

Eucalypt seed collectors: beware of sampling seedlots from low in the canopy!

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Summary

Molecular markers have provided evidence that the canopy position from which open-pollinated seed is harvested in *Eucalyptus globulus* can markedly affect its outcrossing rate. Outcrossing rates were higher and more uniform in upper canopy samples. As expected, no significant differences in outcrossing rate were found between top and bottom canopy samples of self-incompatible trees, but differences were large between top and bottom samples of self-compatible trees. In one tree, 73% of the open-pollinated progenies at the bottom of the canopy were selfed compared to 26% at the top. Such selfing can severely reduce survival and growth. This result clearly indicates the importance of sampling position within the canopy when collecting open-pollinated seed for deployment or genetic evaluation.

Keywords: seed collection seed quality, inbreeding, outcrossing rate, *Eucalyptus globulus*

Introduction

Eucalypts have a mixed mating system which means that a significant proportion of open-pollinated (OP) seed can result from self-fertilisation. While eucalypt flowers are usually protandrous, preventing pollen from one flower fertilizing the ovules of the same flower, the large variation in flowering time within a canopy still provides ample opportunity for self-pollination (House 1997). The rate of self-fertilisation varies markedly between and within species (Potts and Wiltshire 1997), depending upon both genetic and environmental factors (Potts and Savva 1988), with estimates of outcrossing rates, for widespread eucalypt species, ranging from 54% to 96% (Potts and Wiltshire 1997).

It is now well established that variation in outcrossing rates may markedly affect the performance of OP progenies (Burgess *et al.* 1996; Hardner *et al.* 1996). This is due to inbreeding having severe effects on progeny fitness traits such as survival and growth rate (termed inbreeding depression). Inbreeding depression has been reported in numerous eucalypt species (Potts and Wiltshire 1997). For example, in the major plantation species *Eucalyptus globulus*, selfing can reduce stem volume at 43 months by nearly 50% when compared with completely outcrossed plants (Hardner and Potts 1995). Therefore, in theory, for every 10% of the OP progeny that are selfs, overall

plantation productivity will be reduced by approximately 5%. Across the estimated area of *E. globulus* established in 2000 (c. 80 000 ha, P. Gore *pers. comm.*), economic models for a pulpwood objective suggest such a depression could result in a loss of profit at rotation age in the order of \$12 million (L.A. Apiolaza unpublished data).

One factor that may affect outcrossing rate is the position in the tree canopy from which the seed is harvested. The first suggestion that OP seed obtained from the top of a eucalypt tree may have a higher outcrossing rate than OP seed obtained from the lower canopy was provided by Eldridge (1970). In this case, the frequency of a rare curly-leaf mutant, in a seedlot collected from near the top of a tall *E. regnans* tree, was found to be greatly reduced when compared to a seedlot collected from the base of the canopy. Assuming this tree to be heterozygous for the mutant allele, the decreased frequency of its expression at the top of the tree presumably reflects an increase in outcrossing rate. If such an effect is common in eucalypts, then the position from which OP seed is harvested in the tree canopy may significantly affect offspring performance, affecting genetic gains in deployment and breeding value predictions (Borrallho and Potts 1996), and inducing genetic differences between repeat seed samples from the same tree. Given the importance of *E. globulus* as a plantation species, the present study aimed to determine the difference in outcrossing rate between OP seed collected from the top and bottom of individual trees.

Methods

Open-pollinated seed was sampled from the top and bottom of six open-grown *E. globulus* trees in remnant forest in south-eastern Tasmania. Top samples were typically taken at 20-25 m above ground level while bottom samples were taken at 2-5 m. Two of the trees were chosen because they were known to be self-incompatible (trees no. 537 and 546; A. Mitchell and C. Hardner unpublished data) and therefore would not be expected to differ in outcrossing rate between top and bottom samples. Three trees were chosen because they were known to be self-compatible (309, 507 and 541), and would have the potential to exhibit variation in outcrossing rate. The degree of self-incompatibility for trees 537, 546, 507 and 541 was determined by the percentage reduction in seed set following self-pollination compared with that obtained following controlled outcrossing (Hardner and Potts 1995). By this method, fully self-compatible trees would have a self-incompatibility rating of

0% whereas fully self-incompatible trees would rate at 100% (Potts and Wiltshire 1997). Tree 309 was determined to be at least partially self-compatible by the presence of viable seed, following assisted self-pollination (A. Mitchell unpublished data). Tree 739 was of unknown self-incompatibility status and was chosen solely because it possessed a rare isozyme genotype, which is useful in distinguishing selfed progeny from outcrossed progeny.

