TOP GRAFTING TO ACCELERATE SELF-POLLINATION IN *Eucalyptus* BREEDING

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ABSTRACT – The top grafting technique can make early flowering possible and consequently accelerate self-pollination in *Eucalyptus* breeding, reducing the period of each generation. This study aims to establish a methodology to top graft by applying the growth regulator paclobutrazol in self-pollinated *Eucalyptus* genotypes to induce early flowering and accelerate inbred line strategies. A total of 448 top grafts of seven genotypes (three *Eucalyptus urophylla* × *Eucalyptus grandis* hybrids, one *Eucalyptus urophylla*, and three *Eucalyptus grandis*) were performed in two periods of the year: July and October. The top grafting samples were evaluated concerning flower induction and graft development at three-month intervals. A t-test was performed with a 5% significance level for type I error to compare the relevance of paclobutrazol application. A fixed model was also used to analyze the significance of the treatments. The fastest blooming occurred after three months of applying the graft methodology. After two years, the top graftings performed in October presented higher flower bud and fruit production. The top grafting affected the induction of self-pollinated *Eucalyptus* flowers with enough flower buds produced to follow the next cycle of self-pollination in some genotypes. The effectiveness of self-pollinated top graftings varied with the genotype used as the scion. The paclobutrazol improved the flowering of the top grafting samples. The methodology established in this work allows accelerating self-pollination strategies in the globally important industrial crop *Eucalyptus*.

Keywords: Early flowering; Inbred line; Paclobutrazol.

ENXERTIA DE TOPO PARA ACELERAR A AUTOPOLINIZAÇÃO NO MELHORAMENTO DE Eucalyptus

RESUMO – A técnica de enxertia de topo pode possibilitar o florescimento precoce e, consequentemente, acelerar a autopolinização no melhoramento de **Eucalyptus***, reduzindo o período de cada geração. O objetivo deste estudo é estabelecer uma metodologia para enxertia de topo, aplicando o regulador de crescimento paclobutrazol em genótipos de* **Eucalyptus** *autopolinizados, para induzir o florescimento precoce e acelerar estratégias que envolvam a produção de linhagens. Foram realizados 448 enxertos de topo de sete genótipos (três híbridos de* **Eucalyptus urophylla** *×* **Eucalyptus grandis***, um de* **Eucalyptus urophylla** *e três de* **Eucalyptus grandis***) em dois períodos do ano: julho e outubro. As amostras de enxertia de topo foram avaliadas quanto à indução floral e o desenvolvimento do enxerto em intervalos de três meses. Foi realizado um teste t com nível de significância de 5% para o erro tipo I para comparar a relevância da aplicação de paclobutrazol e um modelo fixo também foi usado para analisar a significância entre os tratamentos. A floração mais rápida foi observada após três meses de aplicação da metodologia de enxertia. Após dois anos, as enxertias de topo realizadas em outubro apresentaram maior produção de botões florais e frutos. A enxertia de topo afetou a indução de flores* *de* ***Eucalyptus*** *autopolinizadas devido a ter produzido botões florais suficientes para realizar o próximo ciclo de autopolinização em alguns genótipos. A eficácia das enxertias de topo autopolinizadas variou com o genótipo utilizado como copa. O paclobutrazol melhorou a floração das amostras de enxerto de topo. A metodologia estabelecida neste trabalho permite acelerar as estratégias de autopolinização em* ***Eucalyptus****, que é uma cultura industrial globalmente importante.*

Palavras-Chave: Florescimento precoce; Linhagens; Paclobutrazol.

1. INTRODUCTION

Self-pollination strategies have high potential to increase genetic gains related to wood quality when individuals within the lines are generated, maximizing heterosis for yield traits by crossing line individuals (Cobb et al., 2019; Saxena et al., 2021). Inbred line strategies are commonly used in agronomic crops (Wang et al., 2012; Gasim et al., 2015; Avdikos et al., 2021). This strategy aims to increase the homogeneity of seminal plantings by crossing individuals with elevated levels of homozygosity (Maia, 2010; Salvador et al., 2021). Applying these procedures in *Eucalyptus* can increase the additive genetic variance among inbred lines after self-pollination cycles and reduce the variance within lines (White et al., 2007). The inbred lines could allow for selecting individuals with dominant homozygous alleles for the trait of interest, contrasting with individuals from other inbred lines. The maximum expression of additive effects within lines and heterosis in the crossline genotypes can be explored due to the high additive genetic variance in crossing inbred lines (Reddy et al., 2015; Santos et al., 2016). Therefore, self-pollination strategies can generate considerable gains for the forest industry by generating elite genotypes.

The potential of self-pollination strategies in forest breeding programs is high, but the up to two-to-seven-year time for the trees to mature and flower makes generating inbred lines impractical (Jones et al., 2011; Klocko et al., 2016). The performance of consecutive cycles using traditional breeding is not feasible because obtaining homozygous individuals takes up to six or seven cycles of self-pollination and selection (Ramalho and Araújo, 2011). Self-pollination is common in agronomic crops, but it has not been utilized in the forest sector due to the long generation period. New techniques such as top grafting with early flower induction have the potential to accelerate self-pollination cycles and increase their genetic gain (White et al., 2007; Wong and Bernardo, 2008; Grattapaglia et al., 2018; Castro et al., 2021).

