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Eucalyptus breeding programs: a proposal for the use of inbred progenies

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SILVICULTURE

ABSTRACT

Background: This review aims to discuss alternatives for obtaining new clones of eucalyptus (*Eucalyptus* spp.) with greater efficiency than current methods, as well as the feasibility of obtaining hybrid seeds. The commercial-scale use of eucalyptus hybrid seeds may reduce implantation costs and bring other advantages for forest exploitation. The proposal focuses on using inbred progenies (S_1, S_2...S_g) to improve the species. Self-fertilization releases a greater proportion of undesirable alleles hidden in heterozygotes, increasing selection success.

Results: From the best individuals of the inbred progenies, full-sibs (FS) progenies will be developed, allowing the selection of new clones and conduct recurrent selection programs. The hybrid seeds must be obtained, also from the FS evaluations, in each selfing generation (S_g x S_g). Simultaneously with the conduction of inbreeding generations, information must be gathered aiming to implement the strategy for obtaining hybrid seeds, such as verifying the feasibility of generating double haploid lines and looking for alternatives to facilitate the large-scale production of hybrid seeds.

Conclusion: this review of research results can serve as an initial basis for obtaining new clones of eucalyptus with greater efficiency than current methods, as well as the feasibility of obtaining hybrid seeds.

Keywords: forest breeding, quantitative genetics, heterosis, inbreeding depression, recurrent selection.

HIGHLIGHTS

Feasibility of using inbred progenies in eucalyptus breeding programs.
Inbreeding used simultaneously to obtain hybrid seeds and new clones.
A proposal for a hybrid seed program, with different levels of inbreeding, in forestry exploration.
Discussion of the challenges to be overcome to enable large-scale production of eucalyptus hybrid seeds.

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INTRODUCTION

The huge contribution of using clones in eucalyptus genetic improvement in Brazil is undeniable (Assis et al., 2015). Cloning is the most efficient way to perpetuate a good genotype and thus capitalize on any type of allelic and non-allelic interaction or any other cause of heterosis (Rezende et al., 2014).

However, the use of clones has some restrictions. Although the technology for producing cloned seedlings has greatly evolved (Assis et al., 2015), the cost of clonal seedlings is much higher than the seminal ones. In addition, some clones have poor root development, becoming more vulnerable to abiotic stresses, especially water deficit. Moreover, several species of the genus *Eucalyptus* or *Corymbia* have limitations in asexual propagation. Even in species where asexual propagation is viable, some individuals with good performance for wood volume or other favorable traits have cloning restrictions. Additionally, the low correlation between the individual's performance in the progeny evaluations and the clone derived from it in the clonal tests must be accounted for, which reduces the selection efficiency for new clones (Furtini et al., 2012).

Hybrid seeds represent another option to explore the heterosis found in good combinations. They began to be employed at the beginning of the 20th century with the cultivation of maize and later with numerous other annual species (Bernardo 2020; Andorf et al., 2019; Steeg et al., 2022). The use of hybrid seeds has decisively contributed to increasing grain production across the world.

For perennial plants, including eucalyptus, commercially hybrid seeds have not been used. However, eucalyptus plants, for instance, have some peculiarities that probably justify the investment in research into the feasibility of using hybrid seeds. Supposedly, eucalyptus hybrid seeds have not been effectively obtained due to the time-consuming in generating lines. Obtaining lines requires 5 to 6 generations of self-fertilization – inbreeding – which, in principle, it will require a few decades in eucalyptus plantations. However, currently, there are technologies that can greatly accelerate this process (Castro et.al., 2021). In parallel with the increase in the frequency of homozygotes, inbreeding increases the probability that undesirable alleles, which are hidden in heterozygotes, will manifest, increasing selection efficiency (Ramalho et al., 2021a). Thus, the use of inbreed progenies associated with other breeding methods may contribute to increase the efficiency in the selection of new clones. Additionally, it should be noted that it is not necessary to obtain lines to have good hybrid combinations. Souza Junior (2001) discusses the possibility of obtaining hybrids with partial inbreeding, for example S_1 x S_1 or S_2 x S_2.

It should be emphasized, however, that the use of hybrid eucalyptus seeds, on a large scale, has several other challenges, which are not restricted to obtaining lines. Information has been accumulated showing the feasibility of obtaining lines (double-haploid) in many crops (Niazian and Shariatpanahi, 2020; Maqbool et al., 2020). The eucalyptus plant has numerous traits that allow us to see if it is possible to obtain a double haploid. It has also been shown that it is possible to manually obtain large amounts of seeds in a short time (Assis et al., 2005), indicating that the possibility of inbreeding use in breeding programs and the use of hybrid seeds may be feasible.

