

Impact of inbreeding on growth and development of young open-pollinated progeny of *Eucalyptus globulus*

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The use of open-pollinated seeds from seed orchards is a common strategy for the deployment of genetically improved eucalypts, including *Eucalyptus globulus*, an important pulpwood tree in many temperate climate areas. However, seed quality can be affected by the rate of selfing and to a lesser extent by contamination from pollen outside the orchard. Inbreeding between related parents and especially from self-crosses is known to cause diminished growth and developmental abnormalities in the resulting progeny. This study looks at the magnitude and variation in selfing and the impact in inbreeding depression across several *E. globulus* families collected over the years in a seed orchard. The effects on growth and development of outcrossed and selfed progeny were studied across five progeny trials, after pedigree reconstruction of the open pollinated progeny based on SSR genotyping. An additive genetic mixed linear model was fitted to the data to evaluate the impact of inbreeding on height growth. The results showed a significant inbreeding depression, with a height growth reduction of 15% in selfed progeny, when compared with crosses from unrelated parents. These inbreeding depression values varied among families, ranging between 7% and 24%, evidencing the importance of genetic background. Contamination rates were on average 10% suggesting long distance pollen dispersal was present. A small number of abnormal phenotypes (less than 10%) was observed in the field. This was associated with specific, unrelated, crosses and not to high inbreeding rates such as found among selfed progeny. The relevance of these results for orchard management and parent selection is discussed.

Keywords: Inbreeding Depression, Seed Orchard, Selfing, Abnormal Phenotypes, SSRs, Pedigree Reconstruction

Introduction

Eucalyptus globulus Labill. is a tree species belonging to the Myrtaceae family, native to Tasmania and Victoria, in mainland Australia (Jordan et al. 1993). It has been planted around the world due to its excellent growth and pulpwood characteristics (Alves et al. 2007). Introduced in Portugal in the 19th century, *E. globulus* currently covers around 850,000 ha of plantations supplying the local industry with nearly 6 million cubic meters per year of high-quality pulpwood (CELPA 2019).

In Portugal, breeding programs for this species have been in place for over 40 years, relying mostly on clonal and controlled crossing schemes (Araújo et al. 1997). However, due to their simplicity and low costs, large-scale production of improved stocks has also been based on open pollinated seed orchards (Borralho et al. 2007, Gonzaga et al. 2016, Griffin 2014). Such system however has its own caveats. Although the species is mostly outcrossing and several self-incompatibility mechanisms have been reported (Pound et al.

2002), self-pollination is common (Potts & Wiltshire 1997). This can increase average co-ancestry and the concomitant build-up of inbreeding and inbreeding depression in the seed, resulting in losses of growth, survival and other adverse developmental consequences (Hardner & Potts 1995, Charlesworth & Willis 2009, Costa e Silva et al. 2011, Fuchs et al. 2015, Nickolas et al. 2019, Pupin et al. 2019). These adverse effects however are known to vary among genotypes (McGowen et al. 2010, Costa e Silva et al. 2010). Also, selfing rates can vary due to asynchronous flowering among the parents in the orchard or to low activity of pollinators (Patterson et al. 2004, Hingston et al. 2004a, 2004b, Moncur et al. 1995). Another factor influencing the genetic quality of seeds from seed orchards is the degree of contamination from unimproved pollen from outside the orchard, which depends on the degree of its spatial isolation. Reported contamination rates have ranged between 5% and 15% (Gonzaga et al. 2016, Pupin et al. 2019) in isolated orchards but can reach 40% in smaller or less isolated locations (Chaix et al. 2003, 2010, Rao et al. 2008). Thus, the amount of selfing and contamination from outside pollen in seed orchards are important seed quality parameters to quantify, monitor and if pos-

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sible, to control. Finally, abnormal phenotypes, often observed in the field trials and plantations, have been previously associated with possible high levels of inbreeding, and particularly with selfing. This is expected since selfed plants (*i.e.*, more inbred) tend to express more often, putative deleterious or prejudicial recessive alleles (Klekowski 1988, Fuchs et al. 2015). In *E. grandis*, similar abnormalities, including different leaf shape and excessive lateral branching, have similarly been associated with inbreeding and high levels of homozygosity (Fuchs et al. 2015).

The aims of this study were to: (i) evaluate the selfing rate and impact of inbreeding depression in *E. globulus* and to what extent family genetic background negatively impacted growth in self-crossed progenies; (ii) study the prevalence of outside pollen contamination in progenies collected in a seed orchard; and (iii) assess whether morphological abnormalities detected in some of the offspring were related to their level of inbreeding.

To accomplish this, several field tested open-pollinated family progenies between 2 and 5 years were evaluated for growth rate and presence of abnormalities. Given the relatively young age, height, rather than diameter, was considered a better proxy of later age tree volume. Early height was shown to be strongly correlated with later age volume growth, with correlations ranging between 0.8 and 0.9 (Borrinho et al. 1992). These families were collected in an *E. globulus* seed orchard and their full pedigree was reconstructed based on molecular markers. An individual tree mixed linear model accounting for all pedigree information was fitted to quantify the importance of inbreeding depression and how much it varies between families.

Materials and methods

Seed orchard

The tested *E. globulus* progeny was collected from a clonal seed orchard located at Herdade de Espirra, near the city of Pegões in southern Portugal (38° 41' N, 08° 12' W). The orchard occupies 1.7 ha and was established in 2008. It is located on a flat area, with an initial spacing of 5 × 5 m, and is surrounded by native stone pine (*Pinus*

pinia) and cork oak (*Quercus suber*) stands, with the nearest *E. globulus* trees located at least 600 m away. The orchard consists of 27 *E. globulus* parent trees, with variable number of copies (ramets) per genotype, ranging from 1 to 60. These are first- and second-generation selected material from RAIZ eucalypt breeding program.

