Processing Eucalyptus Pollen for Use in Controlled Pollination

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Summary

Controlled breeding is facilitated if highly viable, clean, pollen is available whenever required in a form which minimises wastage and the risks of contamination or labelling error. Such a pollen handling system is described for *Eucalyptus*.

Anthers are clipped from freshly opened flowers, ground in distilled water, and passed through a double filtration system which leaves the pollen as a deposit on a Milipore filter. After drying over silica gel these filters may be cut into strips and stored in glass bottles at −16° C. When required for pollination individual strips may be extracted, used to brush pollen onto the stigmas, and then discarded.

In vivo viability tests of extracted *Eucalyptus regnans* pollen were carried out after 36 days and again after 12 months storage at room temperature, 5° C, and −16° C respectively. The viability assay involved pollinating bagged emasculated flowers, then harvesting styles 72 hours later. Following squash preparation and staining with methylblue, microscopic examination of styles under U.V. light allowed the numbers of developing pollen tubes to be counted. Storage at room temperature was adequate for the 36 day period, but high viability was only maintained for 1 year at −16° C.

Only the freezer-stored pollens showed any germination in vitro, and further work is required to allow interpretation of such results in terms of capacity to effect adequate seed set.

Viable seed collected following pollination with wet-extracted pollen provided a definitive test of the non-injurious nature of the treatment.

Key words: Pollen extraction, pollen storage, pollen viability testing, controlled pollination, *Eucalyptus* sp.

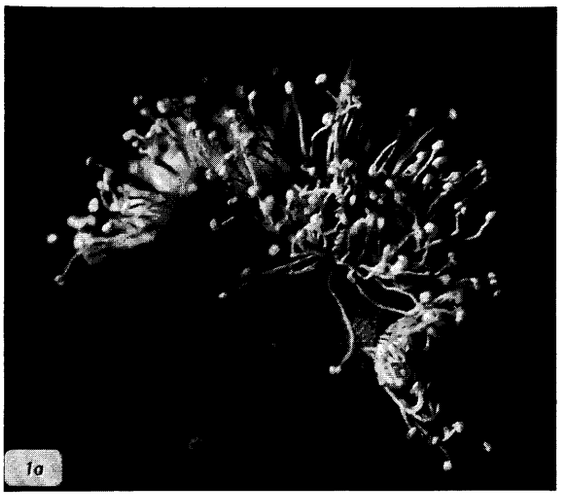
Zusammenfassung

Kontrollierte Kreuzungen werden erleichtert, wenn in hohem Maße lebensfähiger und sauberer Pollen zu jeder Zeit in einer Form zur Verfügung steht, die die Gefahr von Verunreinigung bzw. Fehlbezeichnung auf ein Minimum reduziert. Eine solche Pollenbehandlungsmethode wird für *Eucalyptus* beschrieben.

Die Antheren werden aus gerade geöffneten Blüten herausgeschnitten, in destilliertem Wasser gewaschen und durch ein Doppelfiltersystem gereinigt, welches den Pollen als Niederschlag auf einem Milipore-Filter liefert. Nach erfolgter Trocknung über Kieselgel können diese Antheren streifenweise zerschnitten und in Glasflaschen bei −16° C aufbewahrt werden. Wenn bestäubt werden soll, können die Antheren einzeln aus der Flasche entnommen und der Pollen auf die Narben gebracht werden. Nach 36 Tagen sowie nach 12monatiger Aufbewahrung bei Raumtemperaturen von 5° C bzw. −16° C wurden Keimprüfungen mit gewonnenem *Eucalyptus regnans*-Pollen durchgeführt.

Die Prüfung der Keimfähigkeit erfolgte durch Bestäubung eingetüteter kastrierter Blüten und die Ernte der Griffel 72 Stunden später. Nach Zerquetschen und methylblau-Färbung ließ sich die Anzahl der sich entwickelnden Pollenschläuche durch mikroskopische Untersuchung der Griffel unter UV-Licht bestimmen. Für die 36stündige Aufbewahrung genügte eine Aufbewahrung bei Raumtemperatur, dagegen wurde eine hohe Keimfähigkeit nach der Aufbewahrung über ein ganzes Jahr nur bei −16° C erreicht.