In this context, this study aims to propose a strategy for the use of inbred progenies associated with obtaining new clones and, at the same time, enabling the production of hybrid seeds on a commercial scale for eucalyptus plantations.

Eucalyptus breeding in Brazil

Brazilian eucalyptus breeding programs have employed different selection strategies over time. The first one was the introduction of species and provenances, which continued for several years through mass selection in free-pollinated populations. Subsequently, progeny selection was carried out, especially half-sib (HS). At the end of the 1960s, selection of superior individuals in commercial plantations and their cloning was used. Since then, clonal plantations have been used in large areas.

For some years, efforts were devoted to developing technologies to facilitate artificial hybridizations and, above all, accelerate flowering, achieving tremendous success (Assis et al., 2005; Castro et al., 2021). With the facilities in artificial hybridizations, emphasis was given to obtaining full-sib (FS) progenies. Interpopulation recurrent selection and reciprocal recurrent selection (RRS) were encouraged. However, RRS was not as efficient as desired, mainly due to the delay in completing a selective cycle.

Over time, it was also found that the performance of the selected individual in the progeny and clonal tests had a small correlation (Furtini et al., 2012). Researchers then started to look for new alternatives to speed up the obtention of new clones. The option currently employed is the clonal progeny test (Resende, 2002; Ramalho et al., 2021b). In this case, clones are evaluated without the initial progeny test. Specifically, the seeds obtained in the recombination lot, i.e., seeds of the HS or FS progenies, are cloned in the nursery soon after germination. The clones of the different individuals of each progeny are evaluated and, thus, their performance depends on the performance of the progeny that gave rise to it. The assumption is that the clone is better evaluated and that it is not necessary to perform the progeny test before the clonal test (Ramalho et al., 2021b). Thus, progenies and clones are evaluated simultaneously. Even though there are no results that prove the efficiency of this method, expectations are high.

From what has been reported, it is evident that inbred progenies have not yet been explored to improve eucalyptus or any other forest species, although it has some advantages over HS and FS progenies as the greater release of genetic variance. Considering the long duration of a selection cycle in a perennial plant, it is desirable to explore the greater genetic variance coming from inbreeding. Additionally, evidence indicates that eucalyptus populations likely have a high frequency of undesirable alleles hidden in heterozygous loci. In this situation, inbred progenies can be a good option since, besides releasing greater genetic variability, it exposes the harmful alleles, enabling their selection.

Inbreeding and its effects on eucalyptus populations

Although eucalyptus is considered an allogamous plant, where cross-pollination occurs randomly, it presents a certain frequency of self-pollination. Costa e Silva et al. (2010a) comment that there is an average frequency of 26% of self-fertilization, considering 23 species. This self-fertilization rate differs with species and environmental conditions. Why does a plant with androgynous flowers (both sexes in the same flower) have a high rate of cross-fertilization? It happens mainly due to self-incompatibility (Muñoz-Sans et al., 2020). What is noteworthy, in eucalyptus, is that in many individuals of the population, self-incompatibility is not complete, meaning that even with self-incompatibility, some self-fertilization occurs.

The inbreeding/self-fertilization effect can be observed in some ways. The first is when there is a dominance-type allelic interaction controlling the character. Inbreeding reduces the population average due to a reduction in the frequency of heterozygous loci, compared to what would be expected if the crosses were all random. This effect varies with the proportion of self-fertilization or any other form of related crosses that occurs, the frequency of harmful alleles, and the contribution of dominance to character expression (Falconer and Mackay, 1996).

The effect of self-fertilization on the population average, called inbreeding depression (ID), has been estimated in species of the genus Eucalyptus (Costa e Silva et al., 2010a). The study was carried out with a native population of *E. globulus*, using plants from self-fertilized seeds (SELF), open pollination (OP), and artificial hybridization with a pollen mixture (POL). In this work, data were collected at four, six, and ten years of age. SELF individuals had a lower percentage of survival. The difference between SELF and POL in survival was accentuated as age increased. At ten years, the survival was 21.9% and 82.9% for SELF and POL, respectively. Due to the difference in plant mortality, the growth data were adjusted, and the inbreeding depression due to self-fertilization was estimated at 77% at ten years, in the basal area.

