Artificially induced protogyny: an advance in the controlled pollination of *Eucalyptus*

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Summary

Artificially induced protogyny (AIP) is a new technique for the controlled pollination of *Eucalyptus*, without emasculation. AIP involves cutting off the tip of the operculum of the mature flower bud just prior to anthesis, with the cut positioned so as to remove the stigma, and then applying the target pollen to the exposed cut-surface of the upper style.

In trials in Brazil and Australia, rates of capsule retention and seed yield, and therefore yields of seeds per bud pollinated, have been very similar for AIP and one-stop pollination (OSP). However, AIP has achieved a 3–18-fold increase in productivity over OSP and conventional (three-visit) methods, in terms of seed produced per operator hour.

Contamination levels in the Brazilian experiment ranged from 3.75% in buds pollinated at the ripe/yellow stage, to 0.77% in buds pollinated at the immature/green stage. The yield of 2.0 seeds per bud pollinated with AIP at the immature/green stage was unacceptably low compared with 17.2 seeds per bud at the ripe/yellow stage. Molecular genetic analysis of seedlings produced from one of the *E. grandis* x *E. camaldulensis* crosses in Australia confirmed that all 20 seedlings were from the target cross.

The high operator productivity and relatively low levels of contamination achieved with AIP, across several eucalypt species, make it a potentially attractive technique for operational crossing. Our experiments were carried out in indoor clone banks that contained very few potential insect pollinators, so self-pollination is likely to be the main source of the contamination observed in the Brazilian experiments. Higher levels of contamination from non-target outcross pollen may occur following AIP on trees exposed to normal levels of pollinator activity.

Keywords: plant breeding methods, cross pollination, seed production; techniques, *Eucalyptus*

Introduction

Eucalypts are naturally protandrous, presumably an adaptation to promote outcrossing (Eldridge *et al*. 1993; House 1997). The eucalypt bud is capped by an operculum, derived from fusion of the petals and sepals. Operculum-lift in the mature flower is followed immediately by the spreading of the stamens and anthesis, while the stigma does not become receptive until several days afterwards. The time from operculum-lift and anthesis to the onset of stigma receptivity, and the duration of receptivity, vary from species to species: the stigma is receptive 4–6 d after anthesis in *Eucalyptus grandis* (Hodgson 1976), compared to 10–14 d in *E. regnans* (Eldridge and Griffin 1983). The sequence of anthesis and receptivity is also affected by environmental conditions, proceeding more rapidly at higher temperatures (Hodgson 1976). Individual flowers in an inflorescence and within a tree vary in their timing, so there is ample opportunity for self-pollination, particularly for the later-opening flowers within the crown of a flowering eucalypt (Eldridge *et al*. 1993).

Controlled pollination of eucalypts has been carried out for over five decades (e.g. Pryor 1951; Meskimen 1965) and is an essential tool in modern eucalypt breeding. The first-developed methods took advantage of the natural protandry of the eucalypt flower. The technique developed for *E. grandis* involved at least three visits to an inflorescence: (1) removal of open flowers and young flower buds, the emasculation of about-to-open flowers and exclusion bagging to prevent contamination of these flowers with non-target pollen; (2) controlled pollination at the time of maximum stigma receptivity, involving removal of the bag, application of pollen to the stigmas and re-bagging; and (3) final removal of the exclusion bag a week or so after pollination (Van Wyk 1977).

The development of one-stop pollination (OSP) for *E. globulus* (Harbard *et al*. 1999; Williams *et al*. 1999) was a significant advance. OSP involves emasculating a flower just before operculum lift, cutting or slicing the style, and applying pollen to the freshly cut style surface. Contamination with unwanted pollen can be prevented by covering the cut style with a length of fine plastic tubing, or by bagging the treated flower or inflorescence. The yield of *E. globulus* seeds, per flower pollinated, is as high as that from conventional three-visit controlled pollination (Williams *et al.* 1999). Because OSP involves less labour, the cost per seed produced is reduced. For *E. globulus*, with its large, easily-pollinated flowers and capsules bearing up to 20–30 seeds each, OSP is now used to mass-produce seeds of elite crosses for operational deployment in plantations in Chile, Portugal and Australia (Espejo *et al*. 2001; D. Boomsma, STBA, *pers. comm*. 2004).