Families and field trials

Up to eleven open pollinated families were collected in the orchard, at three consecutive years (2014, 2015 and 2016). These families were selected because either they resulted from the most represented mothers in the orchard, with between 14 and 40 flowering ramets each (see Tab. S1 in Supplementary material for detailed information) or they had progeny with large number of abnormal phenotypes (see below).

The progeny from these families (n=324) was tested across five locations, covering a range of sites in Central and Southern Portugal. All trials were designed as randomized complete blocks with up to 9 replicates, with each family represented in each replicate by 1 to 5 tree plots, with a standard spacing of 0 × 2 m (see Tab. S2 in Supplementary material for trials' edaphoclimatic conditions). As a result of initial variation in progeny numbers and subsequent mortality, each family was represented in the dataset varying between 1 and 24 offsprings per site.

Phenotypic measurements

Survival across all trials was high being on average 95%. The mixed model analysis of growth included all trees with normal phenotype coming from the 7 most representative families of the orchard. They were measured for height, with ages between 1.6 to 4.5 years (n=283 – Tab. 1). Since the percentage of trees in the field trials presented abnormalities were initially low, to increase sample size, four extra families were added to the study (but not included in the mixed model – see below). Normal phenotypes were ascribed to individuals having the typical juvenile eucalypt canopy, with opposite juvenile leaves, lanceolate and symmetrical and with a single axillary bud at the base of each leaf. In contrast, abnormal phenotypes were ascribed to trees having an excessive proliferation of

axillary buds and a loss of apical dominance, resulting in a mallee or bushy appearance.

Molecular analysis

DNA was extracted from leaves of 324 progeny and 27 putative parents in this study, with an optimized CTAB protocol (as described in Grattapaglia & Sederoff 1994). Spectrophotometry was used to calculate DNA concentrations and ensure dilutions equal to an optimal concentration of 10 ng μL^{-1} . A total of 12 microsatellite (SSR) markers were selected, covering all linkage groups in the *Eucalyptus* sp. genome (Ribeiro et al. 2011), and were amplified in four multiplex sets (see Tab. S3 in Supplementary material). The PCR products detection was established by electrophoresis with use of ABI 3130 Genetic Analyzer® (Applied Biosystems, Foster City, CA, USA) followed by alleles length detection using the Peak Scanner software v. 2.0.

Parental assignment (*i.e.*, pedigree reconstruction) was carried out based on the allele data for all the progeny belonging to the seven open pollinated families and considering all putative parental individuals in the orchard. The assignment was conducted using standard maximum likelihood methods implemented in CERVUS® 3.4 (Marshall et al. 1998, Kalinowski et al. 2007). The analysis determined the critical value of Delta (D) for each confidence in the paternity and maternity analysis, defined as the difference between the “LOD score” of the first most likely parent candidate and the “LOD score” of the second most likely candidate. Significance of the paternity tests simulated by CERVUS software was based on a confidence level of 80% (relaxed) to 95% (strict), as suggested by Marshall et al. (1998), using 50,000 repetitions, 0.01 as the ratio of genotyping errors, and 90% as the proportion of pollen donors sampled within each population (given the high degree of isolation of the orchard). The minimum number of loci necessary to determine the paternity of a seed was set at six.

If a seedling had no assigned pollen donor from any of the orchard parental population, pollen parents were assumed to be originated from unrelated trees from outside the orchard, hence resulting in foreign pollen contamination. In pedigree reconstruction for each seed, the possibility of having one single mismatch (or reading error) was considered in the simulation, as recommended in Kalinowski et al. (2007).

Data and statistical analyses

Selfing and contamination rates were estimated for the most representative families in each of the three different seed collection years. Percentage selfing per open-pollinated family (*i.e.*, per maternal parent) was estimated by dividing the number of selfed offspring, by the total number of offspring in that family. Similarly, contamination rates were calculated by dividing the

Tab. 1 - Details of the trial location, age of measurements and experimental design in the five field trials included in the mixed model analysis. Number of Randomized complete blocks and families tested, and the total across family progeny measured are also given (n=283).

Trial Location	Seed year	Age	# Replicates	# Families	Total progeny size
Fundão	2014	4.5	4	4	53
Abrantes	2015	3.2	9	4	75
Oleiros	2015	3.2	9	5	50
Gorda	2016	2.3	9	6	54
Nisa	2016	1.6	9	6	51

number of offspring sired by foreign pollen by the total number of offspring in the family. As mentioned, pollen was considered foreign if it did not match any of the genotypes present in the orchard. The inbreeding coefficient of each offspring (F) was estimated based on the complete reconstructed pedigree. The package “optiSel” in R (Wellmann 2019) was used to calculate the expected F values for each tree. Group co-ancestry (Θ) for parents, progeny and Status Number were calculated as described by Cockerham (1967), Lindgren et al. (1997) and Lindgren & Mullin (1998). Coancestry or coefficient of kinship is the probability that a gene taken at random from an individual will be identical by descent with a random homologous gene from another individual, including identical individuals (self-coancestry). Group coancestry (θ), as used here is the average of all possible pairwise coancestries, including self-coancestry and reciprocals between all trees in the pedigree set. By definition, the status number (N_s) is half the inverse of the group coancestry, thus $N_s = 0.5/\theta$. Only when all seed orchard genotypes contribute equally, in addition to being unrelated and noninbred, will N_s equal the census number in the orchard.