Most ID estimates were obtained from plants not selected by man, evidencing the high frequency of harmful alleles maintained in heterozygosity in these populations. Specifically for artificially selected populations, not many ID estimates were reported. In two experiments located close to each other, Bison et al. (2006) evaluated ten clones (F_1 generation) and the resulting plants from the self-fertilized clones (F_2 generation). At two years of age, ID for the circumference at breast height (CBH) was estimated at 17.5%, on average, ranging from 0% to 29.4%. The difference in survival was also expressive, with values from 2.5% to 45%, indicating that several clones have a high genetic load. It should be emphasized that the ID estimates may be underestimated because due to the fact that the difference in the number of plants in the plot was not considered and that the evaluations were carried out on very young plants, and, as mentioned above, ID increases with age.

In another study, Griffin et al. (2019) concluded that selective elimination of inbred genotypes during the intense intra-specific competition characteristic of the pre-reproductive phase of the life cycle of *E. regnans* results in a fully outcrossed reproductive population, in which self-fertility is comparable with that of its parental generation. Which is comparable to a natural population.

Another aspect related to self-fertilization is the consequences of releasing variability between and within inbred progenies. In this context, Cockerham and Weir (1984) showed that other components of genetic variance occur in addition to additive genetic variance (V_A) and dominance variance (V_D) when there is inbreeding and allele frequencies are different from ½. Souza Junior (1989), based on the work of Cockerham and Weir (1984), wrote a review highlighting the importance of these new components in selection when using inbred progenies. He commented that genetic variance, in the absence of epistasis, can be obtained by:

$$V_G=(1+F_g)\ V_A+(1-F_g)\ V_D+4F_gD_1+F_gD_2+F_g\ (1-I_g\ )\ \breve{H}$$

where: F_g is the inbreeding coefficient and can be obtained by the expression: $F_g=1-(1/2)^{(g-1)}$, where g is the number of inbreeding generations.V_A and V_D have already been defined; D_1 is the genetic covariance between the average (additive) effects of alleles and the dominant effects of homozygous; D_2 is the genetic variance of the dominant effects of homozygous and; $\breve{H}$ is the squared value of inbreeding depression.

Estimates of variance components were obtained from populations originated by SELF, OP, and POL (Costa e Silva et al., 2010b). They estimated the components of genetic variance for the CBH at four years, ie: $V_A= 0.96 \pm 0.32$; $V_D = 1.10 \pm 1.39$; $D_1 = -1.24 \pm 0.82$; $D_2 = 9.71 \pm 3.52$. As noted, the errors associated with the estimates were usually high. Note that the non-additive variances were higher than the additive variance, confirming the effect of inbreeding in the genus *Eucalyptus*.

The D_1 estimate is noteworthy, which is a covariance and thereby can be negative, as was the case. D_1 is part of the numerator in the formula for gain from inbred progeny selection (Gs), S_1 or S_2, as shown by Souza Junior (1989):

$$Gs = k\ [\ (1+ F_P)V_A\ +(\ F_g\ +F_P)\ D1]\ /\ \sqrt{}V_F$$

Where: *k* refers to the standardized selection intensity; F_P is the inbreeding coefficient of the population that will originate the progenies; F_g is the inbreeding coefficient of the progenies being evaluated, and $\sqrt{}V_f$ is the phenotypic deviation between the mean of the progenies being assessed. If S_1 progenies are assessed, F_P will be null because the self-fertilized population was S_0, and F_g will be the inbreeding of the progenies evaluated, in this case, S_1 = ½. When evaluating S_2 progenies, F_P= ½ since the reference population is S_1 and the F_g =3/4. Therefore, we have:

$Gs_{(S1)} = k\ [V_A + (½)D_1]/ \sqrt{V_F}$

and $GS_{(S2)} = k\ [(½)V_A + (5/4)D_1]/ \sqrt{V_F}$

As D_1 is part of the equation numerator, if it is negative, one can even reduce the average of the improved population in relation to the original instead of increasing it, as expected with the selection of the best individuals.

It should also be mentioned that some V_A and V_D estimates have been obtained for some *Eucalyptus* species with non-inbred progenies for characters associated with plant growth (CBH and height). In a survey carried out by Bouve et al. (2009), among 19 estimates of the V_D/V_A ratio for diameter of breast height, only three were not differed from zero. Although the number of progenies or parents involved in the estimates was low in many cases, it can be inferred that there is a dominant effect in the manifestation of this character, as previously mentioned. It would be important to obtain estimates with a lower associated error.

In populations of *E. grandis* and *E. urophylla* already subjected to some selection cycles, estimates of V_D/V_A were not found. However, it should be noted that the good performance of clones found in breeding programs is excellent evidence that there is a contribution of dominance in the loci involved with growth-related characters of eucalyptus plants.

