at 70% RH, both extraction temperature and the moisture content to which pollen was dried, affected its subsequent viability.

While extraction temperature had a limited impact on the germination of freshly extracted pollen (a finding supported by the literature), it very significantly affected the viability of

Table 1. – Effect of storage for 0.9 year on germination percentage (and tube length) of pollen at different MC after extraction at different temperatures.

Extraction temperature °C	Pollen germination % (+ tube length µm)			
	Moisture content (%)	Ambient room storage	Storage at 4°C in refrigerator	Storage at -20°C in freezer
20	10	24 (64)	77 (126)	75 (121)
25	30	0 (-)	0 (-)	15 (53)
25	15	1 (11)	37 (79)	48 (101)
25	10	52 (91)	85 (152)	81 (129)
25	7	45 (94)	88 (141)	85 (125)
25	3	11 (51)	82 (136)	79 (124)
30	10	20 (64)	63 (113)	61 (104)

Within a treatment, differences in germination >10% and in tube length >15 µm are significant at the 5% % level (DUNCAN's test). Behaviour of the highlighted pollen is reported in *table 2.* The lenghts are indicated between brackets.

Table 2. – The effect of post-storage treatment on germination percentage and tube length of pollen stored at 10% MC, after extraction at 25 °C.

	Post-storage treatment			
	No rewarming	Standard rewarming	1 x Thaw & freeze	2 x Thaw & freeze
Germination (%)	77 a	87 b	80 a	81 a
Pollen tube length (µm)	126 b	127 b	137 a	137 a

Values without a common letter in a given column differ significantly at the 5% % level (DUNCAN's test).

pollen after almost one year's storage. An extraction temperature of 25 °C was shown to be the best of the temperatures tested. Extraction temperatures of 20 °C and 30 °C both produced pollen with much lower germination and tube growth after 0.9 year storage. While it is easy to envisage a prolonged high temperature extraction as being detrimental, it is less easy to see why an extraction temperature of 20 °C should lead to a reduced pollen viability after storage at ambient room temperature. Presumably, the answer lies in the longer extraction period required at that temperature (see *Fig. 1*). With the sensitivity that was demonstrated over a relatively small temperature range, it might be useful to explore further what is an optimal extraction temperature. It should be remembered, however, that the RH used in these extractions (in controlled environment cabinets) was higher than that traditionally used, and was also held constant throughout the extraction period. It is possible that these facts influenced the extraction temperature which was proved to be „optimal".

Provided it was extracted and stored well, the germination of *P. radiata* pollen declined relatively little over a period of nearly one year. Pollen extracted at 25 °C for 72 hours had a 93% germination rate. Following extraction its MC was 44%. Subsequent drying to 10% MC did not significantly reduce germination, but a short-term storage in a refrigerator reduced germination to 84%. This value did not change significantly when the storage period was extended to nearly 11 months.

Moisture content is clearly critical to define satisfactory pollen storage procedures. MC's above 10% led to loss of viability during storage, but there were no obvious gains from drying below 10%. Drying to 3% appeared to be somewhat detrimental. While well-dried pollen was relatively insensitive to storage condition, pollen at moisture levels above 10% benefited from storing at –20 °C, rather than at 4 °C.

On the basis of these results, it seems that there is no advantage in storing pollen dried to 10% MC at –20 °C if only one year's storage is involved. Normal refrigerator storage temperature appears optimal.

It can of course be questionable how well *in vitro* measurements of germination and pollen-tube growth reflect the *in vivo* capacity of pollen to germinate in the micropyle and fertilise archegonia? SETIAWATI (1994), showed a clear correlation between *in vitro* pollen viability and seed yields, but, in her experiment, pollen viability was not the major factor limiting seed yield. JETT et al. (1993), working with loblolly pine, reported that 88% of the variability in total seeds per cone was explained by the correlation with percent pollen germination on agar. WEBBER and BONNET-MASSIMBERT (1993) were able to predict seed set in Douglas-fir by using a series of *in vitro* pollen assays. Thus, although the correlation between *in vitro* pollen germination and seed set in conifers may not be perfect, a seed orchard manager cannot ignore the information provided from *in vitro* testing.

In conclusion, extraction temperature, moisture content and storage temperature were confirmed to be the key factors affecting pollen viability. Recommendations with respect to these factors are what follows: i) Pollen should be extracted at 20 °C to 25 °C with a dehumidifying equipment to shorten the extraction time required. A quick germination test may be carried out at this stage to monitor pollen viability; ii) Fresh pollen should be dried to a moisture content of 7% to 10% for long term storage and 30% or less for temporary storage. These levels of moisture contents are important in maintaining a good pollen viability. It is suggested that extraction and drying should be carried out simultaneously, using specifically designated extraction facility. It is also important to test the viability of dried pollen prior to storage as a mean of quality control; iii) Properly dried pollen should be stored in a refrigerator for both temporary and long-term storage; iv) If pollen has been stored in a freezer, a rewarming procedure is not necessary.

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