The top grafting technique allows preserving individuals with relevant botanical, morphological, or ecological characteristics by propagating them efficiently with earlier bloom induction (Almqvist, 2013a; Tabacu et al., 2020). Top grafting uses the canopy of physiologically mature and reproductive adult trees, optimizing an available rootstock (Gaspar et al., 2017). This technique has been used in *Pinus* and fruit growing trees, such as kiwi (Liang et al., 2011; Almqvist, 2013b; Gaspar et al., 2017) and is promising for improving the *Eucalyptus* genetic material in breeding programs. *Eucalyptus* species are long cycle allogamous. The best individuals of the breeding population are crossed, and their best progenies are cloned. However, the possibility of increasing the heterosis and obtaining significant gains in traits of interest can be achieved using inbred lines. Therefore, there is a need to adapt this methodology for *Eucalyptus* trees and obtain the inbred lines in a shorter time. Selection of the best individuals in each cycle with the use of top grafting and molecular markers to accelerate the flowering and the consecutive self-pollination cycles, until achieving the desired homozygosity rate, reduces the necessary time and adverse effects of inbreeding (Bison et al., 2004; Nickolas et al., 2019).

The potential of inbred lines developing in *Eucalyptus* and the problems they present justify establishing a viable methodology to perform top graftings. Therefore, the aim of this study is to establish a methodology to top graft and test whether it can be combined with application of the growth regulator paclobutrazol (PBZ) in self-pollinated genotypes, aiming at induction of early flowering and acceleration of inbred line strategies.

2. MATERIAL AND METHODS

2.1. Environmental conditions and genetic material

The experiment was conducted in the hybridization orchards CENIBRA S.A. (latitude 18º

46′ 30″ S; longitude 42º 55′ 57″ W; altitude 744 m), Minas Gerais, Brazil. The climate is classified as Cwa according to the Köppen and Geiger classification with an annual rainfall of 1,497 mm and average temperature of 19.9 oC. Selected genotypes were self-pollinated using pollen isolation bags, and their seeds were planted in 2012 as the first cycle of inbred progeny tests of the species *Eucalyptus grandis* W. Mill ex Maiden, *E. urophylla* S.T. Blake, and hybrids of *E. urophylla* × *E. grandis*. After the first cycle of self-pollination, seven self-pollinated individuals were selected to be evaluated through top grafting for inbred line production. The choice of these genotypes was determined by their volume and wood density values measured from the inbred progeny test at 5 years of age. Twigs were collected in 2017 from the trees and used as scion in the top graftings.

Rootstocks with the same genetics (maternal parents of the genotypes from the first inbred cycle) as the scion were used to make the compatibility between the graft and the rootstock feasible. The ages of these trees were between 18 to 32 years and each genotype was replicated twice in the company's orchard for the top graftings, totaling 14 rootstocks. These old trees were being underused, so using them as rootstock for this methodology was financially expedient. The effect of the growth regulator (PBZ) in these replicas was evaluated on the survival and development of top graftings. The rootstocks were physiologically mature (blooming) with the potential of producing abundant flowers and fruits due to its good crown formation, size, and phytosanitary status.

2.2. Experimental design

Two effects were evaluated to establish a viable methodology using the graft technique to accelerate self-pollinating generations of *Eucalyptus*: a) the application or absence of PBZ and b) the best period of the year for this procedure. The tested periods were six and three months before the conventional species flowering season (January-March); that is, the grafts were performed in July (period 1) and October (period 2) of 2017, respectively. The lower physiological activity of plants in these periods due to the low temperatures tends to be beneficial to the graft's survival (Gaspar et al., 2017; Perez-Luna 2020) as they only direct their compounds to induce sprouting and flowering when the rainy season starts.

The experimental design was randomized blocks with eight replicates per rootstock, equally divided into the two crown sides with each face representing a time when the grafts were performed. Each replicate was established from the graft of four top graftings in a different rootstock branch. In addition, one of the two replicates of the rootstocks of same genetics was selected to be used for the application of the regulator and another as a control without PBZ. The other treatments used in this study, including pruning, watering, and fertilization of the rootstocks, were the same with or without PBZ. Sixty-four top grafts were made per genetic material, with 32 in each of the rootstock replicates (with and without PBZ). Thus, a total of 448 top graftings [seven self-pollinated genotypes × four top grafts/rep × four reps/period/side of the crown × two periods (six and three months before flowering) × two treatments (with and without PBZ)] were made.

2.3. Flowering acceleration processes

2.3.1. Top graftings of self-pollinated genotypes

The grafting technique used was the fork in full slit (top cleft) method, performed in the best branches of the rootstock canopy with some adaptations. The scion (twigs) were collected and transported to the adult rootstocks. Branches with twigs of the same scion circumference were selected from the rootstocks for grafting. The grafted material was coated with parafilm. The connection between scion and rootstock was pressed with thread-seal tape. Three months after the graftings, the plants were evaluated.

2.3.2. Paclobutrazol application

After performing the top graftings, one of the rootstocks of each genotype was selected in the first period (six months before flowering) and PBZ was applied over its root area. This step started by measuring the circumference of the rootstock trunk base. The ratio used was 1 mL of Cultar 250 SC in concentrated suspension (250 g/L paclobutrazol composition), per centimeter of circumference. The regulator was diluted with five liters of water per rootstock application. The PBZ influence on flowering was evaluated at intervals of approximately three months after the grafts were made for each period (six and three months before flowering). The data from each top grafting per genotype, in rootstocks with and without PBZ, were separated during the evaluations for analysis.