Another aspect to be considered regarding self-fertilization concerns the selection for undesirable alleles. It is particularly advantageous when the population under selection has a low frequency of the recessive undesirable allele. Suppose that resistance to a pathogen is due to the R gene, whose dominant R allele confers resistance at the frequency of p in the population, and the r allele responsible for susceptibility is at the frequency q. In the S_0 population, the genotype frequencies will be: RR = p^2, Rr = 2pq, and rr = q^2. If it is self-fertilized once (S_1), the new frequency will be: RR = p^2 + (½) pq, Rr = pq, and rr = q^2+ (½) pq. Note that the frequency of the genotype to be eliminated with selection (rr) increases with self-pollination, improving the efficiency of the process (Table 1). Supposing a population A undergoing a self-fertilizing generation, and the r allele is at the frequency q = 0.8, the efficiency of self-fertilizing will be: 12.5%. Now suppose another population B in Hardy-Weinberg equilibrium, whose r allele frequency is q=0.1. In this case, it was evident that the frequency of the rr genotype will be much higher in the self-fertilized population than in the equilibrium population. Thus, it is evident that selection will be much more efficient in this self-fertilized population since 450,00% more undesirable individuals can be eliminated (Table 1).

However, as self-fertilization before selection requires another generation, the selection process takes longer. Thus, when the breeder has a population that is little improved, where the frequency of the recessive allele is high, he performs the initial selective cycles without using self-fertilization. However, when the population already has a low frequency of the recessive allele, the improvement in the selection efficiency after self-fertilization compensates for the additional time spent. Nevertheless, especially when using self-fertilized progenies, maximum care must be taken during evaluations to achieve the maximum possible heritability for the trait under selection. In this way, greater efficiency in the self-fertilization of the population will be achieved.

Use of heterosis in Eucalyptus breeding

Heterosis (h) or hybrid vigor is the superiority of the hybrid (F_1 generation) in relation to the average of the parents, that is, $h=F1-((\bar{P}_1+\bar{P}_2)/2)$. In some cases, heterosis is conceptualized as the superiority of the F_1 generation in relation to the best-performing parent. For this situation, the preferred name is heterobeltiosis (Resende et al., 2002). For h to be different from zero, the parents must be divergent (Y), presenting a different genotypic constitution in loci involved in character control and also that dominance occurs, d must differ from zero (Falconer and Mackay, 1996), i.e., h = d $(Y)^2$.

Although heterosis has been explored for decades, with enormous repercussions both in the biological and economic context, its molecular mechanism has not yet been completely elucidated. Numerous hypotheses have been proposed and new ones frequently emerge. Detailed reviews in this regard are found in the literature (Andorf et al., 2019). However, despite the lack of knowledge concerning the molecular mechanism(s) behind this phenomenon, heterosis has been widely used in genetic improvement.

It should be noted, as already mentioned, that in a population of eucalyptus plants crossing randomly, or any other plant or animal that are diploid, all individuals are simple hybrids (SH) - crossing of two lines. This is because every individual comes from the union of two gametes and every gamete, if duplicated, corresponds to a line. Thus, there is a mixture of SH in a randomly crossing population, representing infinite possible combinations of gametes.

As the average of the parents $[(P_1+P_2)/2]$ corresponds to: m+a (Vencovsky, 1987), the average of the SH can also be obtained by: SH = m + a + d. Where, m + a is the contribution of the homozygous loci in the SH, that is, the loci in which the lines/gametes are not divergent have the same alleles; the d or h in

Table 1. Comparison of self-fertilization efficiency in two populations A and Bdiffering in the frequency of the r allele that confers resistance to a given pathogen.

Population	Allele frequency r	Genotype frequency rr in equilibrium	Genotype frequency after self-fertilization	Advantage after one generation of self- fertilization
A	0.80	0.64	0.72	(0.72/0.64) 100 = 112.5 %
B	0.10	0.01	0.055	(0.055/0.01) 100 = 550.0%

turn, regardless of the degree of dominance or other phenomenon involved, come from the heterozygous loci. It is inferred that hybrid performance can be improved through the accumulation of homozygous loci with favorable alleles and with the greatest possible heterosis.

The increase in the frequency of loci with favorable alleles in populations can be achieved through intrapopulational recurrent selection, performed by any of the existing methods (Resende, 2002; Hallauer et al., 2010). Although it seems that there is no formal RS program in the maize hybrid breeding programs from seed companies, it is informally practiced since the best lines within each heterotic group are crossed two by two at each cycle, which characterizes an RS program using lines. This fact is proven maize when comparing the yield performance of SHs obtained in different decades. Those obtained more recently show a greater relative contribution of homozygous loci - (m + a) - than the heterozygous ones - h (Li et al., 2014). In other words, the average of the parent lines of the current hybrids has proportionally grown more than the heterosis among them.