OSP has been used with some success on a range for eucalypt species including *E. grandis* (Harbard *et al*. 2000a,b), *E. dunnii*

(Barbour and Spencer 2000), *E. camaldulensis* and *Corymbia maculata* (CSIRO Forestry and Forest Products, unpublished). However, small-flowered eucalypt species such as *E. nitens* have displayed unacceptably low seed set following OSP, or the method has failed altogether (Williams *et al*. 1999).

Alternative controlled-pollination techniques, particularly for small-flowered eucalypt species, may offer gains in labour productivity and hence reduce the cost of producing control-pollinated seed. An obvious possibility for modification is the emasculation process, as this is time-consuming and holds a high risk of damage to the flower, since it involves cutting away the ring of floral tissue supporting the base of the stamens.

In protogynous species the stigma becomes receptive before pollen is released from the anthers. As with protandry, this temporal separation of male and female receptivity has most likely evolved to increase the likelihood of outcrossing. The advantages of protogyny in breeding programs of bamboo, a naturally protogynous species, have been discussed by Nadgauda *et al*. (1993). Protogyny allows the emasculation process to be omitted, saving time and reducing costs.

OSP for eucalypts involves applying pollen to the cut style before the day when anthesis would have occurred on the flower, had it not been emasculated. Trindade *et al*. (2001) found that immature sliced styles of non-dehisced flowers were superior to normal receptive stigmas in regard to the production of viable seed per capsule. Furthermore, they showed that pollen tubes reached the ovary 1–2 d faster than those from receptive stigmas. These results suggest that OSP could be done before flower maturation with some advantages. Here we report on this modified method, which involves artificially inducing protogyny without emasculation, for controlled pollination of eucalypts.

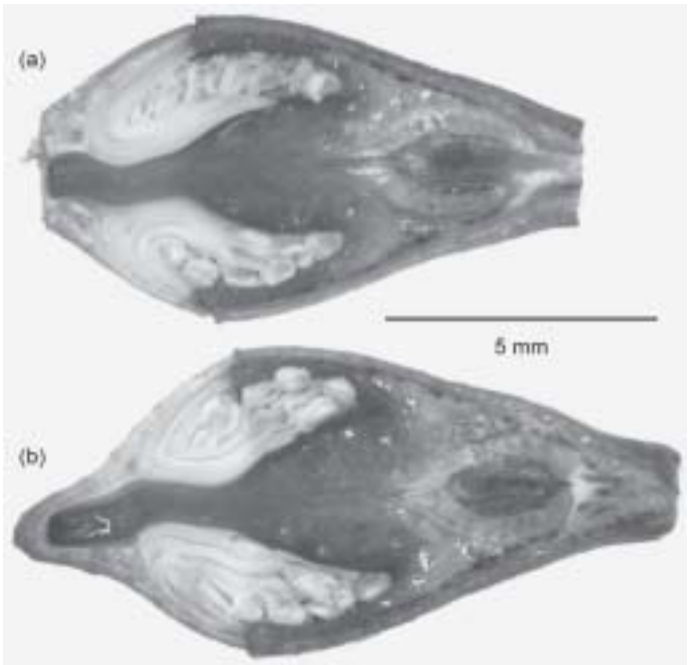

Figure 1. (a) Longitudinal section of *E. grandis* flower immediately after cutting of the operculum tip and style to induce artificial protogyny; (b) longitudinal section of untreated flower

Materials and methods

Two separate series of controlled pollination experiments, the first in Brazil where the technique was conceived, and the second in Australia, were conducted to test the artificial induction of protogyny (AIP) in eucalypts. The experiments produced interspecific hybrid crosses to enable easy confirmation that the correct crosses had been achieved, either by examination of seedling morphology or molecular genetic testing.

The technique applied was to use a scalpel or nail-clipper to cut off the tip of the operculum on a flower bud, a few days before anthesis would have occurred. The cut was placed so as to slice through the tip of the style, about 1 mm below the stigma in *E. grandis*, but not to emasculate the flower. Pollen was then applied immediately to the cut style surface. The umbel or flowering branch could then be bagged to exclude external pollen sources. Figure 1 shows a longitudinal section of an *E. grandis* bud taken immediately after the operculum cut has been made. At this stage of floral development, the stamens of *E. grandis* are still inflexed and the anthers have not yet opened. Figure 2 shows pollen being applied to the style surface of a freshly-cut bud of an *E. grandis* x *E. urophylla* hybrid subjected to the AIP treatment.