Since trials at the time of measurement had different ages, means and variances of height varied considerably between trials. Mean annual height increments, rather than total height, was used in the linear model, to ensure measurements are more comparable and variances are homogeneous across sites. The following mixed model was fitted (eqn. 1):

$$y = Xf + Z_1 a + Z_2 b_{Fi} + e \quad (1)$$

where y is the vector of height increments, f the vector of fixed effects, which includes the effects due to site, replicate within site, and population mean inbreeding depression slope (b_{Fi}), fitted as a covariate; a is the vector of tree's additive genetic effect, with $a \sim \text{NID}(0, A\sigma_a^2)$, where A is the average numerator relationship matrix (a matrix with the additive genetic relationships between all trees in the pedigree) and σ_a^2 is the additive genetic variance. The b_{Fi} is the vector of genetic inbreeding depression slopes, fitted as a random covariate for each genotype, with $b_{Fi} \sim \text{NID}(0, I\sigma_{b_{Fi}}^2)$ where $\sigma_{b_{Fi}}^2$ is the inbreeding depression slope variance and e the vector of residual effects with $e \sim \text{NID}(0, I\sigma_e^2)$, where σ_e^2 is the residual error variance. The X and Z_i are incidence matrices relating observations to the respective effects. To test whether the assumption of homogeneous residual variances could be held across the five sites, the above model was compared with an alternative model with a block diagonal R structure (with one residual section for each site) and fitted as $R = \bigoplus_{j=1}^5 R_j$ where $R_j = I\sigma_{e_j}^2$. We tested the null hypothesis that error variances are equal among sites, using a likelihood ratio test. The $LRT=4.876$ (with

Tab. 2 - Details of the nuclear SSR markers used in the analysis, and the number of alleles, the percentage of null alleles and the expected heterozygosity (H_e) found across the seven open-pollinated families tested.

Microsatellite	No Alleles	Null (%)	H_e
Embra11	17	0.10	0.87
Embra119	14	0.14	0.86
Emcrc8	17	0.04	0.88
Emcrc7	8	0.08	0.77
Embra23	15	0.08	0.90
Embra41	15	0.07	0.86
Embra227	10	0.04	0.78
En15	11	0.03	0.84
Es76	18	0.07	0.90
Eg65	18	0.05	0.89
Embra37	23	0.06	0.91
En12	17	0.08	0.87
Average	15.25	0.07	0.86

4 df; $p=11\%$), hence we accepted the assumption of homogeneous variances across sites. Further significance of random effects (a and b_{Fi}), was also tested based on a Likelihood Ratio Tests, assuming 0.5 df as suggested by Self & Liang (1987). Significance of fixed effects was tested based on a scaled Wald F statistic, following the Kenward & Roger (1997) simplification, as implemented in ASReml v. 4.2 (Gilmour et al. 2021).

This model assumed mean annual height increments from height measurements taken at different ages (as height measurements divided by their corresponding age) would be an expression of the same trait. High genetic correlations between height at different early ages have been reported for *E. globulus* (Borralho et al. 1992).

Across-site heritability for height growth was estimated without explicitly fitting a genotype by site interaction term, since the number of genotypes was small and there was unbalance in their representation across sites. It is expected that such effects, if present, would be captured in the residual term. Heritability was estimated as (eqn. 2):

$$h^2 = \frac{\sigma_a^2}{\sigma_a^2 + \sigma_e^2} \quad (2)$$

Taylor series expansion was used to obtain approximate standard errors for the variance parameters estimated from the linear mixed model, as well as for derived linear combinations or ratios of the variance estimates, as implemented on the “vpredict” function in ASReml v. 4.2 (Lynch & Walsh 1998, Gilmour et al. 2021).

The association between frequency of abnormal phenotypes and inbreeding was tested based on a χ^2 independence test, using the R function “chisq.test” with data from Tab. S4 (Supplementary material) as input.

Results and Discussion

Allele counts and heterozygosity

Amplification was successful across the 12 SSR markers, corroborating their importance in paternity tests in *E. globulus* reported previously (Costa et al. 2017) and in kinship analyses (Ribeiro et al. 2011). Overall, only 1.6% of the individuals had two missing SSR amplifications, 3.7% had three missing microsatellites, 1.2% had four and 0.3% had six. Allele diversity was high, with an average of 15.5 alleles per loci across all SSRs and with most showing eight or more alleles (Tab. 2). Such allelic diversity is somewhat higher than that found for the native land race in Portugal (Freeman et al. 2007) and closer to the values reported across the species natural distribution (Costa et al. 2017). This is expected since parents represented in the orchard are derived from various native provenance backgrounds. Heterozygosity was also high with $H_e = 0.86$ (Tab. 2), and similar to levels reported in domesticated and wild populations of *E. globulus* (Costa et al. 2017).

Seed orchard characterization

In the present study, at the time of measurement, between 2 and 4 years, the degree of selfing across families was on average 30%. This estimate is in the upper range of what has been reported in other orchards (Moran & Bell 1983, Gonzaga et al. 2016). Selfing rates varied considerably between collection years (Tab. 3). In 2014, no selfing was reported. This is a surprising result since it was a year of particularly poor flowering (data not shown), where high rates of selfing would be expected. In the following years, selfing was considerably higher. Since survival in the trial was consistently high at all sites (greater than 95%), these differences are unlikely to have been caused by selective mortality of some of the selfs. Selfing percentages also varied

Tab. 3 - Total number of progenies sampled from each family (ID Mother) and seed collection year, as well as the number and respective percentage of selfed and contamination with foreign pollen. The trial's location where the progenies were tested are also given (in parentheses). The progeny listed only includes the most representative families within each year (n = 311).