Figure 1. — Procedure for extraction, storage, and use of *Eucalyptus* pollen.



a) Flowers with freshly unfolded filaments.

b) Anthers clipped off into glass tissue grinder.

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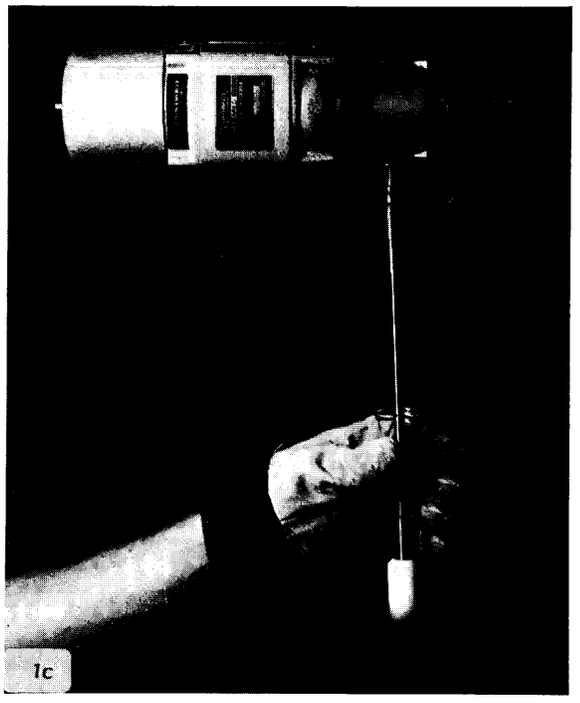
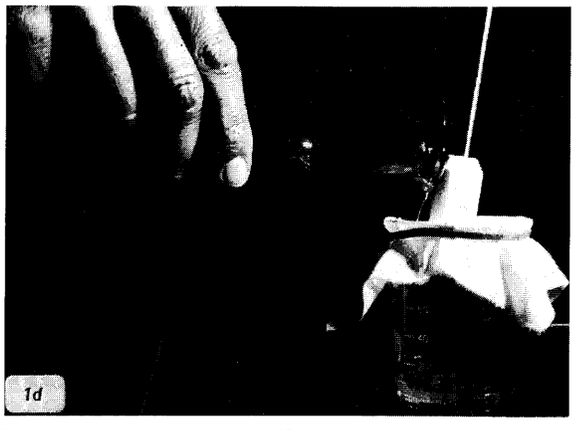
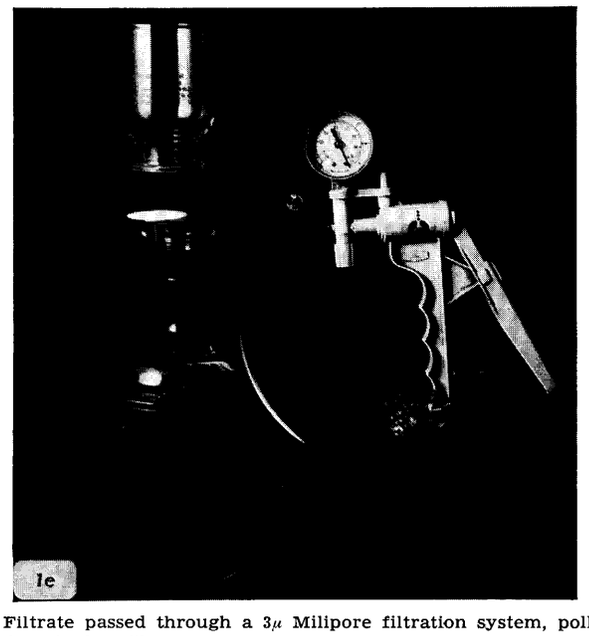