Experiments in Brazil

The first series of experiments was carried out at the nursery of the Aracruz Company near Guaiba, Rio Grande de Sul province, Brazil. Candidate mother plants were grafts of selected clones of *E. urophylla* x *E. grandis* (4 clones) and *E. urophylla* (2 clones). They were exposed to a controlled dose of the growth retardant paclobutrazol (0.25 g of active ingredient per plant, applied in aqueous suspension as Cultar®), 6 mo after grafting to induce early and prolific flowering, then repotted in 20-L pots once paclobutrazol effects were noticed, and grown in an enclosure with a plastic roof and mesh-screened walls. The pollen source was a polymix of pollens from ten *E. globulus* subsp. *globulus* trees.

Once the technique had been developed in preliminary trials (Assis and Jardim 2000), an experiment was set up to compare seed production rates and time taken, using the following methods:

1) conventional three-visit controlled pollination with emasculation and exclusion bagging (as described above)

Figure 2. Flower of the interspecific hybrid *E. grandis* x *E. urophylla* being pollinated following AIP treatment

2) OSP without exclusion bagging
3) AIP on yellow-coloured buds without exclusion bagging
4) AIP on yellow-green buds without exclusion bagging
5) AIP on green buds without exclusion bagging.

In uncut buds of the clones used, the colour changed from green (up to 20–10 d before operculum lift) to yellow–green (10–3 d before operculum lift) to yellow (2–0 d before operculum lift).

At 20–24 weeks after pollination, using a scalpel blade to slice the style, the capsules remaining from each treatment were collected and the capsule retention rate calculated for each treatment. After extraction, the number of seeds per capsule was recorded and used to calculate the number of seeds produced per flower pollinated. Seeds were then germinated to calculate percentage germination rates of the counted seeds.

A second experiment was set up to compare the use of nail clippers and a scalpel blade in making the operculum cut in the AIP controlled pollination method, using yellow-coloured buds. The same operator conducted both treatments to avoid potential confounding effects and, in this experiment, results were followed through only to the stage of capsule retention/loss, 20–24 weeks after pollination.

Once the AIP method had been proven effective, the extent of contamination by non-target pollen was tested. Ramets of four clones of *E. grandis*, four clones of *E. saligna*, four clones of *E. urophylla* and six clones of *E. grandis* x *urophylla*, were raised as described above, and housed in the same screened, plastic-roofed enclosure. Large numbers of pollinations were carried out using the AIP technique. Buds were not isolated after pollination. The resulting seeds were germinated, and seedlings were raised and examined 4 mo after sowing, to identify individuals that were not hybrids with *E. globulus*. Hybrids between *E. globulus* and all four maternal taxa in the experiment were highly distinctive, on the basis of their stem and leaf morphology, being quite different from crosses within and among the taxa used as maternal parents. As there were no *E. globulus* flowering in the vicinity of the mother plants, all seedlings identified as hybrids with *E. globulus* were successful crosses. Individuals that were not hybrids with *E. globulus* were all classed as contaminants.

Finally, a large-scale test with over 3000 buds pollinated per treatment compared the use of nail clippers and scalpel blades for making the cut on the bud for the AIP treatment.

Experiments in Australia

The second series of experiments was carried out at CSIRO Forestry and Forest Products, Canberra, Australia, after the basic technique of AIP had been developed in Brazil. The plants used in this experiment were grafted plants of four selected *E. grandis* clones, and plants raised from cuttings of one selected *E. urophylla* hybrid clone (the hybrid known as U_6 is widely planted in southern China and considered likely, from its leaf and bud morphology, to be a hybrid between *E. urophylla* and either *E. camaldulensis* or *E. tereticornis*). These plants were grown in an artificial soil medium in 75-L bags, and housed in a climate-controlled greenhouse. Paclobutrazol (0.2 g of active ingredient per cm of stem circumference, applied in aqueous suspension as Cultar®) was applied as a soil drench 18–24 mo prior to commencement of the experiment. A temperature range of 15 °C (minimum night temperature) to 35°C (maximum day temperature), was maintained by heating in winter and use of reflective shadecloth in summer to reduce maximum temperatures. Trees were irrigated daily by a computer-controlled dripper irrigation system and treated weekly with a dilute complete nutrient solution. At pollination, trees were flowering heavily, and tree heights were in the range 2–4 m.