Year (Trial)	ID Mother	Progeny	Self	% Selfing	Contaminated	% Contamination
2014 (Fundão)	M74	9	0	0.0	0	0.0
	M07	13	0	0.0	0	0.0
	M13	15	0	0.0	0	0.0
	M18	18	0	0.0	0	0.0
2015 (Abrantes and Oleiros)	M74	27	3	11.1	0	0.0
	M07	31	3	9.7	5	16.1
	M13	34	3	8.8	5	14.7
	M49	27	26	96.3	1	3.7
	M28	17	15	88.2	1	5.9
2016 (Gorda and Nisa)	M74	19	7	36.8	3	15.7
	M07	15	1	6.7	5	33.3
	M13	20	2	10.0	4	20
	M89	24	16	66.7	0	0.0
	M49	21	12	57.1	2	9.5
	M28	21	3	14.3	4	19

F=0. However, some parents in the orchard are second- and third-generation selections, with some degree of kinship among them although not inbred themselves. The average co-ancestry among the 15 orchard parents contributing to the studied progeny, derived from pedigree records, was greater than zero ($\theta_p=0.05$) and corresponds to a Status Number of $N_s=9.28$, less than the census number of $N=15$. Their progeny ($N=283$), on the other hand, included crosses between some of these related parents, as well as from self-pollination. Therefore, its group co-ancestry was higher ($\theta_{off}=0.075$) and the expected inbreeding was greater than zero ($F_{off}=0.17$), although the corresponding Status Number remained similar ($N_{off}=9.27$). This build-up of inbreeding in the orchard's progeny was mostly the result of selfing, since very few progenies have been the result of outcrosses between related parents. There was also a substantial unbalance in the frequency of full- and half-sibs among families and in the relative pollen contribution to the progeny by the putative paternal parents (Fig. 1), with the top three paternal parents contributing to 65% of the progeny. Given the crossing scheme present in the dataset, the observed intra-class correlation amongst open-pollinated (OP) sibs was $r=0.51$, ranging across families between 0.32 and 0.83 (Tab. S5 in Supplementary material). In other words, open-pollinated sibs were on average as related as full-sibs (where $r=0.5$), a clear departure from the often-assumed half-sib kinship in open pollinated progeny studies.

Inbreeding depression for height growth

The reconstructed pedigree for all sampled trees allowed the estimation of the inbreeding coefficient (F) for each tree. Of the 283 trees with normal phenotypes used in the mixed model analysis, 54% had unrelated parents (F=0), 11% had F=0.0625, 4% had F=0.125, 1% had F=0.25 and 30% had F=0.5. Mean annual increment across the five trials was 2.9 m, with site effects and replicate effects within site being significant ($p<0.001$). The population mean inbreeding depression slope (b_f) was significant ($b_f = -0.867$; $p = 0.017$). In other words, for F=1, mean annual height growth is expected to be reduced on average by 0.87 m \pm 0.32. Progeny resulting from self-pollination, which has an F=0.5, would be expected on average to be 0.43 m year⁻¹ smaller in height when compared with outcrossed progenies from unrelated parents (Fig. 2), which corresponds to an inbreeding depression of 14.7%. These are similar to estimates published previously for growth in *E. globulus* (Hardner & Potts 1995, Costa e Silva et al. 2011, Nickolas et al. 2019). However, since the present study is based on young trees, between 1.6 and 4.5 years old, is likely to be an underestimation, as the impact of inbreeding depression is likely to increase with time due to increas-

among different parents, but again the pattern was unclear. In the 2015 seed year, selfing rates were low (at around 10%) for three of the families (M74, M07 and M13), and very high (around 90%) for two other families (M49 and M28). In the following seed year (2016), family M28 showed a relatively low selfing rate (14.3%), much lower than in the previous year. These results suggest other factors such as flower synchrony and the activity of pollinators at the time of stigma receptivity may play a significant role.

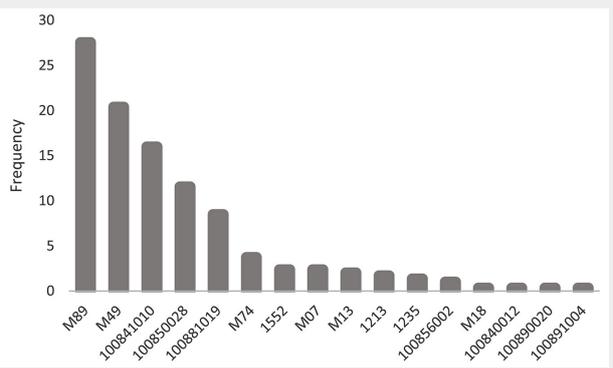
Contamination rates, from pollen outside the orchard, were low in absolute terms (average of 10% across years) but seem to be increasing over time. It was 0% in 2014, 8% in 2015 and 16% in 2016 (Tab. 3). Since *Eucalyptus* is mostly an insect pollinated species, distance of pollen dispersion is more limited than for wind pollinated species and dependent of the involved specific insect populations with maximum recorded pollen dispersal distances of a few hundred meters only (Barbour et al. 2005, Gonzaga et al. 2016, Field et al. 2011).

Considering the distance between the orchard and any other putative *E. globulus* tree in the neighborhood in the present study was over 600 m, the contamination rates found are higher than expected. This highlights the importance to better understand the pollination ecology and in particular the relevance of long-distance pollen dispersal mechanisms in eucalypt seed orchards (Burczyk et al. 2004, Jones et al. 2008).

The causes for the substantial annual variation in selfing and to a lesser extent in contamination rates are hard to interpret since no data on the phenology of trees inside and around the orchard, and insect activity has been recorded in detail. The flowering synchrony, the length and intensity of flowering, the weather conditions at critical pollination periods and the intensity of insect activity are all plausible factors affecting pollen dispersal in a seed orchard, hence influencing outcrossing and contamination rates.

All unrelated base parents in the orchard were assumed to have an inbreeding of

Fig. 1 - Frequency of male contribution (pollen) to the progeny in the field-tested offspring. The counting includes progeny from seed collected over a period of three flowering seasons (2014-2016) and tested in five different locations, with ages ranging between 1.6 and 4.5 years.



Tab. 4 - Variance components for the random effects in the mixed linear model for annual height growth and corresponding likelihood ratio tests (LRT) and probability of significance. Effects include the trees' additive genetic effects (a), the deviation of inbreeding depression slopes per genotype, relative to the population mean slope (b_{Fi}), and the residual effects (e).