2.4. Data collection

The survival rate, flowering, and canopy area were also measured in periodic evaluations at three-month intervals. The survival rate was obtained each evaluation from the count of living top grafts over time. When flowering was detected, the evaluations also consisted of counting the floral buds, flowers, and fruits present. The top graftings' canopy area (m^2) was calculated by multiplying widths and lengths, both in cm, of all living grafts and dividing the result by 10,000 to convert the value to m^2. The growth of the canopy over time was monitored with the area values.

$$Top\ graftings\ canopy\ area\ (m^2) = \frac{length\ (cm) * width\ (cm)}{10{,}000} \quad \text{Eq.1}$$

The top graftings submitted to different effects were compared using the collected data from eight evaluations (from September 2017 to August 2019). The vigor of the rootstocks was also evaluated. Specific recommendations regarding fertilization, orchard management, and graft maintenance and identification were made to improve the material conditions over time.

2.5. Statistical analysis

Different analyses were performed with the data collected using RBio and R software (Bhering, 2017; R Core Team, 2021). A t-test with a 5% significance level for type I error compared the relevance of PBZ application for the effect of fixed treatment. Fixed models were also used to analyze the significance between the treatments. The covariances of matrix structures were assessed to fit the best model, according to Akaike Information Criteria (AIC) and Bayesian Information Criteria (BIC) values.

3 .RESULTS

3.1. Flowering analysis

The top graftings performed six months before natural flowering produced flower buds from the first evaluation, which occurred three months after the grafting procedure. However, the count for flower buds started seven months after this grafting period (Table 1). The last assessment, in August 2019, was used to demonstrate the effectiveness of the methodology. The number of top grafts with flowering was counted among those alive per genotype. The ratio of bloomed top grafts and those alive 25 months after grafting was higher than 83%. The genotype with the early flowering used as a graft is a *E. urophylla* × *E. grandis* hybrid (UROGRA3). 25 months after grafting, the genetic material presented a total of 15 top grafts with flowering (78% of live graftings from this genotype). The flowering of the top graftings for the genotypes UROGRA1, URO1, and GRA3 was not evaluated due to their precocious graft death. The PBZ was efficient in promoting flower buds, fruit production, and fruit development, with a higher number of top grafts flourishing with PBZ than without it over time.

The top graftings performed in October (three months before natural flowering) were successful in the first flowering season as two flourished in the evaluation three months after grafting. URO1 was the genotype with the earliest flowering when used as scion. The evaluation in August 2019 (22 months after grafting) found, in most cases, a high ratio of genotypes that flourished among those alive. A total of 38 top graftings flourished and the highest number was found for the hybrid with PBZ application. The PBZ was efficient in inducing flowering in general. The flowering capacity of the UROGRA1 and GRA3 genotypes was not evaluated, because of the precocious death of the grafts. The other living genotypes flowered for the first time in different periods.

The number of flower buds produced in 2018 was higher for the top graftings carried out in July (Table 1), but the top graftings from the second period stood out. Higher flower bud and fruit production were obtained for the materials grafted in October, compared to those grafted six months before natural flowering. The PBZ induced greater flower bud and fruit production. Eight top graftings produced a considerable quantity of flower buds and fruits during the last evaluations, surpassing two thousand buds in a single top graft. The maintenance of fruits was satisfactory over time, with 34% for the top grafts done in July and 48% for those grafted in October.

Overall, the flowering achieved with top graftings of both periods produced enough material to obtain the second cycle of self-pollination and the

Table 1 – Number of new top graftings bloomed in each evaluation and of flower buds and fruits produced by the top graftings. The total (2018-19) corresponds to the accumulative number of floral materials that remained alive until August 2019.

Tabela 1 – *Número de novos enxertos floridos em cada avaliação e de botões florais e frutos que foram produzidos pelos enxertos. O total (2018-19) corresponde ao número acumulado de materiais florais que permaneceram vivos até agosto de 2019.*

Six months before flowering – Top grafting performed in July										
Months after	grafting	7	10	15	Accrued in	19	22	25	Accrued in	Total
Assessments	PBZ	Feb_18	May_18	Oct_18	2018	Feb_19	May_19	Aug_19	2019	(2018-2019)
Top graftings	With	6	1	3	10	8	-	-	8	18
	Without	6	-	-	6	2	-	-	2	8
Flower buds	With	1883	74	x	1957	11562	153	x	11715	13672
	Without	2966	300		3266	7908	27		7935	11201
Fruits	With	-	-	-	-	-	3434	x	3434	3434
	Without	-	-	-	-	-	3225		3225	3225

Three months before flowering – Top grafting performed in October										
Months after	grafting	3	7	12	Accrued in	16	19	22	Accrued in	Total
Assessments	PBZ	Jan_18	May_18	Oct_18	2018	Feb_19	May_19	Aug_19	2019	(2018-2019)
Top graftings	With	1	2	15	18	8	-	-	8	26
	Without	1	1	2	4	8	-	-	8	12
Flower buds	With	-	178	x	178	13745	156	x	13901	14079
	Without	-	73		73	12796	-		12796	12869
Fruits	With	-	-	-	-	-	6585	x	6585	6585
	Without	-	-	-	-	-	5700		5700	5700

The number of flower buds and fruits produced in some evaluations was not counted (represented by the x), only the amount of how many top graftings bloomed and remained alive.

Não foi contabilizado o número de botões florais e frutos produzidos nessas avaliações (representado pelo x), apenas a quantidade de enxertos de copa que floresceram e permaneceram vivos.