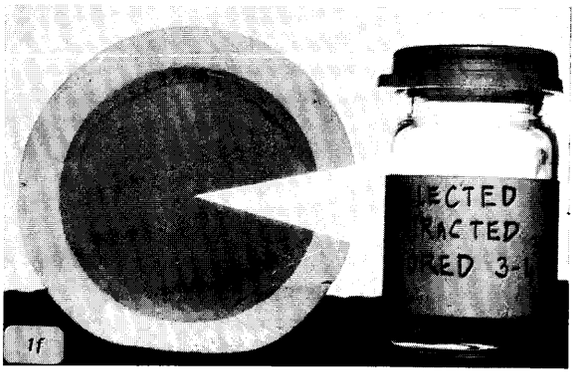
c) Anthers ground in distilled water suspension.



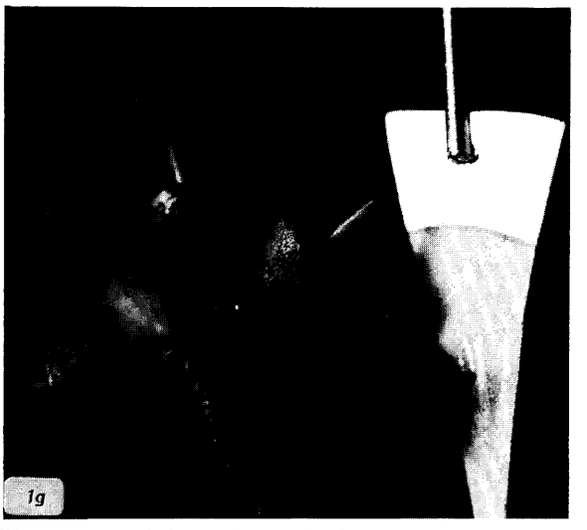
d) Homogenate filtered through 37 μ nylon mesh.



e) Filtrate passed through a 3μ Milipore filtration system, pollen deposited on filter.



f) Pollen deposit after drying. Wedge removed for storage in freezer.



g) Wedge removed from storage bottle and used for pollinating emasculated flowers.

Nur die in der Gefrierkammer aufbewahrten Pollen wiesen in vitro Keimungen auf. Weitere Untersuchungen sind notwendig, um klären zu können, ob eine ausreichende Samenbildung zu erreichen ist.

Die Behandlung des Pollens mit Wasser hatte keine nachteiligen Folgen für die Kreuzungen, aus denen später lebensfähiger Samen hervorgegangen ist.

Introduction

Any serious attempt at plant breeding which involves production of controlled pollinated seed requires development of an efficient pollen handling system.

Desirable features of such a system are that highly viable pollen be available whenever required by the breeder; that the pollen be free from anther filament tissue and other debris so that application rates can be controlled and storage space requirements minimised; and that the handling required between collection and use in pollination is also minimised.

Procedures reported for handling *Eucalyptus* pollen (Boden 1958, Van Wyk 1977, Eldridge 1963) or indeed for most fruit tree species in horticultural use (Janick and Moore 1975), fall short of these requirements in one or more respects. Boden's (1958) recommendation involved collection of flowers at anthesis and storage in a deep freeze (-16^{0} C). This gave viable pollen up to 12 months after collection and hence met the availability requirement, but much unnecessary bulk was also included in storage. The method used in applying pollen to receptive stigmas was not speci-

fically described but presumably was accomplished by using a whole flower as a brush. HODGSON (1975) and VAN WYK (1977) also described the use of fresh flowers in this manner (a method presupposing that flowers from the designated male parent are readily available), and also discussed the removal and crushing of anthers, and subsequent use of a camel hair paint brush to apply pollen. Such a method is apparently used succesfully for apples, pears, peaches etc. (JANICK and Moore 1975), but is dependent upon quantities of dry pollen falling freely from the anthers — this situation does not occur in many species of *Eucalyptus*.