An important question: how to perpetuate an individual with a performance superior to others in the population? One of the alternatives widely used in Brazil was cloning (Rezende et al., 2014). It's easy to imagine why cloning is said to take advantage of all types of interactions, allelic or non-allelic.

The other alternative devised at the beginning of the 20th century was to obtain lines – an individual with all or most loci in homozygosity. Thereby, lines can be maintained with the same genotypic constitution indefinitely. Thus, when some good combination of two lines (SH) is needed, they can be obtained. This alternative has been widely applied in a large number of annual species over time. In addition, the obtention of hybrids using lines expanded the seed industry all over the world.

It should be emphasized that in some countries, especially in Brazil, the hybrid combination of individuals of the species *E. grandis* x *E. urophylla*, as already mentioned, presented enormous heterosis that has been perpetuated through cloning. The possibility of additionally harnessing heterosis through the production of hybrid seeds, via the inbreeding process, is the main focus of this review.

The proposal here presented contemplates this aim. Note that intrapopulation selection is performed using inbred progenies, which is expected to contribute to the elimination of the genetic load more quickly, increasing the contribution of m+a. When evaluating the interpopulation FS, the heterosis from the cross is also harnessed.

However, it is possible to take advantage of at least part of the heterosis using progenies with partial inbreeding. According to Souza Junior (2001), the genetic variance explored for the selection between hybrids, with different levels of inbreeding, is provided by:

$$VHS = ¼ (1+F)V_{AGU} + ¼ (1+F)V_{AUG} + 1/4 (1+F)^2 V_{DGU}$$

Where, V_{AGU} or V_{AUG} is the additive-genetic variance in relation to the population of the heterotic group *E. grandis* when crossed with *E. urophylla* and vice versa, respectively; V_{DGU} is the genetic variance of dominance that is expressed through the crossing of two heterotic groups; F is the inbreeding coefficient of the plant that originated the hybrid parents and not the hybrid itself. Thus, for hybrids from S_1 parents, the F is 0 and not 1/2. For some hybrids from parents with different levels of inbreeding, considering the equal contribution of V_{AGU} and V_{AUG} ($V_{AGU} = V_{AUG}$) we have:

$$V_{(S1G \times S1U)} = (1/2)V_{AGU} + (1/4)V_{DGU}$$

$$V_{(S2G \times S2U)} = (3/4)V_{AGU} + (9/16)V_{DGU}$$

$$V_{(S3G \times S3U)} = (7/8)V_{AGU} + (49/64)V_{DGU}$$

$$V_{(LG \times LU)} = 1 V_{AGU} + (1)V_{DGU}$$

Note that in the last example, the use of lines exploits all the additive and dominance variance present. The same happens when the individual is cloned. Also note that the hybrid with S_3 progenies explores a large part of the total variance, equivalent to a hybrid from a line; however, even with less inbreeding, it harnesses a considerable part of the heterosis

Proposal for the use of inbred progenies in eucalyptus breeding

The proposal presented (Figure 1) offers some improvement strategies without discontinuing clone obtention. It aims to explore good combining ability, for example, involving individuals of *E. grandis* and *E. urophylla* (Van Den Berg, 2015). Furthermore, it will make it possible to study the feasibility of obtaining eucalyptus hybrid seeds, with partial inbreeding or even with the generation of lines at the beginning.

The process starts with two populations: one from *E. grandis* clones (G) and the other from *E. urophylla* (U) clones. These clones are supposed to be the best ones obtained from the two species. Ideally, at least ten clones from each heterotic group should be used. Moreover, even with the availability of many clones, it is not advisable to use more than 20 (Figure 1). The more clones used, the more difficult it is to identify clones with good performance. Furthermore, in the first recombination, if more clones are involved, the alleles of many of them will be lost. An initial challenge is to separate the clones within each group. If it is not possible to separate the groups at first, one may form two distinct heterotic groups in the first evaluation of the hybrid combinations (FS) to continue the process. Even though there are other options to generate the population, we will focus on conducting the two heterotic groups separately.

Within each group, clones must initially be self-fertilized. Each individual/clone comes from the union of two gametes, male and female. Thereby, each clone is equivalent to the F_1 generation of a single hybrid (SH), and the seeds from the self-pollination of each clone will be the SH F_2 generation. This population is in Hardy-Weinberg equilibrium. Furthermore, although it comes from F_1 self-fertilization, its inbreeding coefficient (F) is zero. Thus, the F_2 generation is equivalent to the S_0, receiving this name from now on. If ten clones are self-fertilized, ten different S_0 populations will be obtained for *E. grandis* and ten for *E. urophylla*.