A single *E. camaldulensis* tree was used as the pollen donor for the experiment with *E. grandis* clones. Pollen was collected from unopened flowers and dried in a vacuum freeze-dryer for 24 h. The viability of the pollen was confirmed using the methods described by Moncur (1995). Between 100 and 200 flowers were pollinated per treatment per clone using a scalpel to cut the style where appropriate. A number of branches were selected for each treatment and immature or open flowers were removed to leave only mature flowers, that is, at the stage where emasculation would normally be carried out for controlled pollination. The following six treatments were imposed:

1) Open-pollination
2) Conventional three-visit controlled pollination with emasculation and exclusion bagging
3) OSP, followed by exclusion bagging
4) AIP, followed by exclusion bagging
5) Bud emasculation and cutting the style surface, followed by exclusion bagging, but with controlled pollination delayed until nectar was produced at the floral nectaries. The time taken for nectar production to commence was recorded.
6) Bud emasculation, style cut and exclusion bagging as for treatment 5, but with no pollen applied.

Inflorescences were bagged individually using purpose-made exclusion bags. This provided greater control of the stage at which pollinations were conducted and minimised the amount of flowers cut to waste on branches exhibiting asynchronous flowering. The time taken to carry out a set number of flower pollinations was recorded for treatments 2, 3 and 4. These data, together with capsule retention rates and seed yields per capsule, were used to calculate productivities in terms of seeds produced per hour of effort, for treatments 2, 3 and 4.

A similar study was carried out using the *E. urophylla* hybrid clone U_6 and four pollen sources (two individuals of *E. pellita*, one of *E. grandis* and one of *E. globulus*). In this study only pollination treatments 3 and 4 (OSP and AIP) were applied.

After 28 weeks, the capsules remaining from each treatment were collected and the capsule retention rate calculated. After extraction, the number of seeds per capsule was recorded and viability of sub-samples of counted seeds was confirmed during subsequent germination of progenies. Labour productivity (flowers pollinated per hour, and viable seeds produced per hour) was calculated for the different treatments.

DNA fingerprinting

Seeds produced from the Australian AIP pollination experiment were germinated, and a subset of 20 seedlings from one of the *E. grandis* x *E. camaldulensis* crosses was randomly selected for DNA fingerprinting, along with the two parents. Twelve microsatellite markers (Eg61, Eg65, Eg84, Eg91, Eg94, Eg96,

Eg99, Eg126, Embra2, Embra6, Embra10, Es157) were used for fingerprinting following procedures in Glaubitz *et al*. (2001). Five of these microsatellites were developed from *E. grandis* by Brondani *et al*. (1998), one from *E. sieberi* (Glaubitz *et al*. 2001) and six from *E. globulus* (http:www.ffp.csiro.au/tigr/molecular/eucmsps.html). Allelic profiles at the twelve loci of each progeny were checked to see whether they were consistent with those expected from a cross between the two parents.

Statistical analysis

Rates of capsule retention following the different treatments were compared using the statistical software GenStat (Genstat 2002) and a generalised linear model based on the binomial distribution. Species and different pollination treatments were set as fixed effects. For the Australian experiment with *E. grandis*, where data were collected separately from four different clones, a generalised linear mixed model based on the binomial distribution was fitted (Schall 1991), with treatments as fixed effects and clone identity as a random effect. Seed yields per capsule from the different treatments in the Australian experiment with *E. grandis* were compared using a generalised linear model assuming a normal distribution, with treatments fixed and clone identity random. Because of low numbers of capsules obtained from some treatment-by-clone combinations, this analysis was carried out using the logarithm of (number of capsules harvested + 1) as weights.

Results

Brazilian experiments

The results of the first large-scale trial comparing AIP to other controlled pollination methods in Brazil are shown in Table 1. It can be seen that OSP and AIP with yellow-coloured and yellow–green buds all had very similar and high capsule retention, in the range 78–79% of buds pollinated. Conventional three-visit controlled pollination gave a much lower retention rate of 39.8%, while AIP on green-coloured buds gave the lowest retention of 19.5%. The number of seeds per retained capsule was very similar (23.1–25.7) for all treatments except for AIP on green buds, which was much lower at only 12.8 seeds per capsule. When differences in the germination percentage of seed produced by the different treatments are accounted for, OSP, and AIP with yellow buds, gave the highest viable seed yields per bud pollinated, 18.8 and 17.2 viable seeds per bud respectively. Conventional controlled pollination (10.0 viable seeds per flower) and AIP with yellow–green buds (14.8 viable seeds per flower) also gave good seed yields, but AIP with green buds was unacceptably low at 2.0 seeds per bud pollinated.