Table 2 – T test for the fixed effect (PBZ), for the two variables analyzed in the evaluations of the top graftings and for the two grafting periods (six and three months before flowering). The results related to top graftings were separated in the presence and absence of PBZ. The *tcal* and *p-value* obtained from the t test were performed using the R software.

Tabela 2 *– Teste-t para efeito fixo (PBZ), para as duas variáveis analisadas nas avaliações das enxertias de topo e para as duas épocas de enxertia (seis e três meses antes da floração). Os resultados referentes às enxertias de topo foram separados na presença e ausência de PBZ. Os* ***tcal*** *e* ***p-valores*** *obtidos do teste-t foram realizados usando o software R.*

Traits	Survival (%)		Area (m²)	
Treatments	Six months before	Three months before	Six months before	Three months before
With PBZ	0.221	0.429	0.210	0.223
Without PBZ	0.148	0.372	0.132	0.244
Tcalc	1.567	1.077	2.405	-0.908
p-value	0.120	0.284	0.016*	0.364

*The difference between treatments is significant, considering p-value at (P< 0.05).

**A diferença entre tratamentos é significativa, considerando p-valor em (P<0.05).*

crosses between different $S_1 \times S_1$ genotypes. The S_2 selfed progenies were also generated at CENIBRA's Hybridization Orchard, using the top grafts from this project. In summary, from all graftings made, 64 top grafts presented flowering. Thirty-two were self-pollinated and generated fruit and seeds for the next generation. The seeds were germinated and a total of 529 seedlings (possibly S_2) were produced, which were sent for microsatellite genotyping to confirm S_2 selfed generation. A total of 379 S_2 individuals were confirmed (72% of success). Part of these seedlings were grafted as top graftings to follow the same process and obtain S_3.

3.2. Influence of PBZ on survival and canopy area of top grafting

The top grafting's survival and canopy area growth data were tested with and without PBZ application. Differences between treatments were assessed at each grafting time using the t-test at ($P<$ 0.05) type I error (Table 2).

The PBZ increased flowering in the top graftings. However, the survival and the canopy area increase did not differ with or without PBZ application in most cases (Table 2). The growth of canopy area in the top graftings of six months before flowering was the only trait affected by the PBZ application. For this reason, in most casses its application is not necessary to guarantee higher values for the other two traits.

3.3. Repeated evaluations analysis

The evaluation of the survival and canopy area aimed to detect differences in the development of top grafts in the two grafting periods (six and three months before flowering). The adequate models were selected based on the lower values of Akaike Information Criteria (AIC) and Bayesian Information Criteria (BIC). Considering survival, the selected model with the best covariance structure was AR and for canopy area, the HCS covariance structure.

Based on the selected models, the Genotype effect showed significance ($P<$ 0.05) for the survival of the top graftings performed six and three months before and for the area of top grafts from three months before (Table 3). T-test was not significant for the area of top graftings performed six months before. The Evaluation effect was significant for all the hypotheses, so there is a difference in the development of top graftings

Table 3 – P-values for fixed effects of paclobutrazol (PBZ), genotypes and the interaction between evaluations and genotypes. These probability values were generated from the repeated evaluations model under heterogeneous compound symmetry (HCS) covariance matrix structure for Area (m^2) and auto regressive (AR) for survival (%), for each grafting period.

Tabela 3 *– P-valores para efeitos fixos de paclobutrazol (PBZ), genótipos e interação entre avaliações e genótipos. Valores de probabilidade gerados a partir do modelo de medidas repetidas sob estrutura de matriz de covariância de simetria composta heterogênea (HCS) para Área (m^2) e autoregressiva (AR) para sobrevivência (%), para cada período de enxertia.*

Traits	Survival (%)		Area (m²)	
Treatments	Six months before	Three months before	Six months before	Three months before
Genotypes	< 0.0001*	< 0.0001*	0.4137	< 0.0001*
Evaluation	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*
Genotypes × Evaluation	< 0.0001*	0.6853	< 0.0001*	0.004*

*The difference between treatments is significant, considering p-value at (P< 0.05).

**A diferença entre tratamentos é significativa, considerando p-valor em (P<0.05).*

over evaluation time. The Genotype × Evaluation interaction was statistically significant for most scenarios except for the survival in grafting conducted three months before natural flowering. The overall conclusion demonstrates that considering all the effects analyzed, performing grafts of self-pollinated individuals three months before natural flowering is the most appropriate period for *Eucalyptus* species, to obtain satisfactory survival and development rates.

4. DISCUSSION

4.1. Flowering and paclobutrazol influence upon top grafting development

The flowering started three months after grafting with subsequent fruit production, confirming that self-pollinated genotypes presented early flowering and retained the successfully produced flower buds. The top graftings from bolth periods flourished, but the top grafting of six months before natural flowering (performed in July) can be carried out to obtain short-term results. In this period the top grafting has adequate time to develop and start producing a higher number of flower buds in the first year of development after grafting.

The top grafting performed three months before (October) guaranteed greater flower bud and fruit production in the second year after grafting. The superior performance of the top graftings performed in this period occurred in the rootstocks with PBZ application. Also, this period's higher long-term survival rate contributed to the higher flowering production. These top graftings maintained and developed until their second flowering season, producing more flower buds and fruits. Thus, despite requiring a longer period (almost two years after grafting), the three-months before period is recommended, ensuring a higher number of living genotypes and abundant flowering.