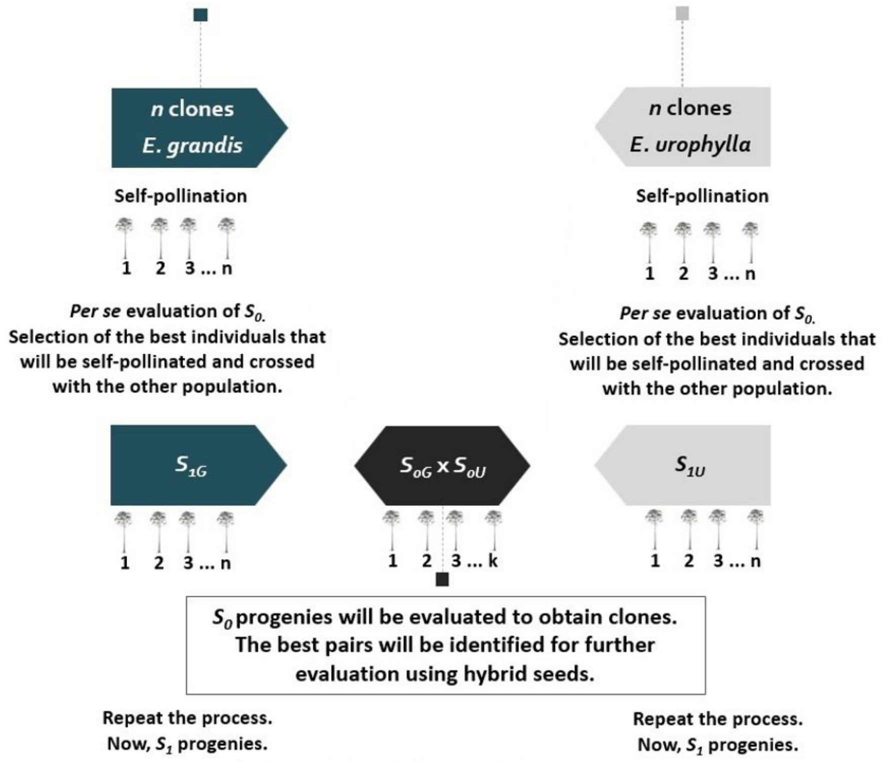

Figure 1. Schematic representation of the proposal for producing eucalyptus hybrid seeds simultaneously conducting the clonal program.

One might ask how many S_0 individuals will be initially required to provide a good representation of each S_0. In reality, this number does not have to be large since each S_0 plant has the average potential variability of all possible plants in an S_0 population. However, since each S_0 will be evaluated and selected within, a larger number should be used, 1000 individuals, for example. In addition, it would be good to evaluate the S_0 in experiments with replicates. Thus, the average of each one would be additional information to guide the selection of the best S_0 individuals. The S_0 evaluation must be carried out as soon as possible. Once selected, the best plants will be induced to flower.

Another question concerns the number of plants selected within each S_0 (parental clone) In principle, it would be nice to have representatives from all S_0. However, it would be more interesting to select more plants from the better performing S_0 (Castro et al., 2019). It should be noted that it would not be feasible to self-fertilize all individuals. In theory, 10 to 20 plants of each S_0 would be fine. Remember that it may be difficult to get some clones to flower. In the end, if at least 20 plants are obtained, two from each group, on average, would be enough.

Each of the plants mentioned above must be self-fertilized, obtaining the S_1's. At the same time, they should be crossed with plants from the other heterotic group, generating FS, S_{0G} x S_{0U}. In this case, a partial diallel must be performed. Thus, if all combinations were obtained, there would be 400 FS progenies (20 of G x 20 of U).

From this point onwards, there is a step similar to the one already described when the S_0 populations were evaluated. The S_1 populations of G and U would now be evaluated. The same observations made before are valid now. In this step, the progenies of FS, S_{0G} x S_{0U} must be evaluated as well. As previously mentioned, the proposal would be to obtain 400 FS; however, hardly all combinations would be obtained. Supposing it was possible to obtain 120 FS progenies, at least two options would be possible in the evaluations. The first involves the assessment of FS in experiments in experimental design, if possible, in more than one location. From the obtained data, one should select the best individuals to be cloned. The second option is to get clones from the seedlings of each FS progeny, performing a clonal progeny test. Experimental details on each of these options are not the goal of this study. However, it is necessary to emphasize that the proposal allows to simultaneously obtain clones, like any other improvement strategy that companies have adopted.