Data collected on productivity of the three pollination methods (Table 2) are based on the seed yields per capsule shown in Table 1, assuming that yellow-coloured buds are pollinated in the AIP treatment.

The levels of contamination in the Brazilian study are shown in Table 3. Statistical analysis established that the differences in levels of contamination among species, among bud stages and the interaction effect between species and bud stage were all highly significant ($P < 0.001$). Levels of contamination were clearly lowest (0.77% across all species) at the green bud stage, intermediate for yellow–green buds (1.16%) and highest (3.57%) for yellow buds.

The subsequent large-scale test comparing blades found that use of nail clippers gave 70.8% capsule retention while use of a scalpel gave 67.1%, a slight but significant ($P < 0.001$) reduction (data not shown).

Australian experiments

The capsule retention and seed set obtained in the Australian experiment with *E. grandis* are shown in Table 4. Productivity estimates based on time taken to apply the different treatments are also displayed.

Table 1. Capsule retention and seed yields obtained from different pollination methods in Experiment 1, Guaiba, Brazil

Treatment	No. of buds pollinated	Mean capsule retention (%)	Mean no. of seeds per capsule	Seed germination (%) per flower pollinated	No. of viable seeds
Conventional 3-visit pollination	479	39.8 a	25.7	97.5	10.0
OSP with emasculation	1500[b]	79.2 b	24.3	97.5	18.8
AIP, yellow buds	1500[b]	78.9 b	23.5	92.5	17.2
AIP, yellow-green buds	1500[b]	78.1 b	23.1	82.0	14.8
AIP, green buds	1500[b]	19.5 c	12.8	80.0	2.0

[a]Different letters denote significant ($P < 0.001$) differences in percentage capsule retention
[b]250 buds of each of the six clones were pollinated

Table 2. Productivity of three controlled pollination methods at Guaiba, Brazil

Pollination method	Flowers pollinated per person per hour	Per cent capsule retention	Seed production (seeds per person per hour)
Conventional controlled pollination with emasculation and isolation using pollination bags	35	39.8	358
OSP with subsequent pollen application and no individual isolation (inside screened enclosure)	105	79.2	2021
AIP with subsequent pollen application and no individual isolation (inside screened enclosure)	360	78.9	6675

Table 3. Levels of contamination with non-target pollen in seedling progeny detected from morphological examination in crosses between a range of female parents and *E. globulus* (male parent), at several bud colour stages at Guaiba, Brazil

Female parent taxon	Yellow buds		Yellow–green buds		Green buds	
(No. of clones)	No. of seedlings	Contamination (%)	No. of seedlings	Contamination (%)	No. of seedlings	Contamination (%)
E. grandis x *E. urophylla* (6)	7 000	1.46	7 000	0.81	7 000	0.62
E. saligna (4)	4 000	5.00	4 000	2.08	4 000	1.17
E. urophylla (4)	4 000	0.22	4 000	0.10	4 000	0.05
E. grandis (4)	4 000	7.60	4 000	1.66	4 000	1.25
Mean	19 000	3.57	19 000	1.16	19 000	0.77

Table 4. Mean capsule retention, seed set and productivity of different pollination methods[a] in Australian experiments with four *E. grandis* clones and an *E. camaldulensis* pollen source

Treatment	Total no. of buds treated	Mean capsule retention[b] (%)	Mean no. of seeds/capsule[b]	Mean no. of seeds per bud treated	Buds pollinated per person/hour	Seed production (seed/person/hour)
1. Open pollination	243	10.8 a	1.7 b	0.1	na	na
2. Conventional pollination (3 visits)	264	21.2 b	7.5 a	1.3	75	98
3. OSP	622	44.3 d	5.0 a	1.8	133	240
4. AIP (yellow bud stage)	250	30.2 c	6.7 a	2.2	240	528
5. Emasculated, style cut, isolated, pollination at nectar production	206	14.2 ab	7.3 a	0.6	na	na
6. Emasculated, style cut, isolated, no pollen	299	7.7 a	0 b	0	na	na