Several years of controlled breeding work with *Eucalyptus regnans* F. MUELL. in Victoria, Australia, led us to conclude that (at least for this species which has a single well defined flowering season (GRIFFIN 1980)), it is impracticable to rely on fresh flowers as a pollen source. The use of brushes or stored whole flowers for pollen application also presented difficulties and an alternative pollen handling system was therefore developed. This paper describes the system, based on a new wet extraction method developed by one of us (K. K. CHING); reports results indicating that viable seed can be produced with fresh pollen so treated; and that viable seed can be produced with fresh pollen so treated; and that in vivo pollen germination can be achieved with pollen stored for at least 12 months in a deep freeze.

Methods

Extraction and Handling

On the assumption that natural pollen shedding is the best indication of pollen ripeness, it is desirable to harvest branches from donor trees when the flowers are just about to shed their opercula — and to complete the process by standing the branches in water in the laboratory or glasshouse. All flowers which have already shed opercula should be removed to avoid the possibility of contamination. Pollen may already be shedding from the anthers at anthesis (VAN WYK 1977) and this certainly occurs once the filaments have unfolded to expose the anthers — a process which takes from several hours to two days or more depending on species.

Anthers are clipped from flowers with scissors and pollen extracted using a double filtration procedure *(Fig. 1 a-e)*. The anthers are placed in a glass tissue grinder with 5 ml of distilled water, and then ground with a pestle. The resulting homogenate is filtered through a nylon cloth of 37 μ pore size. This allows the pollen grains (14—35 μ, depending on species, PIKE 1956) to pass through while retaining most tissue debris. About 100 ml of water is needed to wash the pollen through this stage of filtration.

The filtrate is then run through a Milipore filtration system with a 3 μ pore filter. A hand pump is used to partially evacuate the system and hasten removal of the filtrate. The filter plus deposited pollen is finally removed to dry in a desiccator over silica gel.

If the pollen is to be used for controlled pollination work the filter should be backed with an adhesive label, as it can become rather brittle on drying.

For storage and subsequent use in pollination it is convenient to cut the filter into segments and store these in small (10 ml) plastic capped bottles *(Fig. 1 f)*. These can be carried into the field and segments extracted as required for use. The broad end of a segment which does not carry pollen may be grasped with the fingers or forceps allowing application of pollen to receptive stigmas *(Fig. 1 g)*. The pollen cakes onto the filter and therefore does not fall off, so pollination can be carried out at any angle. The act of rubbing onto a stigma will generally remove more than sufficient pollen grains, but if desired the surface of the caked pollen can also be loosened by scraping gently with a scalpel blade. After pollination the filter segment may be discarded.

Storage

Storage at low temperature generally prolongs the period over which pollen viability is retained (JANICK and MOORE 1975). BODEN (1958) recommended storage of *Eucalyptus* pollen in a deep freeze (−16° C), while BORGES, DA SILVA, and FERREIRA (1973) favoured 4° C. It was important to determine whether pollen extracted by our method required immediate freeze storage or whether viability would be retained at higher temperatures. Pollen from 3 trees was extracted on 1st of April 1981. After drying over silica gel for 48 hours the filters were cut into segments and placed in sealed glass phials. Phials of each pollen were then stored at room temperature in the laboratory; in a refrigerator (5° C); and a deep freeze (−16° C).

Viability of samples was tested in vivo after 36 days — a period over which pollen might be stored for use the same flowering season, and again after 12 months. In vitro tests were also carried out after 20 months.

In vivo germination

Tests were carried out in vivo, applying pollen to the receptive stigmas of emasculated *E. regnans* flowers, and harvesting styles 72 hours later for microscopic examination of pollen tube development. Preparation (based on the method of MARTIN, 1959) involves the following steps:

1. Fix excised styles in a 1:3 acetic acid/ethanol solution for 1—3 hours, after which they may be stored indefinitely in 70% ethanol.
2. Rinse in distilled water, and autoclave for 10 minutes at 120° C in an aqueous sodium sulphite solution (50 g/litre) to soften the tissues. Rinse free of sodium sulphite with more distilled water.
3. Place style on a glass slide and slit the epidermis longitudinally using a scalpel, or more effectively microsurgical bow scissors.
4. Stain with 0.1% methyl blue in 0.1 M tri-potassium ortho-phosphate including 10% glycerine, which has been decolourized by storing in darkness for 24 hours prior to use (CURRIER 1957). This fluorochrome is used because of its specificity for the callose deposited in pollen tubes.
5. Place cover slip over dissected styles, and a second glass slide over that; squash with minimal lateral movement and remove upper slide. If desired seal preparation with clear nail polish.
6. Observe using a reflected light fluorescence microscope. If using Zeiss equipment a suitable series of filters and beam splitters are supplied for epi-condenser II F1, with the 05 filter being best suited to the methyl-blue stain. A total magnification of about 80x is suitable for general scanning, and 200x for detailed observation and counting of numbers of pollen tubes per style.

In vitro germination

For simplicity and convenience it would be preferable to be able to test pollen quality in vitro or with a chemical test. The literature regarding in vitro testing contains

Table 1. — Numbers* of pollen tubes growing in single styles+ following pollination with pollens stored for 36 days at different temperatures.

Temperature	Pollen Parent Tree N3	N20	N26	Mean over pollen parents
Freezer (-16°C)	100+,100+	60,100+,16	8,36,1	53+
Fridge (5°C)	3	9,15	45,17	18
Room (10-35°C)	100+,28	4	38,6	35+

* Direct counts for < 50 tubes: Estimates to nearest 10 tubes for 50 < 100; Any number in excess of 100 designated as 100+.
+ 3 style preparations made for each treatment, but some damaged and not scored.

numerous references to the effects of collection, storage and handling procedures, and also minutiae of technique, on the germination results obtained (Stanley and Linskens 1974). It is therefore perhaps not unexpected that we have experienced difficulty in applying the hanging drop procedure advocated for *Eucalyptus* by Boden (1958) to the wet-extracted pollen. An alternative approach of staining with fluorescein diacetate (Heslop-Harrison and Heslop-Harrison 1970) also proved inappropriate for *Eucalyptus* pollen and effort was again concentrated on an in vitro assay. The following simple procedure has proved to give repeatable results: Place a pollen bearing filter segment in a 2 dram glass phial and introduce 3 drops of 30% sucrose solution containing 1.5 ppm boron (as sodium tetraborate). Gently scrape pollen from the filter with a sterile needle. Cap phial and incubate at 30° C for 24 hours. Pipette solution onto a slide, cover with cover-slip and count the proportion of germinated grains under a microscope at x125 magnification. The temperature regime, test period, and test solution were determined in preliminary trials based on general literature reviewed by Stanley and Linskens (1974) and experience with *Eucalyptus* by Boden (1958) and Borges, da Silva and Ferreira (1973).

The samples used in the in vivo tests were immediately returned to their respective storage regimes and were tested in vitro 8 months later i.e. after a total storage period of 20 months.

Capacity for seed set

Finally an unequivocal demonstration that pollen extracted by our method can be used to produce viable seed, was obtained from an experimental controlled pollination programme in 1980.

Results

In the short term (< 36 days) viability of pollen, as judged in vivo, was unaffected by storage temperature *(Table 1).* Pollens to be used in the season of collection can therefore be kept satisfactorily in a desiccator at room temperature. However this is not the case if it is desired to keep the pollen from one flowering season to the next. Within 12 months viability of pollen stored at room temperature was completely lost *(Table 2, Figure 2a)* whereas that kept at —16° C retained its viability *(Figure 2b).* Some of the samples stored at 5° C still had a reduced capacity to germinate. Since care was taken to carry out all pollinations with the same technique it is reasonable to assume that the variation in numbers of pollen tubes reflects the proportion of viable pollen grains in each sample.

Under our test regime 7 of the 9 freezer stored pollen samples showed some in vitro germination *(Table 2),* but no germinating tubes were obtained from any of the 5° C or room stored samples.