Effects	Variance	LRT	Prob
a	0.017	7.12	0.008
b_{Fi}	0.476	6.16	0.013
e	0.064	-	-

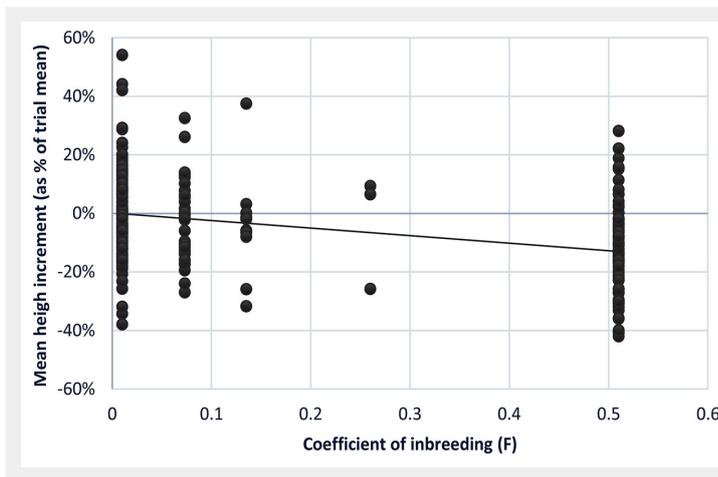


Fig. 2 - Mean annual height increments (given as percentage of the trial means) for all trees in the five-progeny trial ($n=283$), relative to their corresponding inbreeding coefficient.

ing inter-tree competition (Tomé et al. 1994, Weiner & Damgaard 2006, McGowen et al. 2010, Costa e Silva et al. 2011, Zhang et al. 2017). This highlights the importance of ensuring self-pollination rates are kept to a minimum in seed orchards.

The inbreeding depression slopes for each of the seven maternal parents studied (b_{Fi}) showed a consistent decrease in growth with inbreeding (*i.e.*, they all have a negative coefficient), with its magnitude varying from $b_{Fi} = -0.4$ to -1.4 (Tab. 4). For example, progeny from parent M74 was substantially more impacted by inbreeding, with an estimate annual height loss of -1.4 m when $F=1$ (an inbreeding depression in selfs of 24%), whereas M13 was more tolerant to inbreeding with $b_{Fi}=-0.43$ (or 7% depression in selfs – Tab. 5).

Despite this large variation in the inbreeding depression slope between parents, an effect fitted as a random term in the model, differences between them and the mean population slope $b_F = -0.867$ were only moderate (LRT = 6.16; $p=0.013$; $df = 0.5$; and variance of $\sigma_{b_{Fi}}^2 = 0.476$). Although indicative of a genetic effect underlying the inbreeding depression slopes, the statistical power of the test was surely affected by the relatively low number of offsprings per parent and per class of inbreeding. Further evidence of a genetic effect on inbreeding depression should be confirmed in future studies.

Across-site additive genetic effects for height were significant, with a variance of $\sigma_a^2 = 0.017$ (LRT=7.12; $p=0.008$ – Tab. 4), which corresponds to an estimated heritability for height of $h^2=0.21$ (standard error=0.16), an estimate well within the reported for the species and trait (Borralho et al. 1992, Hardner & Potts 1995, Potts & Wiltshire 1997, Costa e Silva et al. 2010). Given the very limited sample size and unbalance in the crossing scheme, the linear model fitted only accounted for additive genetic effects and did not include dominance or other non-additive genetic effects. Since dominance effects are known to be of importance for growth traits in eucalypts (Costa e Silva et al. 2004, Araújo et al. 2012), and if unaccounted, likely to

cause an upward bias in the heritabilities (Borralho et al. 1992), these estimates are expected to be somewhat over-estimated.

Abnormalities

Among the 324 trees assessed, 30 were classified as having abnormal phenotypes, showing dwarfism and a mallee appearance, as a result of partial or total loss of apical dominance and excessive proliferation of axillary buds. However, it was clear from the pedigree records that there was no obvious association between such abnormalities and selfed individuals, as many abnormal phenotypes were found in unrelated outcrossed progeny (where $F=0$). The chi-square test confirmed the independency

observed between abnormal phenotypes and estimated levels of inbreeding (null hypothesis: $\chi^2_{[4]} = 2.4277$; $p = 0.6576$). To clarify any putative association between abnormal phenotypes and parents, we looked at crosses with 10 or more offsprings. This analysis showed however that abnormal phenotypes were more frequent in specific, but otherwise unrelated, crosses (Tab. 6). For example, cross between M49 and M28 produced seven abnormal phenotypes out of a total of 22 trees (an incidence of 32%), whereas selfs from the same mother tree (M49) only had one abnormal phenotype out of 38 trees present in the trials. Also, cross between parent trees 100841010 and M18, although with a

Tab. 5 - Estimates of the solutions for the overall and site mean, the parent's additive genetics effects (a), the mean population inbreeding coefficient slope (b_F), and the individual parent inbreeding coefficient slopes (b_{Fi}) for the seven parents in the study.

Effects	Mother ID	Estimate	Standard error
Mean	-	2.929	0.145
Site	394	2.929	0.145
	407	2.367	0.156
	416	3.355	0.210
	421	2.853	0.185
	423	1.596	0.193
	Parents breeding value (a)	M74	0.231
M18		0.032	0.106
M49		0.097	0.099
M28		0.013	0.099
M07		-0.117	0.087
M13		-0.024	0.086
M89		-0.012	0.080
b_F			-0.867
b_{Fi}	M74	-1.404	0.392
	M18	-1.310	0.628
	M49	-0.795	0.375
	M28	-0.580	0.392
	M07	-0.743	0.460
	M13	-0.432	0.423
	M89	-0.724	0.386

Tab. 6 - Progeny with normal or abnormal phenotypes resulting from crosses with 10 or more offspring present in the five progeny trials (aged between 1.6 and 4.5 years). Parent A and Parent B columns are not indicative of female or male parents and merely represent the cross between the two clones.