The predisposition and the time needed to start flowering differed in the self-pollinated genotypes used as a scion. This difference in plants is complex and involves genetic interactions, environmental factors, and development stages (Ha, 2014; Van Eeuwijk et al., 2019). The physiological aspect, for instance, refers to the plant's ability to transport nutrients, phytohormones, and organic compounds from the root to the crown and vice versa (Nanda and Melnyk, 2018). The affinity between the scion and the rootstock is also important to ensure the morphological, anatomical, physiological, and biochemical compatibility between them (Tedesco et al., 2022). This aspect was considered in the present methodology since the rootstock is the maternal parent of the genotype that originated the scion material. The success of grafting to induce flowering also varied for cassava genotypes (Ceballos et al., 2017).

Factors such as environmental adaptation (average temperature and photoperiod) and the expression of proteins affects the predisposition of genotypes to the flowering phenomenon (Turck et al., 2008; Amasino, 2010; Yeoh et al., 2011; Ha, 2014; McClung et al., 2016; Sharif et al., 2021). The *Flowering locus* T -FT protein is a mobile signal produced in the leaves and transported via the phloem to the apical meristem, where it interacts with other transcription factors to initiate floral development (Amasino, 2010; Lee and Lee, 2010; Min and Kramer, 2020). The induction of FT expression in the leaves and its movement towards the apex trigger flowering (Wigge, 2011; Yeoh et al., 2011). Graft techniques such as top grafting can take advantage of protein mobility to induce flowering (Wu et al., 2022). This protein is naturally produced and translocated to the top grafts canopy because the rootstock is in an advanced development stage.

The relevance of PBZ is another influence on flowering and the application of this growth regulator induced early flowering/fruiting in *Mangifera indica* (Srivastav et al., 2010; Kumar et al., 2021). However, its effect varies with species, age, and concentration of phytohormones used (Wei et al., 2018). The PBZ application also increases ABA and cytokinin concentrations in *Solanum trilobatum*. The higher concentration of cytokinin improved the chloroplasts differentiation and chlorophyll biosynthesis, preventing the degradation of these pigments (Nivedithadevi et al., 2015).

The flowering of the top graftings was greater when performed in rootstocks with PBZ due to reduced growth, accelerated maturity, chlorophyll concentration, and greater translocation of photoassimilates caused by this growth regulator (Hajihashemi, 2018). The production and growth of plants depend on photosynthetic rates, essential for plant growth and development. PBZ application guarantees greater photosynthetic yield per unit of

Figure 1 – Use of top grafting for inbred lines production. A and B- Graft methodology using twigs collected from self-pollinated individuals; C and D- Paclobutrazol application over the rootstock's root area; E- Top grafting blooming three months after grafting; F and G- Flower bud and flower production was satisfactory in many top graftings; H, G, and J- Top grafts were able to provide fruit development until the seed harvest period.

Figura 1 *– Utilização da enxertia de topo para produção de linhagens. Metodologia A e B-Metodologia de enxertia utilizando galhos coletados de indivíduos autopolinizados; C e D- Aplicação de Paclobutrazol sobre a área radicular do porta-enxerto; E- Enxertia de topo florescendo três meses após a enxertia; F e G- A produção de botões e flores foi satisfatória em muitos enxetos; H, G e J- Os enxertos foram capazes de proporcionar o desenvolvimento dos frutos até o período de colheita das sementes.*

leaf area and better partition of the photoassimilates (Xia et al., 2018). Thus, this greater photoassimilate production allowed higher flower bud production (Figure 1).

The choice of branches in rootstock can affect the flowering rates and survival of top graftings. The repetitions were based on four grafts in the same branch. The crown growth of those grafted on the main branches was higher than using the laterals, as the laterals tend to stagnate and die. The effect of branch senescence in the survival rate has also been described for *Pinus elliottii* var. *elliottii*. Higher graft survival rates were observed in the middle portion of the crown, followed by the apex and lower percentages in the basal portion (Perez et al., 2007). Also, the maintenance of vigor (pruning and fertilization) can contribute to the success of top grafting, guarantying the development of those that live and adequate flowering production (Almqvist, 2013b). The vigor also favored the grafting survival in young branches of *Araucaria angustifolia* (Wendling, et al., 2017).

Top grafting also allows the seedlings obtained at each cycle of self-pollination in the field or a nursery (young seedlings selected by the Genome-Wide Selection- GWS) to be grafted, producing the subsequent cycle (Castro et al., 2021). Its use will rule out the exclusive use of traditional breeding programs to obtain superior clones as the inbred lines will be obtained with self-pollination cycles, tending to revolutionize tree improvement. The GWS can also contribute selecting the best individuals of each generation at an early stage for grafting (Lebedev et al., 2020). This will solve the problem of genetic improvement programs, related to the time needed to complete each generation. Thus, the inbred lines can be accomplished by using two different methodologies: top grafting and GWS.

The depression for traits due to inbreeding may affect this process as the generations advance, since *Eucalyptus* species are allogamous (Cobb et al., 2019; Berlan, 2018; Nickolas et al., 2019). However, the effect of inbreeding depression on ten commercial *Eucalyptus* clones showed a small magnitude for circumference at breast height [mean depression of 17.5% (P< 0.05)] and, especially, for wood basic density (Bison et al., 2004). It is expected to avoid the effects of inbreeding by selecting and grafting the best self-pollinated individuals per generation, which will make it possible to achieve the high rate of homozygosity expected for the inbred lines produced.