[a] Refer to materials and methods section for more details of treatments
[b] Treatments followed by different letters differ significantly ($P < 0.05$)
na = not applicable

Comparing Tables 1 and 4 it can be seen that the capsule retention rates for *E. grandis* in Australia were lower than those obtained in Brazil, but the rankings of the different treatments were similar. OSP (44.3%) and AIP (30.2%) gave significantly better capsule retention rates, relative to conventional three-visit controlled pollination (21.2%). The numbers of seeds per capsule harvested for OSP and AIP (5.0 and 6.7 respectively) were slightly lower than that for conventional pollination (7.5 seeds per capsule), but differences among these treatments were not statistically significant ($P > 0.05$). Taking into account the longer time taken to carry out conventional pollination and OSP, AIP gave the best productivity at 528 seeds per hour, more than double that for OSP (240 per hour), and five times that for conventional controlled pollination (98 seeds per hour). The clones differed significantly ($P < 0.05$) in their rates of capsule retention and in their yields of seed per capsule. *Eucalyptus grandis* styles that had been cut on emasculated flowers according to the OSP protocol, with buds isolated until nectar production (between 4 and 10 d after emasculation; approximately 2–8 d after anthesis would have occurred), and pollen then applied (treatment 5) yielded a mean of 14.2% capsule retention and 7.3 seeds per capsule harvested. This showed that the cut style surface on some buds was still receptive up to 7 d after the cut had been made. Emasculated buds that had been isolated and the styles cut, but with no pollen applied (treatment 6), yielded no seeds.

The *E. urophylla* hybrid clone U_6 gave much better capsule retention than the four *E. grandis* clones, for both OSP and AIP. Table 5 summarises capsule retention rates across all four different pollen sources (one *E. grandis*, one *E. globulus* and two *E. pellita* individuals), and seed yields and productivity averaged across the *E. globulus* and *E. pellita* pollen sources only. Statistical analysis showed that OSP gave a significantly ($P < 0.001$) higher capsule retention rate (78.6%) than did AIP (58.1%), while differences among pollen sources were small, albeit significant ($P < 0.05$). Seed yields per capsule, and productivity, were much higher than obtained with *E. grandis*. Because of the greater number of flowers pollinated per hour, and higher yield of seeds per capsule, AIP gave almost twice the productivity (in terms of seeds produced per operator hour) of OSP, and a rate similar to that obtained in Brazil (more than 6500 seeds per operator hour, cf. Table 2).

Table 5. Mean capsule retention, seed set and productivity of different pollination methods in Australian experiments with *E. urophylla* hybrid clone U_6 and four pollen sources (combined data shown)

Treatment	Total no. of buds treated	Mean capsule retention[a] (%)	Mean no. of seeds/capsule[b]	Mean no. of seeds per bud treated	Buds pollinated per person/hour	Seed production (seed/person/hour)
3. OSP	159	78.6 a	36.1	29.4	133	3920
4. AIP (yellow bud stage)	117	58.1 b	45.4	27.2	240	6530

[a]Different letters denote significant differences in capsule retention ($P < 0.001$)
[b]Mean of *E. pellita* and *E. globulus* pollen sources only

Molecular genetic testing of *E. grandis* x *E. camaldulensis* cross

Analysis of the allelic data from the microsatellite markers, obtained from the male and female parent trees and the progeny, showed allelic profiles of all 20 progeny of the *E. grandis* x *E. camaldulensis* cross were fully consistent with their being descended from this cross (data not shown). All 20 offspring displayed alleles derived from both parents (ruling out self-fertilised offspring) and there were no alleles foreign to both parents, such as would be detected from seedlings derived from non-target external pollen sources. This result, across all 12 micro-satellite markers known to be polymorphic for the two parent species, confirmed that the 20 offspring were from the desired cross.

Discussion

The results from these experiments show that AIP and OSP produce similar rates of capsule retention and seed yields per capsule. This is not surprising, as pollinations are conducted at a similar developmental stage in both methods, and a receptive surface on the cut style is produced in a similar way.