Parent A	Parent B	Normal phenotype	Abnormal phenotype
M49	M28	15	7
M49	M49	37	1
100841010	M18	6	4
M07	M89	12	0
M07	100841010	11	1
M07	100881019	13	0
M13	M89	33	1
M89	M89	13	3
M28	M28	18	0
M74	M89	16	0
M74	100841010	10	0
M74	M74	10	0

lower progeny size, showed four abnormal trees out of a total of 10. Genetically, this could be a consequence of particular deleterious effects between specific alleles present in the two parents. If these alleles are rare, this would be highly unlikely to occur across several independent crosses, as found in our study. This question regarding abnormal phenotypes deserves further clarification with an experiment that includes a larger sampling size.

Conclusion

The studied open-pollinated *E. globulus* seed orchard progeny presented a substantial degree of self-pollination, accounting for an average of 30% in the 2- to 4-year-old progeny from seed crop collected over three years. Such selfing resulted in an average inbreeding depression of 14.7% in height growth. However, the magnitude of inbreeding depression differed amongst genotypes, ranging between 7% and 24% when comparing selfed with outcrossed progeny from unrelated parents. Preferential harvesting of seed from maternal parents less affected by inbreeding depression should therefore be a useful measure to improve seed quality from orchards. Another important result was that no relation has been found between the presence of anomalous phenotypes and selfing. Instead, a high frequency of abnormalities has been found in specific but unrelated crosses. These findings highlight the importance of understanding the reproductive biology, the genetic basis of reproductive success, the gene flow in orchards and the merits of using genetic markers to regularly monitor factors such as rates of contamination, selfing and inbreeding depression.

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Conflicts of Interest

The authors declare no conflicts of interest.

References

- Alves A, Pereira J, Silva J (2007). A introdução e a expansão do eucalipto em Portugal [Introduction and expansion of eucalypt in Portugal]. In: "O Eucalipto em Portugal: Impactes Ambientais e Investigação Científica" (Alves AM, Pereira JS, Silva JMN eds), ISA Press, Lisboa, Portugal, pp. 13-24. [in Portuguese]
- Araújo JA, Borralho NMG, Dehon G (2012). The importance and type of non-additive genetic effects for growth in *Eucalyptus globulus*. *Tree Genetics and Genomes* 8: 327-337. - doi: [10.1007/s11295-011-0443-x](https://doi.org/10.1007/s11295-011-0443-x)
- Araújo JA, Lemos L, Ramos A, Ferreira JG, Borralho NMG (1997). The RAIZ *Eucalyptus globulus* breeding program: a BLUP rolling front strategy with a mixed clonal and seedling deployment scheme. In: Proceedings of the IU-FRO conference on "Silviculture and Improvement of Eucalypts". Salvador (Bahia, Brazil) 24-

- 29 Aug 1997. EMBRAPA, Brazil, pp. 371-378.
- Barbour RC, Potts BM, Vaillancourt RE (2005). Pollen dispersal from exotic eucalypt plantations. *Conservation Genetics* 6: 253-257. - doi: [10.1007/s10592-004-7849-z](https://doi.org/10.1007/s10592-004-7849-z)
- Borralho NMG, Kanowski PJ, Cotterill PP (1992). Genetic control of growth of *Eucalyptus globulus* in Portugal. I: Genetic and Phenotypic Parameters. *Silvae Genetica* 41: 39-45.
- Borralho NMG, Almeida MH, Potts BM (2007). O melhoramento do eucalipto em Portugal [Eucalypt improvement in Portugal]. In: "O Eucalipto em Portugal: Impactes Ambientais e Investigação Científica" (Alves AM, Pereira JS, Silva JMN eds). ISA Press, Lisboa, Portugal, pp. 61-110. [in Portuguese]
- Burczyk J, DiFazio SP, Adams WT (2004). Gene flow in forest trees: how far do genes really travel? *Forest Genetics* 11 (3-4): 179-192.
- CELPA (2019). Boletim Estatístico da CELPA [CELPA Statistical Bulletin]. CELPA - Associação da Indústria Papeleira, Lisboa, Portugal, pp. 112. [in Portuguese]
- Chaix G, Gerber S, Razafimaharo V, Vigneron P, Verhaegen D, Hamon S (2003). Gene flow estimation with microsatellites in a Malagasy seed orchard of *Eucalyptus grandis*. *Theoretical and Applied Genetics* 107: 705-712. - doi: [10.1007/s00122-003-1294-0](https://doi.org/10.1007/s00122-003-1294-0)
- Chaix G, Vigneron P, Razafimaharo V, Hamon S (2010). Improved management of Malagasy *Eucalyptus grandis* seed orchards using microsatellite and paternity assignment. *Journal of Tropical Forest Science* 22: 271-280. [online] URL: <http://www.jstor.org/stable/23616656>
- Charlesworth D, Willis JH (2009). The genetics of inbreeding depression. *Nature Reviews Genetics* 10: 783-796. - doi: [10.1038/nrg2664](https://doi.org/10.1038/nrg2664)
- Cockerham CC (1967). Group inbreeding and co-ancestry. *Genetics* 56: 89-104. - doi: [10.1093/genetics/56.1.89](https://doi.org/10.1093/genetics/56.1.89)
- Costa e Silva J, Borralho NMG, Potts BM (2004). Additive and non-additive genetic parameters from clonally replicated and seedling progenies of *Eucalyptus globulus*. *Theoretical and Applied Genetics* 108 (6): 1113-1119. - doi: [10.1007/s00122-003-1524-5](https://doi.org/10.1007/s00122-003-1524-5)
- Costa e Silva J, Hardner C, Potts BM (2010). Genetic variation and parental performance under inbreeding for growth in *Eucalyptus globulus*. *Annals of Forest Science* 67: 606. - doi: [10.1051/forest/2010019](https://doi.org/10.1051/forest/2010019)
- Costa e Silva J, Hardner C, Tilyard P, Potts BM (2011). The effects of age and environment on the expression of inbreeding depression in *Eucalyptus globulus*. *Heredity* 107: 50-60. - doi: [10.1038/hdy.2010.154](https://doi.org/10.1038/hdy.2010.154)
- Costa J, Vaillancourt RE, Steane DA, Jones RC, Marques C (2017). Microsatellite analysis of population structure in *Eucalyptus globulus*. *Genome* 60: 770-777. - doi: [10.1139/gen-2016-0218](https://doi.org/10.1139/gen-2016-0218)
- Field DL, Ayre DJ, Whelan RJ, Young AG (2011). The importance of pre-mating barriers and the local demographic context for contemporary mating patterns in hybrid zones of *Eucalyptus aggregata* and *Eucalyptus rubida*. *Molecular Ecology* 20: 2367-2379. - doi: [10.1111/j.1365-294X.2011.05054.x](https://doi.org/10.1111/j.1365-294X.2011.05054.x)
- Freeman JS, Marques CM, Carocha V, Borralho N, Potts BM, Vaillancourt RE (2007). Origins