4.2. Repeated evaluations analysis

A set of a priori candidate models have been defined as possibilities to analyze the survival and canopy area data. The AIC (Akaike, 1974) and BIC (Schwarz, 1978) values were used to compare these models and define the best option based on their results for each scenario. After selecting the HCS model, it was used to analyze the Genotypes and Evaluation effects upon the survival and development of canopy area. Only the Genotype effect presented no significance for the area trait in top graftings performed six months before natural flowering. This result indicates no differences among development and survival for those top graftings, so it would not need to be measured several times as the result was uniform.

The results of the Genotype effect also show that for survival, some genotypes are more likely to remain alive, and either six or three months before flowering are periods that can be recommended for grafting. In both periods the result was similar for the genotypes that remained alive. However, the temperature fluctuation is greater in July than October and this factor tends to affect the initial bloom and shoot development of *Eucalyptus* grafts, increasing early mortality. The graft period also influences the survival rate for *A. augustifolia* (Gaspar et al., 2017). Thus, choosing the three months before natural flowering provides better survival.

The analyses of the Evaluation effect demonstrate that recurrent evaluations are necessary to make an effective definition of the best genotype for grafting made in both periods. The initial evaluations would not make it possible to ranking the genotypes, determining which would present good compatibility and tendency for satisfactory development to continue with the self-pollination cycles. The interaction Genotypes × Evaluations also shows the necessity of monitoring all the development stages of top grafting until flowering and development of fruits is confirmed. Therefore, the definition of self-pollinated genotypes more conducive to the canopy performance should be done after evaluations confirming the adequate grafting period for the species.

5. CONCLUSIONS

The top grafting technique is viable to induce early flowering of *Eucalyptus*. The number of flower buds and fruits produced was satisfactory in the two grafting periods to obtain the second cycle of self-pollination and the crosses between different $S_1 \times S_1$ genotypes. However, the number of flower buds and fruits produced were higher for the period three months before natural flowering, this being the period recommended for the methodology's execution.

The application of paclobutrazol increased the flowering of the top grafting but, in general, its application does not affect the survival rate and the development of the canopy area of the grafts.

The appropriate choice of genotypes apt for grafting can guarantee the survival of a greater number of top graftings and the highest growth rates in the canopy area. Also, the constant monitoring and maintenance of the graft canopy quality is necessary to ensure its vigor.

AUTHOR CONTRIBUTIONS

Carla Aparecida de Oliveira Castro: Methodology, Formal analysis, Data collection, Text writing – Original Draft, Visualization, Project management; Gleison Augusto dos Santos: Conceptualization, Methodology, Resources, Writing - Review & Editing, Funding acquisition; Elizabete Keiko Takahashi: Conceptualization, Methodology, Resources, Writing - Review & Editing, Funding acquisition; Andrei Caíque Pires Nunes: Software, Validation, Writing - Review & Editing; Genaina A. Souza: Resources, Writing - Review & Editing; Marcos Deon Vilela de Resende: Conceptualization, Methodology, Software, Validation; José Cola Zanuncio: Writing - Review & Editing.

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7. REFERENCES

Akaike H. A new look at the statistical model identification. IEEE Transactions on Automatic Control. 1974;19(6):716-723. doi: 10.1109/TAC.1974.1100705.

Almqvist C. Interstock effects on topgraft vitality and strobili production after topgrafting in *Pinus sylvestris*. Canadian Journal of Forest Research. 2013a;43(6):584-588. doi:10.1139/cjfr-2012-0507

Almqvist C. Survival and strobili production in top grafted scions from young *Pinus sylvestris* seedlings. Scandinavian Journal of Forest Research. 2013b;28(6):533-539. doi:10.1080/02827581.2013.803598

Amasino R. Seasonal and developmental timing of flowering. Plant J. 2010;61:1001-1013. doi:10.1111/j.1365-313X.2010.04148.x

Avdikos ID, Tagiakas R, Tsouvaltzis P, Mylonas I, Xynias IN, Mavromatis AG. Comparative Evaluation of Tomato Hybrids and Inbred Lines for Fruit Quality Traits. Agronomy. 2021; 11(3):609. doi:10.3390/agronomy11030609

Berlan JP. Hybrid corn and the unsettled question of heterosis. Journal of genetics. 2018;97(5):1075-82. doi:10.1007/s12041-018-1037-2

Bhering LL. Rbio: A Tool for Biometric and Statistical Analysis Using the R Platform. Crop Breeding and Applied Biotechnology. 2017;17:187-190.

Bison O, Aguiar AM, Rezende GDSP, Ramalho MAP. Inbreeding depression in *Eucalyptus* clones. Crop Breeding and Applied Biotechnology, Madison. 2004;4(4):459-464.

Castro CAO, Santos GA, Takahashi EK, Nunes ACP, Santos GA, Resende MDV. Accelerating *Eucalyptus* breeding strategies through top grafting applied to young seedlings. Industrial Crops and Products. 2021;171:113906. doi:10.1016/j.indcrop.2021.113906.