Individual capsules from the 1998 flowering season were randomly collected, at the four cardinal points around each tree (depending upon shape and accessibility), and from the highest and the lowest region of each of the six selected trees. A minimum sample of 20 capsules from each collection point was used. From each capsule the percentage of inviable (aborted) seed and the number of viable seed (seed set) were determined following Hardner and Potts (1995). The significance of the difference between top and bottom samples was then tested for both traits with a one-way analysis of variance using the GLM procedure of SAS (SAS 1990). The exception to this was tree 739, where a bulk seed collection was obtained from the bottom and the top of the tree and no analysis of variance was performed.

For five of the six trees, outcrossing estimates were obtained using isozyme analysis of seedlings (Moran and Bell 1983). Seed was germinated such that the number of seeds sampled per capsule was generally kept constant between top and bottom. Seedlings were assayed for up to five polymorphic loci using starch gel electrophoresis (Wendel and Weeden 1989). Seven- to nine-day-old seedlings were ground in 0.2 M Tris-HCl, pH 8.0, containing 10% glycerol, 10% PVP-40, 0.2% Triton X100 and 7 mM dithiothreitol. A lithium-borate, pH 8.5/ Tris-citrate, pH 8.2 system was used to assay the enzyme systems EST (esterase) and AAT (aspartate aminotransferase); and a morpholine-citrate, pH 6.1 system to assay PGD (phosphogluconate dehydrogenase), PGI (phosphoglucose isomerase) and SDH (shikimate dehydrogenase) (Moran and Hopper (1983) for PGD, PGI, SDH and AAT except that 10 mg of MTT was used in place of NBT, when required, and PVP was omitted from the AAT staining reaction. EST staining was performed as in Wendel and Weeden (1989), method 1, except that the β -naphthyl acetate was omitted.

Outcrossing rates and pollen allele frequencies were then estimated from the OP progeny isozyme genotypes using maximum likelihood estimation with the multi-locus mating system program, MLTR (Ritland 1990). Family-based estimates of outcrossing rate were determined for each tree where top and bottom were assigned to separate families and the maternal genotype of the tree was included (as determined from pollen samples and progeny arrays). Standard errors were then calculated by performing 100 bootstraps. In the case of tree 309, a single locus outcrossing estimation was performed for the top and bottom samples, based upon the varying frequency of a chlorophyll-deficient phenotype encoded at a single locus (Patterson *et al.* 2000). Contingency χ^2 tests were used to determine the significance of the difference in the estimated proportion of self and outcross between top and bottom samples of each tree, and in pollen allele frequencies between top and bottom for each self-incompatible tree.

Results

Outcrossing estimates obtained for the bottom canopy samples, for the six individual trees, were found to vary from 27% to 99% (Table 1), with an average of 71%. In contrast, estimates from the top of the canopy, for the same six trees, ranged from 74% to 94%, averaging at 87%. For the self-incompatible trees (546 and 537), outcrossing estimates between top and bottom samples were not significantly different, but in all three self-compatible trees (541, 507 and 309) highly significant differences were observed ($P < 0.005$, Table 1). At the top of each self-compatible tree, outcrossing rates approached those calculated for the self-incompatible trees, ranging from 74% to 90%. In contrast, at the bottom, estimates varied from 27% to 66%, indicating a marked increase in selfing at the bottom of all three trees. Interestingly, where the degree of self-incompatibility of the tree had been determined (541 and 507), the outcrossing estimates were found to reflect this. The less self-compatible of the trees (541, 98% SI) had higher levels of outcrossing and the more self-compatible (507, 92% SI) had lower levels. In addition, the difference between top and bottom samples increased as the degree of self-compatibility increased. Outcrossing estimates for tree 739, where the self-compatibility status was unknown, were determined to be 94% at the top and 96% at the bottom. From the clear trend observed between the known self-compatible and the self-incompatible trees, we predict that this tree is likely to be close to 100% self-incompatible.