- and diversity of the Portuguese landrace of *Eucalyptus globulus*. *Annals of Forest Science* 64 (6): 639-647. - doi: [10.1051/forest:2007042](https://doi.org/10.1051/forest:2007042)
- Fuchs MCP, Tambarussi EV, Lourenção JC, Nogueira LM, Bortoloto TM, González ER, Oda S, Marino CL (2015). Molecular marker associated with a deleterious recessive anomaly in *Eucalyptus grandis* seedlings. *Annals of Forest Science* 72: 1043-1052. - doi: [10.1007/s13595-015-0502-9](https://doi.org/10.1007/s13595-015-0502-9)
- Gilmour AR, Gogel BJ, Cullis BR, Welham SJ, Thompson R (2021). ASReml user guide release 4.2: functional specification. VSN International Ltd, Hemel Hempstead, HP2 4TP, UK. [online] URL: <http://www.vsnl.co.uk>
- Gonzaga JMS, Manoel RO, Sousa ACB, Souza AP, Moraes MLT, Freitas MLM, Sebbenn AM (2016). Pollen contamination and nonrandom mating in a *Eucalyptus camaldulensis* Dehnh seedling seed orchard. *Silvae Genetica* 65: 1-11. - doi: [10.1515/sg-2016-0001](https://doi.org/10.1515/sg-2016-0001)
- Grattapaglia D, Sederoff R (1994). Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudo-testcross: mapping strategy and RAPD markers. *Genetics* 137: 1121-1137. - doi: [10.1093/genetics/137.4.1121](https://doi.org/10.1093/genetics/137.4.1121)
- Griffin AR (2014). Clones or improved seedlings of *Eucalyptus*? Not a simple choice. *International Forestry Review* 16: 216-224. - doi: [10.1505/146554814811724793](https://doi.org/10.1505/146554814811724793)
- Hardner CM, Potts BM (1995). Inbreeding depression and changes in variation after selfing in *Eucalyptus globulus* ssp. *globulus*. *Silvae Genetica* 44: 46-54.
- Hingston AB, Potts BM, McQuillan PB (2004a). The swift parrot *Lathamus discolor* (Psittacidae), social bees (Apidae), and native insects as pollinators of *Eucalyptus globulus* ssp. *globulus* (Myrtaceae). *Australian Journal of Botany* 52: 371-379. - doi: [10.1071/BT03018](https://doi.org/10.1071/BT03018)
- Hingston AB, Potts BM, McQuillan PB (2004b). Pollination services provided by various size classes of flower visitors to *Eucalyptus globulus* ssp. *globulus* (Myrtaceae). *Australian Journal of Botany* 52: 353-369. - doi: [10.1071/BT03002](https://doi.org/10.1071/BT03002)
- Jones ME, Shepherd M, Henry R, Delves A (2008). Pollen flow in *Eucalyptus grandis* determined by paternity analysis using microsatellite markers. *Tree Genetics and Genomes* 4: 37-47. - doi: [10.1007/s11295-007-0086-0](https://doi.org/10.1007/s11295-007-0086-0)
- Jordan GJ, Potts BM, Kirkpatrick JB, Gardiner C (1993). Variation in the *Eucalyptus globulus* complex revisited. *Australian Journal of Botany* 41: 763-785. - doi: [10.1071/BT9930763](https://doi.org/10.1071/BT9930763)
- Kalinowski ST, Taper ML, Marshall TC (2007). Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology* 16: 1099-1106. - doi: [10.1111/j.1365-294x.2007.03089.x](https://doi.org/10.1111/j.1365-294x.2007.03089.x)
- Kenward MG, Roger JH (1997). Small sample inference for fixed effects from restricted maximum likelihood. *Biometrics* 53: 983-997. - doi: [10.2307/2533558](https://doi.org/10.2307/2533558)
- Klekowski EJ (1988). Genetic load and its causes in long-lived plants. *Trees* 2: 195-203. - doi: [10.1007/BF00202374](https://doi.org/10.1007/BF00202374)
- Lindgren D, Gea LD, Jefferson PA (1997). Status number for measuring genetic diversity. *International Journal of Forest Genetics* 2: 69-76.
- Lindgren D, Mullin TJ (1998). Relatedness and status number in seed orchard crops. *Canadian Journal of Forest Research* 28: 276-283. - doi: [10.1139/x97-217](https://doi.org/10.1139/x97-217)
- Lynch M, Walsh B (1998). *Genetics and analysis of quantitative traits*. Sinauer Associates Inc, Sunderland, MA, USA, pp. 807. [online] URL: http://www.investar.org.co/redcostera1/investar/docs/RinconLiterario/2011/febrero/AG_8.pdf
- Marshall TC, Slate J, Kruuk LEB, Pemberton JM (1998). Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology* 7: 639-655. - doi: [10.1046/j.1365-294x.1998.00374.x](https://doi.org/10.1046/j.1365-294x.1998.00374.x)
- McGowen MH, Vaillancourt RE, Pilbeam DJ, Potts BM (2010). Sources of variation in self-incompatibility in the Australian forest tree, *Eucalyptus globulus*. *Annals of Botany* 105: 737-745. - doi: [10.1093/aob/mcq036](https://doi.org/10.1093/aob/mcq036)