From the S_{0G} x S_{0U} progeny test results, some strategies can be used, besides clone obtention. The first is to repeat the previously performed process, self-fertilizing the best S_1 individuals to obtain the S_2 generation and the FS of S_{1G} x S_{1U} once again. The process would continue, as shown in the scheme. The other option would be to conduct a reciprocal RRS program in traditional ways using the best descendants of the S_0 (S_1) plants that generated the FS for recombination, or using the best clones identified at the end of each cycle. This recombination can be performed manually or in an isolated field. Recombination would be performed separately for the two heterotic groups, obtaining the cycle I population of *E. grandis* and the cycle I population of *E. urophylla*.

Another alternative for genetic improvement using the FS (S_{0G} x S_{0U}) data would be identifying at least the five best combinations for a more intensive evaluation in other conditions. The S_{0Gi} x S_{0Ui} seeds will be more intensely evaluated through seedlings obtained via hybrid seeds in some places. In these experiments, in addition to the average performance of the hybrids concerning growth and wood quality, the existence of variability within their plots would be observed. In principle, high variability is expected, which is not desirable. However, at least one S_0 x S_0 hybrid that can be used commercially is expected.

The remaining steps of the proposal will be similar to what was previously reported. What needs to be highlighted is that the frequency of heterozygous loci reduces with each inbreeding generation. In the S_0 generation, for example, 50% of the loci are homozygous. However, this generation is considered, as already mentioned, of zero inbreeding, F=0; in S_1, we have F= ½; in S_2, F=¾; and in S_3, F=7/8, and so forth. As the program progresses, S_{1G} x S_{1U}, S_{2G} x S_{2U}, S_{3G} x S_{3U}, and other hybrids can be obtained. With each advance in inbreeding, more uniformity is expected between the developed hybrid plants.

Challenges in the production of eucalyptus hybrid seeds

The obtention of self-fertilized progenies is the first challenge to produce eucalyptus hybrid seeds. Eucalyptus plants present self-incompatibility (SI); however, it seems that it is not complete in the different species. Numerous studies were carried out to obtain information about the efficiency of self-pollen, due to self-fertilization, or allo-pollen, cross-fertilization, in several eucalyptus species. Nonetheless, the results were not conclusive, and most of them focused on verifying whether self-incompatibility occurs before or after fertilization, that is, if it occurs through pre- or postzygotic mechanisms (Costa e Silva et al., 2010a).

Genetic self-incompatibility is a common phenomenon in many species from different families and genera (Muñoz-Sanz et al., 2020). Some studies mention the occurrence of a gene (SI) that is expected to have a large number of alleles. However, other SI-related genes have been identified, especially in Brassicas. Muñoz-Sans et al. (2020) discuss strategies in which plants with self-incompatibility can become compatible with self-pollen. One of them is the naturally occurring self-compatibility alleles already identified in some species, including perennial ones. The induction of self-compatibility has also been investigated with physical mutagens (irradiation). The authors also comment on other molecular biology strategies to transform SI plants into self-compatible ones.

In summary, although there is no information on the genetic control of self-incompatibility in eucalyptus, allo-pollen is preferred over self-pollen for fertilization. There may be some difficulties in obtaining inbred progenies in eucalyptus, but it seems viable, especially when self-fertilization is performed artificially, in the absence of competition from allo-pollen. Nevertheless, the greatest number of plants of a population or clones and flowers of the same plant must be self-fertilized to obtain inbred progenies. However, Assis et al., (2013) comment on a methodology that facilitates the realization of artificial pollinations.

The second challenge is the time needed to obtain the lines after 4 to 6 inbreeding generations. In this case, some comments should be made. Castro et al. (2021) show that it is possible to obtain flowers from a eucalyptus plant approximately 150 days after seed germination. Thus, making this type of procedure feasible on a large scale, the process of obtaining lines will be greatly accelerated, similar to what occurs in annual crops.

Furthermore, concerns what was discussed in the improvement proposal; time itself will not be very important for the research program since new clones can be obtained in each generation, with practically the same time devoted to other non-inbred progenies as half- and full-sibs. Another comment is that, it will not be necessary to obtain lines to have hybrid seeds initially. Hybrids of S_1 x S_1 and S_2 x S_2, for example, can be obtained in each inbreeding generation. However, the aim is for inbreeding to continue until most loci are homozygous.

The third comment is that an attempt should be made to obtain double haploid (DH) lines, as occurs in several species of annual plants. The DH technology develops lines with 100% of the loci in homozygosity through chromosome duplication of haploid individuals. The spontaneous occurrence of haploid plants has been known since 1920 in cotton (Maqbool et al., 2020). However, it was only from the 1990's that efforts to generate DH lines were intensified. Available information shows that there is already possibility of obtaining DH in about 200 species (Yan et al., 2017).