Ceballos H, Jaramillo JJ, Salazar S, Pineda LM, Calle F, Setter T. Induction of flowering in cassava through grafting. Journal of Plant Breeding and Crop Science. 2017;9(2):19-29. doi:10.5897/

JPBCS2016.0617

Cobb JN, Juma RU, Biswas PS, Arbelaez JD, Rutkoski J, Atlin G, Hagen T, Quinn M, Ng EH. Enhancing the rate of genetic gain in public-sector plant breeding programs: lessons from the breeder's equation. Theoretical and applied genetics. 2019 Mar;132(3):627-45. doi:10.1007/s00122-019-03317-0

Gasim S, Hamad SA, Abdelmula A, Mohamed Ahmed IA. Yield and quality attributes of faba bean inbred lines grown under marginal environmental conditions of Sudan. Food Science & Nutrition. 2015;3(6): 539-547. doi:10.1002/fsn3.245

Gaspar RGB, Wendling I, Stuepp CA, Angelo AC. Rootstock age and growth habit influence top grafting in *Araucaria angustifolia*. CERNE. 2017;23(4):465-471. doi:10.1590/01047760201723042447

Grattapaglia D, Silva-Junior OB, Resende RT, Cappa EP, Müller BS, Tan B, Isik F, Ratcliffe B, El-Kassaby YA. Quantitative genetics and genomics converge to accelerate forest tree breeding. Frontiers in Plant Science. 2018;9:1693. doi:10.3389/fpls.2018.01693

Ha TM. A review of plants‟ flowering physiology: the control of floral induction by juvenility, temperature, and photoperiod in annual and ornamental crops. Asian Journal of Agriculture Food Science. 2014;2:186-195. Available at: https://192.99.73.24/index.php/AJAFS/article/view/1267 (Accessed: 9 June 2022).

Hajihashemi SJ. Physiological, biochemical, antioxidant and growth characterizations of gibberellin and paclobutrazol-treated sweet leaf (*Stevia rebaudiana* B.) herb. Journal of Plant Biochemistry and Biotechnology. 2018;27:237-240. doi:10.1007/s13562-017-0428-4

Jones RC, Vaillancourt RE, Gore PL, Potts BM. Genetic control of flowering time in *Eucalyptus globulus* ssp. *globulus*. Tree Genetics & Genomes. 2011;7(6):1209-1218. doi:10.1007/s11295-011-0407-1

Klocko AL, Ma C, Robertson S, Esfandiari E, Nilsson O, Strauss SH. FT overexpression induces precocious flowering and normal reproductive development in *Eucalyptus*. Plant Biotechnology Journal. 2016;14(2):808-819. doi:10.1111/pbi.12431

Kumar A, Ram S, Bist LD, Singh CP. Paclobutrazol Boost Up for Fruit Production: A Review. Annals of the Romanian Society for Cell Biology. 2021;25(6):963-80. Retrieved from https://www.annalsofrscb.ro/index.php/journal/article/view/5582

Lebedev VG, Lebedeva TN, Chernodubov AI, Shestibratov KA. Genomic selection for forest tree improvement: Methods, achievements, and perspectives. Forests. 2020 Nov;11(11):1190. doi: 10.3390/f11111190

Lee J, Lee I. Regulation and function of SOC1, a flowering pathway integrator. J. Exp. Bot. 2010;61:2247-2254. doi:10.1093/jxb/erq098

Liang H, Hu Y, Pang W, Liu W, Yang M. Studies on kiwifruit improvement by multiple top grafting. Acta Hortic. 2011;913:365-371. doi:10.17660/ActaHortic.2011.913.48

Maia MCC. Sistema reprodutivo de populações alógamas e autógamas: modelo básico e equilíbrio. Embrapa Acre-Artigo em periódico indexado (ALICE). 2010.

McClung CR, Lou P, Hermand V, Kim JA. The Importance of ambient temperature to growth and the induction of flowering. Frontiers in Plant Sciences. 2016;7:1266. doi:10.3389/fpls.2016.01266

Min Y, Kramer EM. Transcriptome profiling and weighted gene co-expression network analysis of early floral development in *Aquilegia coerulea*. Scientific reports. 2020 Nov 12;10(1):1-5. doi:10.1038/s41598-020-76750-7

Nanda AK, Melnyk CW. The role of plant hormones during grafting. Journal of Plant Research. 2018;131(1):49-58. doi:10.1007/s10265-017-0994-5

Nickolas H, Harrison PA, Tilyard P, Vaillancourt RE, Potts BM. Inbreeding depression and differential maladaptation shape the fitness trajectory of two co-occurring *Eucalyptus* species. Annals of Forest Science. 2019;76(1):10. doi:10.1007/s13595-018-0796-5

Nivedithadevi D, Somasundaram R, Pannerselvam R. Effect of abscisic acid, paclobutrazol and salicylic acid on the growth and pigment variation in *Solanum* *trilobatum* (l). Int. J. Drug Dev. Res. 2012;4(3):236-246. doi:10.25081/jmb.2017.v1.51

Perez AMM, White TL, Huber DA, Martin TA. Graft survival and promotion of female and male strobili by topgrafting in a third-cycle slash pine (*Pinus elliottii* var. *elliottii*) breeding program. Canadian Journal of Forest Research. 2007;37(7):1244-1252. doi:10.1139/X07-004

Pérez-Luna AL, Wehenkel CH, Prieto-Ruíz JÁ, Lopez-Upton J, Solis-Gonzalez S, Chávez-Simental JA, Hernández-Díaz JC. Grafting in conifers. A review. Pakistan Journal of Botany. 2020;52(4):1369-78. doi:10.30848/PJB2020-4(10)

R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2021. URL: <https://www.r-project.org/>

Ramalho MAP, Araújo LCAD. Breeding self-pollinated plants. Crop Breeding and Applied Biotechnology. 2011;11(SPE):1-7. doi: 10.1590/S1984-70332011000500002