Table 1. Outcrossing estimates at top and bottom of the canopy of individual *Eucalyptus globulus* trees with the number of loci used for each estimate (Loci), associated standard errors (s.e.), number of progeny tested (n) and the probabilities of the contingency χ^2 test (P) of the difference in proportion of selfs in OP progenies between the two sampling positions. Percentage of self-incompatibility (if tested) and designated status are also shown (SI=self-incompatible, SC=self-compatible).

Tree	SI	Loci	Bottom			Top			P
			Outcrossing (%)	s.e. (%)	n	Outcrossing (%)	s.e. (%)	n	
546	SI (100%)	5	87	2	198	90	4	90	0.47 ns
537	SI (100%)	3	99	0	101	94	6	90	0.055 ns
739	SI?	5	96	4	128	94	4	122	0.47 ns
541	SC (98%)	5	66	12	74	90	8	87	0.00 ***
507	SC (92%)	5	27	3	132	74	4	142	0.00 ***
309	SC	1	49		513	77		280	0.003 **

At the top and bottom of each tree where the outcrossing rates were constant (i.e. in self-incompatible trees), pollen allele frequencies were found to be significantly different in 8 out of 13 pair-wise tests at the 0.01 probability level (data not shown). This indicates that the pool of pollen contributing to OP outcross progeny differs within the canopy.

The pattern of variation in seed set and proportion of inviable seed between the top and bottom of individual trees did not follow as clear a trend as that observed for outcrossing rates. Significant differences in seed set per capsule between top and bottom were found for three out of five trees, and in all cases this involved samples collected from the upper canopy having more seed per capsule than those sampled from the lower canopy (Table 2). The increased seed set, however, did not always correlate with high levels of outcrossing. The proportion of inviable seed was found not to vary significantly in either of the self-incompatible trees (i.e. where outcrossing rates were constant, Table 2). However, there was an overall trend for

greater seed inviability at the bottom of the self-compatible trees, suggesting that more seed is being aborted here where selfing is higher.

Table 2. The average number of viable seed per capsule (Seed set) and the proportion of the total seed which is inviable (Inviability) at the top and bottom of individual *Eucalyptus globulus* trees of known self-incompatibility status (SI = self-incompatible, SC = self-compatible) and the probabilities of the contingency χ^2 test (*P*) of the difference between the two sampling positions.

Tree	SI	Seed set			Inviability		
		Bottom	Top	P	Bottom	Top	P
546	SI	7.6	7.7	0.96 ns	0.30	0.30	0.88 ns
537	SI	3.6	4.8	0.017 *	0.18	0.16	0.47 ns
541	SC	3.1	9.3	0.00 ***	0.38	0.17	0.00 ***
507	SC	4.0	9.2	0.00 ***	na	na	
309	SC	5.7	6.2	0.72 ns	0.37	0.25	0.073 ns

Discussion

Consistent with the initial suggestions of Eldridge (1970), outcrossing rates estimated from samples obtained from the tops of *E. globulus* trees were higher and more uniform than those from samples collected from the bottom of the canopy. As expected, self-incompatible trees have outcrossing rates that approach 100% and do not differ significantly within the canopy. The outcrossing rates of self-compatible trees, however, were up to 47% higher at the top of the canopy compared to the bottom of the same tree.