The ultimate test of utility of a pollen sample is the seed yield following pollination. Freshly extracted pollen was used for cross-pollination in a number of genetic experiments in March 1980, and an average of 4.1 full seed/capsule yielded in December 1980 *(Table 3.)*

Over several years of crossing on 6 trees in the same stand, using fresh flowers for pollination, mean full seed yield per capsule was 3.6. Tree N4, which was also used in the test of extracted pollen *(Table 3)* yielded 2·8 seed/capsule with the old pollination method (unpublished data, A. R. Griffin) and 3.1 with the new. It is concluded that extracted pollen gave at least as good a seed yield as the alternative method. Although not specifically documented it is likely that application from filter strips increases the quantity and uniformity of pollen applied to each stigma — the apparent mean increase of 0.5 seed/capsule may therefore be a real consequence of increased efficiency.

Discussion

A possible disadvantage of a wet extraction method is that immersion in water can cause rapid elution of proteins from the walls of some pollens (Stanley and Search 1971) — and viability may be reduced. For example in studies of pear *(Pyrus communis)* pollen germination was not significantly reduced after one 15 sec. elution, but after

Table 2. — Numbers* of pollen tubes growing in single styles following pollination with pollens stored for 1 year at different temperature, with estimates of in vitro germination % of the same samples after 20 months storage.

		Pollen Parent Tree No. N3			N20			N26			Overall mean	
Temperature	Sample No.	# tubes a)	# tubes b)	% viable grains	# tubes a)	# tubes b)	% viable grains	# tubes a)	# tubes b)	% viable grains	# tubes	% viable grains
Freezer (-16°C)	1	50	100+	0	100+	100+	4	100+	-	7		
	2	100+	100+	0	43	26	13	100+	50	8		
	3	50	80	17	60	60	2	60	70	6		
	Mean No./%	80.0		5.7	74.8		6.3	76.0		7.0	73.5+	6.3
Fridge (5°C)	1	1	4	0	12	5	0	28	9	0		
	2	33	3	0	6	18	0	4	0	0		
	3	0	0	0	27	32	0	0	0	0		
	Mean No./%	6.8		0	16.7		0	6.8		0	10.1	0
Room (10-35°C)	1	0	0	0	0	0	0	0	0	0		
	2	0	0	0	0	0	0	0	0	0		
	3	1	0	0	0	0	0	0	0	0		
	Mean No./%	0.2		0	0		0	0		0	0.1	0

* Direct counts for < 50 tubes; Estimates to nearest 10 tubes for 50 < 100; Any number in excess of 100 designated as 100+. Two style preparations (a, b) per stored pollen sample.