Reddy VR, Jabeen F, Sudarshan MR. Heterosis studies in diallel crosses of maize for yield and yield attributing traits in maize (Zea mays L.) over locations. International Journal of Agriculture, Environment and Biotechnology. 2015;8(2):271-283. doi:10.5958/2230-732X.2015.00034.0

Salvador FV, dos Anjos RS, Marçal TD, Rocha JR, Pereira GD, de Paula RG, Carneiro PC, Carneiro JE. Accounting for heterogeneous genotypic variance of progenies within populations in autogamous plant breeding: An evaluation in common bean. Crop Science. 2021;61(6):3947-3961. doi:10.1002/csc2.20590

Santos GA, Nunes ACP, de Resende MDV, Silva LD, Higa A, de Assis TF. An index combining volume and Pilodyn penetration to study stability and adaptability of *Eucalyptus* multi-species hybrids in Rio Grande do Sul, Brazil. Australian Forestry. 2016;79(4):248-255. doi:10.1080/00049158.2016.1237253

Saxena RK, Jiang Y, Khan AW, Zhao Y, Kumar Singh V, Bohra A, Sonappa M, Rathore A, Kumar CS, Saxena K, Reif J. Characterization of heterosis and genomic prediction-based establishment of heterotic patterns for developing better hybrids in pigeonpea. The Plant Genome 2021: Early-View. 2021. doi:10.1002/tpg2.20125

Schwarz G. Estimating the dimension of a model. The annals of statistics. 1978;1:461-464. doi: 10.1214/aos/1176344136

Sharif R, Raza A, Chen P, Li Y, El-Ballat EM, Rauf A, Hano C, El-Esawi MA. HD-ZIP gene family: potential roles in improving plant growth and regulating stress-responsive mechanisms in plants. Genes, 2021;12(8), 1256. Doi:10.3390/genes12081256

Srivastav M, Kishor A, Dahuja A, Sharma RR. Effect of Paclobutrazol and Salinity on Ion Leakage, Proline Content and Activities of Antioxidant Enzymes in Mango (*Mangifera indica* L.). Science Horticulture. 2010;125:785-788. doi:10.1016/j.scienta.2010.05.023

Tabacu AF, Butcaru AC, Lehman J, Stănică F. Top grafting response of some pawpaw (Asimina triloba Dunal) genotypes. Scientific Papers. Series B, Horticulture. 2020:198-203. Online ISSN 2286-1580.

Tedesco S, Fevereiro P, Kragler F, Pina A. Plant grafting and graft incompatibility: A review from the grapevine perspective. Scientia Horticulturae. 2022 Jun 1;299:111019. doi:10.1016/j.scienta.2022.111019

Turck F, Fornara F, Coupland G. Regulation and identity of florigen: *FLOWERING LOCUS T* moves center stage. Annual Review in Plant Biology. 2008;59:573-594. doi:10.1146/annurev.arplant.59.032607.092755

Van Eeuwijk FA, Bustos-Korts D, Millet EJ, Boer MP, Kruijer W, Thompson A, Malosetti M, Iwata H, Quiroz R, Kuppe C, Muller O. Modelling strategies for assessing and increasing the effectiveness of new phenotyping techniques in plant breeding. Plant science. 2019;282:23-39. doi:10.1016/j.plantsci.2018.06.018

Wang X, Jiang GL, Green M, Scott RA, Hyten DL, Cregan PB. Quantitative trait locus analysis of saturated fatty acids in a population of recombinant inbred lines of soybean. Molecular Breeding. 2012;30(2):1163-1179. doi:10.1007/s11032-012-9704-0

Wei XJ, Ma J, Wang K, Liang XJ, Lan JX, Li YJ, Li KX. Early flowering induction in Golden Camellia seedlings and effects of paclobutrazol. HortScience. 2018;53(12):1849-1854. doi:10.21273/HORTSCI13676-18

Wendling I, Zanette F, Rickli-Horsti H, Constantino V. Produção de mudas de araucária por enxertia. In: WENDLING, I.; ZANETTE, F. Araucária: particularidades, propagação e manejo de plantios (Embrapa Florestas-Capítulo em livro científico). Brasília – DF, Embrapa. 2017.159p.

White TL, Adams WT, Neale DB. Forest Genetics. Cambridge, MA: CABI Publishing. 2007.682p. doi:10.1079/9781845932855.0000

Wigge PA. FT, a mobile developmental signal in plants. Current Biology. 2011;21(9):R374-R378. doi:10.1016/j.cub.2011.03.038

Wong CK, Bernardo R. Genome wide selection in oil palm: increasing selection gain per unit time and cost with small populations. Theoretical and Applied Genetics. 2008;116(6):815-824. doi:10.1007/s00122-008-0715-5

Wu YM, Ma YJ, Wang M, Zhou H, Gan ZM, Zeng RF, Ye LX, Zhou JJ, Zhang JZ, Hu CG. Mobility of FLOWERING LOCUS T protein as a systemic signal in trifoliate orange and its low accumulation in grafted juvenile scions. Horticulture Research. 2022 Mar 7. doi:10.1093/hr/uhac056

Xia X, Tang Y, Wei M, Zhao D. Effect of paclobutrazol application on plant photosynthetic performance and leaf greenness of herbaceous peony. Horticulturae. 2018;4(1):5. doi:10.3390/horticulturae4010005

Yeoh CC, Balcerowicz M, Laurie R, Macknight R, Putterill J. Developing a method for customized induction of flowering. BMC Biotechnology. 2011;11(1):36. doi:10.1186/1472-6750-11-36