A similar trend has been reported in several pine species, with cones lower in the crown experiencing more selfing than those higher in the crown (e.g. Fowler 1965). As pines are wind pollinated and pollen-bearing male strobili (catkins) tend not to extend as high into the crown as the female strobili (conelets), the closer proximity of the lower cones to the self pollen source provides a likely explanation for this effect (Fowler 1965; Linhart 2000). Although animal pollination predominates in *E. globulus* (House 1997; Hingston and Potts 1998), it is possible that the situation where pollen showers down through the canopy (Eldridge 1970) results in the increase in selfing we observed in self-compatible trees. However, such 'additional' pollination could be expected to generate a greater seed set per capsule at the bottom of the tree and this was not observed.

An alternative explanation derives from observations of pollinator behaviour and the significant variation in pollen allele frequencies observed between top and bottom samples of individual self-incompatible trees. In *E. globulus*, many bird species have been observed to spend most of their time in the upper half of the canopy (A. Hingston unpublished data), and it appears that in self-incompatible trees, the presence of birds can increase the seed set per capsule (A. Hingston unpublished data). Seed set data for two of the self-incompatible trees revealed a significant increase at the top of one tree, but not at the other. This variation in the seed set data may be due to small sample size or alternatively it could be indicating that the system is complex with pollinators and/or pollinator behaviour varying in different trees.

Insects have also been identified as pollinators of *E. globulus* (Hingston and Potts 1998), but their movement between trees is likely to be less than that of birds and therefore they may often

promote selfing rather than outcrossing. The role of nocturnal animals in *E. globulus* pollination has yet to be studied, but it may further confound the situation. While pollinator behaviour appears to provide the most likely explanation for the variation in outcrossing rates within a tree, pollen rain and possibly physiological mechanisms such as differential abortion of self seed (James and Kennington 1993; Kennington and James 1997) may be involved. It is clear from this study that pollination of a *E. globulus* tree should not be considered to be a consistent and homogeneous process across the canopy.

Implications

Open-pollinated seed collected from various eucalypt species in the wild are the basis of numerous breeding programs throughout the world (Eldridge 1993). Progenies grown from OP seed are evaluated in field trials in order to estimate the genetic worth (breeding value) of the mother. Variation in outcrossing rates will have a marked effect on the estimated genetic worth of mothers because of the confounding effects of inbreeding depression (Borralho and Potts 1996; Hardner *et al.* 1996). While it has been established that outcrossing rates vary from tree to tree (Hardner *et al.* 1996), in part due to variation in stand density (Borralho and Potts 1996), the effect of variation in outcrossing rates within a canopy, even within the same flowering year, has not been appreciated. Indeed, our finding shows that it is possible for different estimates of the genetic worth of a single tree to be obtained simply by sampling OP seed from different positions in the canopy. While it is often inconvenient and more costly to collect seed from high in the canopy, this is warranted if the OP seed is being used for genetic evaluation and the level of self-incompatibility of a tree is unknown. Outcrossing rates in upper canopy samples will be higher and more uniform amongst trees, thereby reducing variation in inbreeding depression and providing better estimates of genetic worth.

With the massive increase in plantation establishment in Australia, there is still a high demand for seed collected from native trees of proven better provenance (e.g. Jeeralang; Dutkowski and Potts 1999). Our findings suggest that a strategy of sampling from the top of trees will ensure the highest quality seed for plantation establishment. Two-thirds of the *E. globulus* trees tested to date are self-compatible to varying degrees (B. Potts unpublished data) and would thus potentially show positional effects. The positional effect may be a problem only if there is significant flowering, and thus seed set, in the lower canopy. The open-grown trees sampled in the present study were from remnant forest, comparable in form to woodland eucalypts which flower from the top to the bottom. How the positional effect in outcrossing rates relates to trees in other forest types (e.g. tall closed forest) or to trees from different strata in the forest (e.g. dominant versus sub-dominant) is yet to be determined. The canopy structure of the trees in this study is more likely to be akin to seed orchard trees, where wide spacing favours heavy flowering (Eldridge 1993). As a growing proportion of the plantations in Australia will be established from seed orchard OP seed, a challenge for the future will be to develop management strategies to minimise inbreeding so as to maximise the genetic quality of this seed.

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