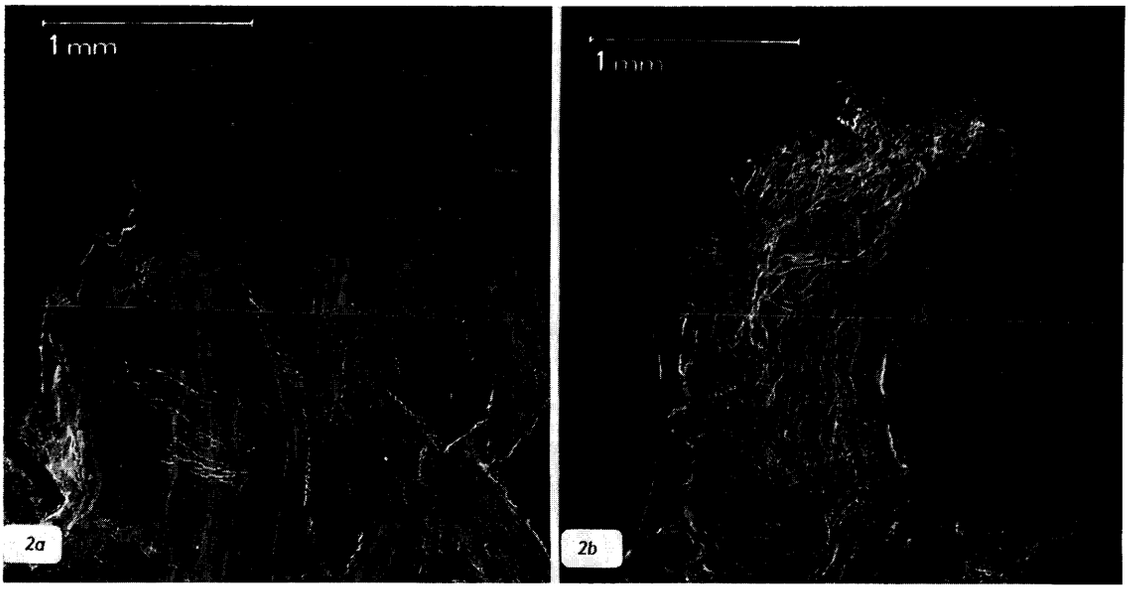


Figure 2. —
a) Ungerminated pollen grains on stigma 72 hours after pollination with N3 pollen stored at room temperature for 12 months.
b) Stigma and terminal portion of style showing 100+ germinated pollen tubes. Fixed 72 hours after pollination with N3 pollen stored at −16° C for 12 months.

four successive repeats of this treatment the pollen was essentially non-viable. Rate of elution of proteins varied considerably between plant species tested.

From the data presented in this paper it can be inferred that the wet extraction process does not materially affect viability of *E. regnans* pollen. It would nevertheless seem good practice to minimise the time during which pollen is in suspension. We have not systematically looked for possible deleterious effects on other species of *Eucalyptus* but freshly extracted samples of *E. amplifolia, E. pauciflora, E. dives, E. grandis, E. melliodora, E. dwyeri,* and samples of *E. obliqua, E. fastigata* and *E. tereticornis* stored in a freezer for 22 months, have all germinated in vitro (Ching and Burgess unpubl. data). Including *E. regnans* 5 species of sub-genus Monocalyptus (Pryor and Johnson 1971) and 5 of Symphyomyrtus have thus been tested, and the procedures appear to have general utility.

The number of pollen tubes per style within storage treatments was more variable in the 36 day test *(Table 1)* than for those carried out after 1 year. We suggest that this is probably due to improved pollen application and style preparation techniques in the latter experiment, rather than real effects of the storage treatment, and therefore conclude that room storage is adequate for pollen to be used within one season. However, if a freezer is available then nothing is lost by storing that way immediately, and there is the advantage that if problems occur with the crossing programme, then the pollen may be retained for a further year.

Table 3. — Mean seed yield/capsule for three trees of *Eucalyptus regnans* following pollination with fresh wet-extracted pollen.

Female Parent	No. capsules Harvested	No. viable Seed/capsule
N4	24	3.1
N5	81	4.1
N10	53	4.5
	Weighted mean	4.1

The in vivo testing technique provides excellent data on pollen viability, but has several practical disadvantages. It is necessary that receptive flowers are available (although the possibility of using flowers of related species which flower at different times is being explored); the technical skills or equipment necessary for the microscopic examination may not be readily available; and the rate of flower development (Griffin and Hand 1979) dictates that the whole process from emasculation of test flowers through to preparation of styles cannot be completed in less than about 16 days. Development of a reliable in vitro test of pollen quality is therefore a desirable objective. We did not test in vitro until 8 months after our in vivo test and therefore cannot discount the possibility that the low germination percentages obtained were due to a decline in condition during that period. It is however clear that a low in vitro germination is not necessarily associated with reduced capacity to effect seed set. Boden (1958) cited Olmo (1942) as finding that stored grape pollen with in vitro germination of $> 6\%$ gave satisfactory fruit set, and Callaham and Duffield (1961) found that 10—30% viable pollen was sufficient for maximum seed set in *Pinus* species. Boden (1958) also cited results of Pryor (unpubl.) which suggested a positive association between in vitro viability and ability to set capsules in *E. pulverulenta,* though one sample with only 10% germination also gave good capsule set.

In order to resolve this important final step in Eucalypt pollen handling methodology a further comparative series of in vivo and in vitro tests will be required.