In our trials, AIP was clearly more productive than OSP in terms of seeds produced per hour of operator effort. There was a two- to three-fold productivity increase across the species that have been tested to date in both Brazil and Australia, the female being *E. grandis*, *E. saligna*, *E. urophylla* and interspecific hybrids involving these species. Consequently, this new method of controlled pollination can significantly reduce the costs of controlled pollination programs. Use of AIP may reduce the cost of control-pollinated seed production to the point where operational planting of elite family crosses is economically feasible for species not readily amenable to clonal forestry such as the spotted gums (*C. maculata* and *C. citriodora* subsp. *variegata*), and *E. dunnii* and *E. nitens*. It may also help to bring control-pollinated turnover of large breeding populations of *Eucalyptus* species within economic reach. The OSP and AIP productivity rates in Australia, but not in Brazil, included the time taken for exclusion bagging carried out after pollination. Greater productivities would have been obtained in the Australian experiments if exclusion bagging had been omitted.

The level of contamination by non-target pollen is an important issue in any controlled pollination method. Harbard *et al*. (2000b) found that OSP in seed orchards of *E. globulus* and *E. grandis*, without any isolation measures, produced contamination rates of 4% and 12% respectively. The rates of contamination following AIP, as indicated by morphological examination of seedlings, were low in the Brazilian study reported here. The highest fraction of non-target seedlings was 7.6% in *E. grandis* at the yellow bud stage. The average across four female parent taxa studied was 3.6% at the yellow bud stage and substantially lower (1.6%) at the yellow–green bud stage, suggesting this is the best bud stage for AIP to maintain good seed yields and at the same time reduce contamination. Seed yields from green buds (only 2 seeds per bud pollinated) were too low for this stage to be recommended for AIP. In the Australian experiments AIP was carried out at the ripe (yellow bud colour) stage, about 3 d before the operculum separates from the hypanthium. Pollen applied to cut style surfaces on *E. grandis* buds that had been emasculated, their styles cut and isolated, and then pollinated at the time of nectar production some 4–6 d after anthesis would have occurred (Treatment 6) produced some viable seeds (mean of 0.6 seeds per bud pollinated), showing that contamination potential may exist for at least 7–10 d after the style is cut. Similarly, Harbard *et al*. (2000b) found that styles were still receptive 4 d after cutting. The natural duration of stigma receptivity can vary between clones and is affected by environmental conditions (Hodgson 1976), and similar variability may be anticipated for the receptivity of cut style surfaces.

There is no obvious explanation for the higher levels of contamination in *E. grandis* and *E. saligna* than in other maternal taxa in the Brazilian study. Differences in self-compatibility, flower morphology or flowering phenology after AIP are possible causes.

The estimates of contamination levels in Brazil did not enable us to differentiate between contamination from selfing and from non-target external pollen sources. As insect pollinators were likely to have been almost completely excluded, and the roof and mesh screening reduced air movement within the facility, pollen movement between clones would have been restricted and it seems likely that contamination by selfing was more important. Flowers pollinated with the AIP method might be prone to cleistogamous self-fertilisation (i.e. self-fertilisation with pollen from the same flower) in self-compatible genotypes, because flowers are not emasculated, and anthesis proceeds normally a few days after pollination. Uncut stamens open normally following AIP and thus contamination may have occurred just after flower opening, when the cut style was still receptive and self pollen at its maximum viability. Personnel making crosses might contribute to self-pollination by moving or shaking branches and causing pollen to drop on the cut styles. Self-pollination could occur even in flowers pollinated by AIP and subsequently bagged for isolation, as in the Australian experiments.

Out of 20 progeny in the Australian experiments, DNA fingerprinting showed that there was no contamination and all were the desired crosses. However, controlled self-pollinations of the *E. grandis* clone used as a mother, carried out prior to the AIP study, failed to produce any seed. The potential for self-fertilisation following AIP could be higher in other, more self-compatible, genotypes.

The overall level of contamination in our clone banks was well below 10% for all the combinations studied, whether buds were isolated or not. It suggests the AIP method should be acceptable for mass supplementary pollination to produce elite control-pollinated families for operational planting, or for field trials to screen for elite genotypes. In both the Brazilian and the Australian studies reported here, the activity of animal pollinators was minimal. The trials were carried out inside well-screened breeding facilities that effectively excluded bees and flies, reported to be the principal insect pollinators of eucalypts (Eldridge *et al*. 1993; House 1997). A few smaller insects were observed occasionally around the flowering trees. Higher levels of outcross pollen contamination on non-isolated flowers following AIP might well occur in an open-air clone bank or seed orchard with more intense pollinator activity. Levels of contamination on isolated and non-isolated flowers following AIP under these conditions remain to be determined.

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