- Moncur MW, Mitchell A, Fripp Y, Kleinschmit GJ (1995). The role of honey bees (*Apis mellifera*) in eucalypt and acacia seed production areas. *The Commonwealth Forestry Review* 74: 350-354. [online] URL: <http://www.jstor.org/stable/42608329>
- Moran GF, Bell JC (1983). *Eucalyptus*. In: "Isozymes in Plant Genetics and Breeding, Part B" (Tanskley SD, Orton TJ eds). Elsevier, Amsterdam, Netherlands, pp. 423-441.
- Nickolas H, Harrison PA, Tilyard P, Vaillancourt RE, Potts BM (2019). Inbreeding depression and differential maladaptation shape the fitness trajectory of two co-occurring *Eucalyptus* species. *Annals of Forest Science* 76: 10. - doi: [10.1007/s13595-018-0796-5](https://doi.org/10.1007/s13595-018-0796-5)
- Patterson B, Vaillancourt RE, Pilbeam DJ, Potts BM (2004). Factors affecting variation in outcrossing rate in *Eucalyptus globulus*. *Australian Journal of Botany* 52: 773-780. - doi: [10.1071/BT03186](https://doi.org/10.1071/BT03186)
- Potts BM, Wiltshire RJE (1997). *Eucalypt* genetics and geneecology. In: "Eucalypt Ecology: Individuals to Ecosystems" (Williams J, Woinarski J eds). Cambridge University Press, Cambridge, pp. 56-91. [online] URL: <http://books.google.com/books?id=ksymh8ocS5QC>
- Pound LM, Wallwork MAB, Potts BM, Sedgley M (2002). Self-incompatibility in *Eucalyptus globulus* ssp. *globulus* (Myrtaceae). *Australian Journal of Botany* 50: 365-372. - doi: [10.1071/BT01076](https://doi.org/10.1071/BT01076)
- Pupin S, Sebbenn AM, Cambuim J, Da Silva AM, Zaruma DUG, Silva PHM, Rosse LN, Souza ICG, Marino CL, Moraes MLT (2019). Effects of pollen contamination and non-random mating on inbreeding and outbreeding depression in a seedling seed orchard of *Eucalyptus urophylla*. *Forest Ecology Management* 437: 272-281. - doi: [10.1016/j.foreco.2019.01.050](https://doi.org/10.1016/j.foreco.2019.01.050)
- Rao H, Patterson B, Potts BM, Vaillancourt RE (2008). A microsatellite study of outcrossing rates and contamination in a *Eucalyptus globulus* breeding arboretum. *Journal of Forestry Research* 19: 136-140. - doi: [10.1007/s11676-008-0023-6](https://doi.org/10.1007/s11676-008-0023-6)
- Ribeiro MM, Sanchez L, Ribeiro C, Cunha F, Araújo JA, Borralho N, Marques CM (2011). A case study of *Eucalyptus globulus* fingerprinting for breeding. *Annals of Forest Science* 68: 701-714. - doi: [10.1007/s13595-011-0087-x](https://doi.org/10.1007/s13595-011-0087-x)
- Self SG, Liang KY (1987). Asymptotic properties of maximum likelihood estimators and likelihood ratio tests under nonstandard conditions. *Journal of the American Statistical Association* 82: 605-610. - doi: [10.1080/01621459.1987.10478472](https://doi.org/10.1080/01621459.1987.10478472)
- Tomé M, Tomé JA, Araújo MC, Pereira JS (1994). Intraspecific competition in irrigated and fertilized eucalypt plantations. *Forest Ecology and Management* 69: 211-218. - doi: [10.1016/0378-1127\(94\)90230-5](https://doi.org/10.1016/0378-1127(94)90230-5)
- Weiner J, Damgaard C (2006). Size-asymmetric competition and size-asymmetric growth in a spatially explicit zone-of-influence model of plant competition. *Ecological Research* 21: 707-712. - doi: [10.1007/s11284-006-0178-6](https://doi.org/10.1007/s11284-006-0178-6)
- Wellmann R (2019). Optimum contribution selection for animal breeding and conservation: the R package optiSel. *BMC Bioinformatics* 20: 25. - doi: [10.1186/s12859-018-2450-5](https://doi.org/10.1186/s12859-018-2450-5)
- Zhang Z, Papaik MJ, Wang X, Hao Z, Ye J, Lin F, Yuan Z (2017). The effect of tree size, neighborhood competition and environment on tree growth in an old growth temperate forest. *Journal of Plant Ecology* 10: 970-980. - doi: [10.1093/jpe/rtw126](https://doi.org/10.1093/jpe/rtw126)

Supplementary Material

Tab. S1 - Number of ramets of the parent trees included in the orchard and progeny analysed in each field trial.

Tab. S2 - Field trials brief characterization, including year of establishment, lithology, soil classification according to FAO, range of annual precipitation and temperatures.

Tab. S3 - Microsatellite identification, forward and reverse primer sequences, fragment size and multiplexing.

Tab. S4 - Contingency table with the number of normal and abnormal phenotypes per class of inbreeding coefficient.

Tab. S5 - Values of within family group co-ancestry and corresponding intraclass correlation for the seven open pollinated families sampled in the *E. globulus* seed orchard.

Link: Faia_4012@suppl001.pdf