Acknowledgements

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Provenance trial with Pinus caribaea Morelet and P. pseudostrobus Lindl. in Orissa, India

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Summary

Results on height and diameter, mortality, foxtail, and stem form are presented from a provenance trial at Koraput, Orissa, with 8 provenances of *Pinus caribaea* var. *hondurensis*, 1 provenance of *P. caribaea* var. *bahamensis*, and 1 provenance of *P. pseudostrobus* at the age of 9 years. The two var. *hondurensis* provenances Limones (Honduras) and Santa Clara (Nicaragua) show fastest growth. Mortality of most of the provenances was high in the first year after planting and should be reduced by improved nursery and planting techniques. Foxtail frequency and stem form varied greatly between the var. *hondurensis* provenances. Some provenances had many foxtails and individuals of poor form. The var. *bahamensis* provenance from Andros Island had the least number of foxtails and excellent stem forms. The trial shows also that *P. pseudostrobus* cannot be recommended for plantations in Orissa because of a low height and diameter growth, high mortality, many foxtails, and poor stem form.

Key words: Pinus caribaea, P. pseudostrobus, height growth, diameter, mortality, foxtail, stem form.

Zusammenfassung

In einem Provenienzversuch bei Koraput, Orissa, mit 8 Herkünften von *Pinus caribaea* var. *hondurensis*, 1 Herkunft von *P. caribaea* var. *bahamensis* und 1 Herkunft von *P. pseudostrobus* wurden Höhe und Durchmesser, Mortalität, Foxtail-Häufigkeit und Stammform bis zum Alter von 9 Jahren untersucht. Bestes Wachstum zeigten die beiden var. *hondurensis*-Herkünfte aus Limones (Honduras) und Santa Clara (Nicaragua). Die Mortalität war bei den meisten Herkünften im ersten Jahr nach der Pflanzung sehr hoch. Dies kann sicherlich durch verbesserte Anzucht- und Pflanzverfahren verringert werden. Hinsichtlich der Foxtail-Häufigkeit und der Stammform bestand zwischen den var. *hondurensis*-Herkünften eine große Variationsbreite. Besonders die gutwüchsigen Herkünfte enthielten viele Foxtail-Bäume. Die geringste Foxtail-Rate und ausgezeichnete Stammform besaß die var. *bahamensis*-Herkunft Andros Island. *P. pseudostrobus* kann aufgrund geringer Wüchsigkeit, hoher Mortalität sowie vieler Foxtail-Bäume und schlechter Stammformen nicht für den weiteren Anbau in Orissa empfohlen werden.

1. Introduction

Pinus caribaea MORELET became the most important lowland timber tree species being planted in tropical countries (LAMB, 1973). It is also a promising pine species for Orissa, India (DAS, 1971, 1982). Large scale plantations with *P. caribaea* are in progress.

In Orissa the first trials with these pines were established in Kalinga Research Centre with the two varieties namely var. *caribaea* MORELET and var. *hondurensis* BARR. and GOLF. Subsequently in 1967 *Pinus caribaea* var. *bahamensis* BARR. and GOLF. was tried at Daringbadi Research Centre.

In the local environment it was noticed that *P. caribaea* var. *hondurensis* showed the best suitability. Among the various provenance trials the one from Mountain Ridge (Belize) gave the best performance in height and diameter growth. *Pinus caribaea* var. *bahamensis* exhibited good form at higher elevations around 900 m.

In subsequent field experiments in Kalinga, Daringbadi, Koraput, Pottangi and Maliput research centres various provenances of *P. caribaea* var. *hondurensis* from Belize, Guatemala, Australia, Nicaragua and Honduras were also tried with varying results (DAS, 1971, 1982). Two provenances of *P. caribaea* var. *bahamensis* (Bahamas and Andros Island) were tried in Daringbadi and Koraput Research Centre in 1967 and 1972.

For a better appreciation of the intraspecific variation of *P. caribaea* provenance trials are necessary to choose the best varieties and provenances for large scale plantation.

In the following paper results on performance, mortality and stem form are presented from a provenance trial at the research centre at Koraput, Orissa, with 9 provenances of *Pinus caribaea* and 1 provenance of *P. pseudostrobus* LINDL. at the age of 9 years.

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