The induction of HD lines can be performed 'in vitro' or 'in vivo'. The most used 'in vitro' methodology has been through another culture (Niazian and Shariatpanahi, 2020). This technology should be tried on eucalyptus for a few reasons: plants have an infinite number of flowers, with large amounts of pollen that are relatively easy to store, without losing viability; and it seems that the recovery of haploid individuals via tissue culture is feasible due to the good asexual propagation capacity of most species of the genus *Eucalyptus*.

The 'in vivo' technology can be carried out through pollination with inducing lines, as widely used in maize. Induction can produce gynogenetic (maternal) haploids when the inducer line is used as the male parent or androgenetic haploids when the inducer is used as the female. In maize, the first inducing lines had a low induction rate, around 2.5%. Selection was successfully carried out to increase this rate. Currently, it is possible to obtain an induction rate close to 15% (Andorf et al., 2019). The induction of the haploid individual 'in vivo' can also be achieved through interspecific crossings (Hancock et al., 2015).

After haploid induction, the following steps are to identify and select haploid individuals and then double their chromosome number to obtain DH lines. The early identification of haploid individuals is one of the most critical phases and constitutes a bottleneck in the HD-obtaining process in some species. It can be performed mainly by phenotypic markers, chromosome counting, flow cytometry, or the use of molecular markers (Fritsche-Neto et al., 2012).

Chromosomal duplication is another crucial step in the process of obtaining DH. It is most often accomplished by applying antimitotic agents, which prevent the formation of spindle fibers during mitosis. Colchicine was the primary agent used in the past; however, breeding programs are currently looking for other duplication alternatives. Natural duplication may also occur at a relatively high rate, as in maize (Mehran et al., 2021).

No study reporting DH obtention in *Eucalyptus* spp. was found. Apparently, if any attempt was made, little effort was devoted. It should be noted, as mentioned before, that there are probably many loci in heterozygosis in eucalyptus, in many cases with harmful alleles hidden in the heterozygotes and, thus, the genetic load must be enormous. In this condition, wide segregation among the DHs is expected, and most of the lines will not be used, as in other species. The selection of unviable lines already occurs soon after duplication in the laboratory, after transplanting to the greenhouse, and among the plants that produce seeds in this last condition, go to the field. Thus, one must try to obtain the maximum of DHs to proceed with intense selection. Despite the expected difficulties, efforts should be directed toward obtaining DHs in *Eucalyptus*. Any success obtained will enable the use of lines in the selection, representing a huge advantage. In turn, it will facilitate the obtention of hybrids.

Once the S_1 and S_2 inbred progenies or lines are obtained and their combining ability is evaluated, the next step is to obtain the hybrid seeds of each favorable combination. From this moment on, the question is: would it be possible to obtain enough hybrid seeds to plant a few thousand hectares? Unfortunately, the obtention of hybrid seeds using self-incompatibility is not viable since it is not complete in most studied eucalyptus species, as already mentioned. The male sterility (MS) occurs in eucalyptus. Apparently, it is controlled by nuclear genes, indicating genetic MS. Genetic or cytoplasmic, MS would be difficult to transfer from one MS line to another line to be used as a female in the hybrid-obtention process. It would have to be performed through a few, three to four, successive backcrosses (Ramalho et al., 2021), which is practically unfeasible with eucalyptus plants due to the time needed to perform this number of backcrosses in a perennial plant.

The first option would be manual pollination. Due to all the advances made to accelerate flowering in eucalyptus plants, and the induction of flowering in small plants cultivated in pots or orchards, Assis et al., (2005) comment on hybrid seed production strategies manually in which it is possible to obtain up to 6000 hybrid seeds per hour/operator. The operation becomes similar to what occurs in annual crops. Thus, a high number of hybrid seeds can be obtained. This strategy has been proposed to generate hybrid seeds in coffee - *Coffea arabica* - (Georget et al., 2019), which would be more dificult.

Finally, meeting the annual demand for a huge number of hybrid seeds in companies is a great challenge. However, as Max Weber affirmed, cited by Sen (1985), "Certainly all historical experience confirms the truth – that man would not have attained the possible unless time and again he had reached out for the impossible".

CONCLUSION

The proposal of genetic improvement strategy presented should contribute to greater efficiency in obtaining new clones, while at the same time showing the feasibility of using hybrid seeds, on a commercial scale, in eucalyptus plantations.

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